

Distribution Statement

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Accomplishments

What were the major goals and objectives of the project?

The overall objective of this project is to explore the adaptive capacity of spleen to enhance diving capabilities in people without breath-hold diving ancestry. This objective was accomplished by examining the following three Aims.

Aim 1: Test the hypothesis that spleen volume and function are enhanced with repeated breath-hold diving like exposures.

Aim 2: Test the hypothesis that HPT-axis activation modifies the extent of splenic adaptations.

Aim 3: Test the hypothesis that the magnitude of splenic adaptation is enhanced when the breath-hold diving like exposures are conducted in cold water.

What was accomplished towards achieving these goals?

Data collection on all three cohorts of participants for the primary study has been completed. Specimen and image analyses are mostly complete, and final statistical analyses will be completed shortly after specimen and image analyses have been completed.

This project required three years for data collection before the three Aims could be formally examined. Therefore, supplemental studies have been conducted to investigate related questions that are fundamental to the overall objective of this project. The research questions that were examined are listed below. The manuscripts and/or conference abstracts for each of these research questions have been included in [Appendix 1](#).

- **Does water immersion modify spleen volume and/or apneas-induced splenic contraction?**
 - Peer-reviewed manuscript published in American Journal of Physiology.
 - Keeler JM, Hess HW, Tourula E, Baker TB, Kerr PM, Greenshields JT, Chapman RF, Johnson BD, **Schlader ZJ**. Increased spleen volume provoked by temperate head out water immersion. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology* 323: R776-R786, 2022.
 - Peer-reviewed abstracts (underline indicates trainee):
 - Keeler JM, Tourula E, Baker TB, Logan R, Hess HW, Kerr PM, Greenshields JT, Chapman RF, Johnson BD, **Schlader ZJ**. Acute elevations in apnea-induced spleen contraction provoked by thermoneutral head out water immersion. Experimental Biology, Philadelphia PA, USA 2022.
 - Keeler JM, Tourula E, Baker TB, Logan R, Hess HW, Kerr PM, Greenshields JT, Chapman RF, Johnson BD, **Schlader ZJ**. Spleen volume is increased during thermoneutral head-out water immersion. American College of Sports Medicine Annual Meeting, San Diego, CA, USA, 2022.
- **Is there a relation between spleen volume and apneas-induced elevations in hemoglobin mass?**

- Peer-reviewed manuscript published in Undersea and Hyperbaric Medicine Journal.
 - Keeler JM, Hess HW, Tourula E, Chapman RF, Johnson BD, **Schlader ZJ**. Relation between resting spleen volume and apnea-induced increases in hemoglobin mass. *Undersea and Hyperbaric Medicine Journal* 51: 59-69, 2024.
- Peer-reviewed abstracts (underline indicates trainee):
 - **Schlader ZJ**, Hess HW, Baker TB, Logan R, Kerr P, Tourula E, Greenshields JT, Chapman RF, Johnson BD, Keeler JM. Preliminary evidence that resting spleen volume is related to the magnitude of apnea-induced increases in total hemoglobin mass in humans without a history of breath-hold diving. Undersea and Hyperbaric Medicine Annual Scientific Meeting, Reno, NV, USA, 2022.
 - Keeler JM, Hess HW, Tourula E, Baker TB, Kerr PM, Greenshields JT, Chapman RF, Johnson BD, **Schlader ZJ**. Is there a relation between spleen volume and increases in hemoglobin mass following apneas in people without breath-hold diving experience? International Conference on Environmental Ergonomics, Niagara Falls, ON, Canada, 2022.
- **Does hypoxia modify activation of the hypothalamus-pituitary-thyroid axis during cold water immersion?**
 - Peer-reviewed manuscript in revision in Journal of Applied Physiology.
 - Keeler JM, Greenshields JT, Goss GS, Baker TB, Chapman RF, Johnson BD, **Schlader ZJ**. Acute moderate normobaric hypoxia does not modify circulating thyroid hormone concentrations induced by one hour of head out cold-water immersion. *Journal of Applied Physiology* In Revision.
 - Peer-reviewed abstracts (underline indicates trainee):
 - Keeler JM, Greenshields JT, Goss CS, Baker TB, Chapman RF, Johnson BD, **Schlader ZJ**. Modification of thermoregulation during cold water immersion by normobaric hypoxia. Undersea and Hyperbaric Medicine Annual Scientific Meeting, Virtual, 2021.
 - Greenshields JT, Goss CS, Keeler JM, Baker TB, Logan RT, Chapman RF, Johnson BD, **Schlader ZJ**. HPT-axis activation during cold head out water immersion is not modified by moderate hypoxia. Experimental Biology, Philadelphia PA, USA 2022.
- **Can hemoglobin mass be accurately measured non-invasively via carbon monoxide rebreathing?**
 - Peer-reviewed abstracts (underline indicates trainee):
 - Tourula E, Keeler JM, Baker TB, Logan R, Hess HW, Carter SJ, Johnson BJ, Chapman RF, **Schlader ZJ**. Pulse oximetry carboxyhemoglobin saturation overestimates carbon monoxide rebreathing derived measures of total hemoglobin mass in health adults. American College of Sports Medicine Annual Meeting, San Diego, CA, USA, 2022.
 - Lignier MJ, Tourula E, Keeler JM, Hite MJ, Regester A, Carter SJ, Johnson BD, Chapman RF, **Schlader ZJ**. Determination of total hemoglobin mass by pulse CO-oximetry in health men and women. American College of Sports Medicine Annual Meeting, Boston, MA, USA, 2024.

These studies were led by postdoctoral fellows and/or doctoral students with support from undergraduate students.

The following abstracts have been presented or accepted for presentation at national/international conferences (underline indicates trainee). These abstracts are derived from the primary data collection under this project. The full-length abstracts for each listed below have been included in [Appendix 2](#).

- Keeler JM, Hess HW, Tourula E, Hite MJ, Chapman RF, Johnson BD, **Schlader ZJ**. The combined effect of 14 days of hypoxia and apnea training on hemoglobin mass and reticulocyte counts. American Physiology Summit, Long Beach, CA, USA, 2023
- Walker C, Keeler JM, Tourula E, Hite MJ, Chapman RF, Johnson BD, **Schlader ZJ**. Fourteen days of hypoxia and apnea training does not modify pulmonary gas exchange efficiency. American College of Sports Medicine Annual Meeting, Denver, CO, USA, 2023

- Keeler JM, Hite MJ, Hess HW, Tourula E, Chapman RF, Johnson BD, **Schlader ZJ**. The effects of hypoxia and apnea training on hypoxemic and hypercapnic tolerance. American College of Sports Medicine Annual Meeting, Denver, CO, USA, 2023
- **Schlader ZJ**, Keeler JM, Hite MJ, Hess HW, Tourula E, Chapman RF, Johnson BD, Thom SR. Preliminary evidence that fourteen days of apnea and hypoxia exposures reduces circulating pro-inflammatory microparticles. Undersea and Hyperbaric Medicine Annual Scientific Meeting, San Diego, CA, USA, 2023
- Skutnik BC, Keeler JM, Hite MJ, Hess HW, Tourula E, Walker C, Chapman RF, Johnson BD, **Schlader ZJ**. Fourteen days of cool water immersion does not modify an indirect index of brown adipose tissue activation. American Physiology Summit. Long Beach, CA, USA, 2024
- Keeler JM, Hite MJ, Hess HW, Tourula E, Chapman RF, Johnson BD, Ilardo M, Thom SR, **Schlader ZJ**. No relation between increases of spleen volume and reductions in circulating pro-inflammatory microparticles following fourteen days of apnea and hypoxia exposures. American Physiology Summit. Long Beach, CA, USA, 2024
- Keeler JM, Hite MJ, Hess HW, Tourula E, Chapman RF, Johnson BD, Ilardo M, Thom SR, **Schlader ZJ**. Hypoxia and apnea training during water immersion (but not air) increases spleen volume. American College of Sports Medicine Annual Meeting, Boston, MA, USA, 2024.
- Bobbitt G, Keeler JM, Hite MJ, Hess HW, Tourula E, Thom S, Ilardo M, Chapman RF, Johnson BD, **Schlader ZJ**. No effect of water immersion on training-induced improvements in apnea duration. American College of Sports Medicine Annual Meeting, Boston, MA, USA, 2024.
- **Schlader ZJ**, Keeler JM, Hite MJ, Hess HW, Tourula E, Chapman RF, Ilardo M, Johnson BD, Thom SR. No correlation between increases in spleen volume and change in circulating pro-inflammatory microparticles following fourteen days of apneas and hypoxia exposures. Undersea and Hyperbaric Medicine Annual Scientific Meeting, New Orleans, LA, USA, 2024.

Appendix 3 contains figures related to the initial findings. Some of these data have been presented at conferences. Peer-reviewed manuscripts resulting from these data are in preparation, and we expect to publish at least two peer-reviewed manuscripts within the next twelve months.

What opportunities for training and professional development did the project provide?

Jo Hite was hired after graduating with her bachelor's degree to serve as the research technician on this project. Jo worked as an undergraduate student working in the lab before being hired. This project directly aided her professional development by giving her the skills necessary to successfully go to graduate school in a STEM field. In fact, while working on this project Jo took prerequisite courses – leveraging the resources available through Indiana University.

Jason Keeler was the postdoctoral fellow supported by this project. This project has provided Jason with the training necessary to become an independent investigator and aided in his ability to transition to a faculty position in a STEM-related program at a research-intensive university.

Joel Greenshields was a doctoral student working on this project. Joel has transitioned to a role with the Canadian Institute of Sport.

Erica Tourula was a PhD student worked on this project. Erica is now in the third year of her PhD.

Ben Skutnik was a PhD student that worked on this project. Ben recently transferred to my lab and is interested in the physiology of cold-water immersion.

Max Lignier was a PhD student that worked on this project. Max is in his first year of his PhD.

Payton Kerr was an undergraduate student that worked on this project. Payton is attending medical school this year.

Graham Bobbitt was an undergraduate student that worked on this project. Graham is applying to graduate school this year.

Charlie Walker was an undergraduate student that worked on this project. Charlie will attend medical school next year.

Molly Heikkinen was an undergraduate student that worked on this project. Molly is just accepted a position as a research assistant at USARIEM (Natick, MA).

Leo Sweitzer was an undergraduate student working on this project. Leo is a sophomore and will continue working in the lab through graduation.

Marley Owen was an undergraduate student that worked on this project. Marley is applying to physician assistant school.

Jon Peterson was an undergraduate student working on this project. Leo is a sophomore and will continue working in the lab through graduation.

How were the results disseminated to communities of interest?

The primary community of interest is Navy personnel (including the diving and undersea medicine community) and the scientific community (physiology and/or biomedical sciences). Thus, the obtained information has been disseminated at ONR Undersea Medicine program review meetings, as conference presentations, and as peer-reviewed publications. References for the conference proceedings and peer-reviewed publications are included above. The documents accompanying these proceedings and publications have been included in Appendix 1 and Appendix 2.

What do you plan to do during the next reporting period to accomplish the goals and objectives?

This is the final reporting period. However, we are presenting preliminary results from this project at conference presentations in 2024 and 2025, and at ONR Undersea Medicine program review in spring 2024. Peer-reviewed manuscripts are in preparation, and we expect to publish at least two peer-reviewed manuscripts within the next twelve months.

What honors or awards were received under this project?

Jason Keeler (postdoc) won the following awards for research directly related to this project.

- 2021 President's Award for Best Overall Oral Presentation, Undersea and Hyperbaric Medicine Society
- 2022 Exercise and Environmental Physiology Section, Charles (Tip) Tipton Postdoctoral Research Award, American Physiological Society

- 2024 Exercise and Environmental Physiology Section, Partnership for Clean Competition Anti-Doping Postdoctoral Research Award, American Physiological Society

Erica Tourula (PhD student) won the following awards during the funding period.

- 2022 P.E.O. Scholar Institutional Nominee, P.E.O. International
- 2023 Hinton, Stager and Tanner Women's Health Research Fellowship, Indiana University School of Public Health – Bloomington
- 2023 Updyke/President's Challenge Fellowship, Indiana University School of Public Health – Bloomington

Charlie Walker (undergraduate student) and Molly Heikkinen were both selected as the 2024 SPH-B Nominee for IU-Bloomington Provost's Award for Undergraduate Research and Creative Activity.

Zac Schlader (PI) won the Outstanding Senior Researcher award in the School of Public Health at Indiana University – Bloomington during this reporting period.

Participants

Period: June 1, 2023-December 21, 2023

1. Type: Most senior project role: Post Doctoral Fellow
2. Prefix (optional)
3. First Name: Jason
4. Last Name: Keeler
5. Middle Name (optional): Michael
6. Suffix
7. Nearest person month worked (a person month equals approximately 160 hours of effort, regardless of funding source): 6 months
8. National Academy Member?: N
9. Country if participant is a foreign collaborator; N/A
10. Country(ies) of international travel and duration of stay. N/A

1. Type: Most senior project role: Research Analyst
2. Prefix (optional)
3. First Name: Jo
4. Last Name: Hite
5. Middle Name (optional):
6. Suffix
7. Nearest person month worked (a person month equals approximately 160 hours of effort, regardless of funding source): 4 months
8. National Academy Member?: N
9. Country if participant is a foreign collaborator; N/A
10. Country(ies) of international travel and duration of stay.

Appendix 1

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Does water immersion modify spleen volume and/or apneas-induced splenic contraction?

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RESEARCH ARTICLE

Don't Deny Your Inner Environmental Physiologist: Investigating Physiology with Environmental Stimuli

Increased spleen volume provoked by temperate head-out-of-water immersion

Jason M. Keeler, Hayden W. Hess, Erica Tourula, Tyler B. Baker, Payton M. Kerr, Joel T. Greenshields, Robert F. Chapman, Blair D. Johnson, and Zachary J. Schlader

H. H. Morris Human Performance Laboratories, Department of Kinesiology, School of Public Health, Indiana University, Bloomington, Indiana

Abstract

This study tested the hypotheses that 1) spleen volume increases during head-out-of-water immersion (HOWI) and returns to pre-HOWI values postdiuresis, and 2) the magnitude of apnea-induced spleen contraction increases when preapnea spleen volume is elevated. Spleen volume was measured before and after a set of five apneas in 12 healthy adults (28 ± 5 yr, 3 females) before, during (at 30 and 150 min), and 20 min after temperate temperature ($36 \pm 1^\circ\text{C}$) HOWI. At each time point, spleen length, width, and thickness were measured via ultrasound, and spleen volume was calculated using the Pilsström equation. Compared with pre-HOWI (276 ± 88 mL), spleen volume was elevated at 30 (353 ± 94 mL, $P < 0.01$) and 150 (322 ± 87 mL, $P < 0.01$) min of HOWI but returned to pre-HOWI volume at post-HOWI (281 ± 90 mL, $P = 0.58$). Spleen volume decreased from pre- to postapnea bouts at each time point ($P < 0.01$). The magnitude of reduction in spleen volume from pre- to postapneas was elevated at 30 min of HOWI (-69 ± 24 mL) compared with pre-HOWI (-52 ± 20 mL, $P = 0.04$) but did not differ from pre-HOWI at 150 min of HOWI (-54 ± 16 mL, $P = 0.99$) and post-HOWI (-50 ± 18 mL, $P = 0.87$). Thus, spleen volume is increased throughout 180 min of HOWI, and whereas apnea-induced spleen contraction is augmented after 30 min of HOWI, the magnitude of spleen contraction is unaffected by HOWI thereafter.

apneas; breath hold; plasma volume; splenic contraction; water immersion

INTRODUCTION

The human spleen serves as a repository of erythrocytes that can be jettisoned into circulation upon sympathetic stimulation (i.e., apnea, exercise, or hypoxia), which has been theorized to be important when operating in the aquatic environment (e.g., breath-hold diving) (1). Splenic contraction increases circulating red blood cells in diving mammals (e.g., seals) during deep dives by up to $\sim 50\%$ (2). This splenic contraction plus bradycardia and peripheral vasoconstriction are components of the mammalian “dive reflex,” which is an innate protective reflex that preserves oxygen and directs blood flow to vital tissues (3, 4). Similarly, splenic contraction has been repeatedly observed in humans during voluntary apneas (5) and when breathing hypoxic gas mixtures, but the increase in circulating red blood cells estimated from increases in hemoglobin concentration and hematocrit is often less in magnitude when compared with that observed in other mammals (~ 10 – 15%) (2, 5). That said, associative evidence supports that a larger spleen and splenic contraction are beneficial for breath-hold diving (1, 6). Specifically, native diving populations have larger spleens

compared with their close geographic neighbors (1), and elite apneic divers have greater splenic contraction during apneas compared with nonapneic diving controls, with no statistical difference in preapnea spleen size (5, 7). In theory, a larger splenic contraction would increase the oxygen-carrying and pH/CO₂-buffering capacities of the blood. These responses could improve gas exchange during immersion and upon resurfacing. Previous research has demonstrated improved apnea times that coincide with observations of greater splenic contraction (8–11), and it was not until recently that splenic widths have been measured during water immersion and apneas, which demonstrated decreased splenic widths post-apnea (12). Thus, to our knowledge, no measurements of spleen volume have been performed in humans during immersion. This fundamental gap in the literature potentially undermines the assumption that the spleen is an important contributor to breath-hold diving performance in humans (1).

Upon immersion in water, hydrostatic pressure causes a translocation of blood to the thorax, which is accompanied by hyperperfusion of tissues and organs (13). This is highlighted by data obtained during 20 min of head-out-of-water



immersion in dogs that found that splenic blood flow increased disproportionately more than the rise in cardiac output (14). However, after 80 min of head-out-of-water immersion, splenic blood flow in the dogs returned to baseline, which was likely due to immersion-induced diuresis and the reestablishment of body fluid balance in the presence of hydrostatic pressure (14). Although spleen volume was not measured in the dogs, the increases in splenic blood flow observed during immersion indirectly suggest that spleen volume, and perhaps the ability of the spleen to contract, may be modified by immersion in humans.

The primary purpose of this study was to test the hypothesis that spleen volume increases during acute (30 min) temperate head-out-of-water immersion (HOWI) and returns to pre-HOWI levels after prolonged (150 min) immersion and subsequent diuresis. Furthermore, the effect of HOWI on spleen contraction has only recently been explored in humans (12), but measures of spleen volume have never been obtained. Thus, a secondary purpose of this study was to test the hypothesis that spleen contraction in response to apnea is elevated during HOWI, when preapnea spleen volume is increased.

METHODS

Experimental Design

Before any study activities commenced, this study was approved by the Institutional Review Board at Indiana University (IRB No. 11855) and was subsequently performed in accordance with the standards set by the latest revision of the Declaration of Helsinki, except for the registration in a database. This study used a within-subject design. Participants reported to the laboratory on two separate occasions, with at least 24 h between visits and an average time between visits of 6 ± 4 days. *Visit 1* included consent process, screening process, familiarization (practice apneas), and a carbon monoxide rebreathing procedure. *Visit 2* was the experimental trial. Before participation, all participants provided written informed consent after being fully informed of the experimental procedures and risks.

Participants

An a priori power analysis was carried out with G-Power version 3.1.9.4 software. To our knowledge, there were no direct data to inform the expected magnitude of differences in spleen volume and apnea-induced contraction with water immersion. Thus, data demonstrating the magnitude of expected splenic contraction with repeated apneas were used, which revealed a Cohen's d_z effect size of 1.35. (15). Using this effect size and assuming a moderate positive correlation among repeated measures ($r = 0.5$), an estimated sample size of 12 participants was needed to detect a significant interaction for changes in spleen volume via two-way repeated-measures ANOVA (apnea $\times$ immersion time) using standard parameters of $1 - \beta = 0.80$ and $\alpha = 0.05$. Therefore, 12 healthy adults (28 ± 5 yr, 3 females) without breath-hold diving or competitive apnea experience were recruited and completed the study. Participant characteristics were 181 ± 14 cm height, 90.0 ± 11.6 kg weight, and 27.7 ± 2.8 kg $\cdot$ m⁻² body

mass index. Participants self-reported being moderately active, nontobacco users, and free from any splenic, hematological, cardiovascular, pulmonary, and autoimmune diseases. Females self-reported normal menstruation and were confirmed not to be pregnant via a pregnancy test. Given the single-day experimental design, females were tested across the menstrual cycle (18 ± 14 days postmenses).

Instrumentation and Measurements

Body weight (kg) and height (cm) were measured using a stadiometer (Holtain Limited, Seritex, Wales, UK) and a scale (Sauter, Balingen, Germany). Urine specific gravity was measured using a refractometer (Atago, Tokyo, Japan). Heart rate (beats/min) and an estimate of arterial oxyhemoglobin saturation (SpO₂, %) were measured continually via pulse oximetry (Nellcor N-600X, Medtronic, Inc.) with the sensor placed on the forehead. The SpO₂ nadirs that occurred during or within 60 s postapneas were averaged over each apneic bout to quantify the hypoxemic minimum. Blood pressure was measured outside the water using an automated blood pressure cuff (Suntech CT40, SunTech Medical) on the left brachium. Oral temperature was measured using a clinical-grade thermometer (Covidien Filac 3000 Thermometry Module, Covidien AG Corporation, Switzerland) with an accuracy of $\pm 0.2^\circ\text{C}$ and a measured within-subject coefficient of variation of 0.5%. Oral temperature, and not a more accepted measure of internal body temperature (e.g., rectal or esophageal temperature; 16), was measured to provide a minimally invasive estimate of changes in internal body temperature, which was expected not to change throughout the protocol. After the hand was warmed via a heating pad, using the aseptic finger-prick method, blood samples were collected to assess hemoglobin concentration (HemoQue Hb 201+, DM System, ängelholm, Sweden) and hematocrit (microcentrifugation). Hemoglobin concentration and hematocrit measurements were made in duplicate and averaged. Finger-prick blood samples were considered to be acceptable in this study despite the expectation of apnea-induced peripheral vasoconstriction because of the vasodilatory effect of temperate HOWI (17) and the use of a heating pad to promote blood flow to the fingers.

Out of or while in the water, spleen volume was always measured with participants in a seated position with their feet out in front of them and on the dorsal side of the body via ultrasound (Philips Lumify, Philips North America, Corp.), by a trained sonographer (J. M. Keeler) with a spleen volume test-retest (within subject) coefficient of variation of $2 \pm 2\%$. Spleen volume was measured using a similar ultrasound imaging technique described by Schagatay et al. (18) and Holmstrom et al. (19). With participants in the seated position, best individual sites for spleen measurement were identified and marked with a permanent marker to allow repeatable measures. All spleen images were captured within 90 s of the measurement time point and 90 s before and after an apnea bout. Each spleen volume measurement consisted of a set of two frozen images, with one image including maximal spleen length (L) and thickness (T) and the other image including maximal width (W). Spleen volume was calculated using the

Pilström formula [$L\pi(WT - T^2)/3$] (19). To minimize probe placement errors and pulsatile changes in spleen volume, a minimum of two sets of images were used to calculate the mean spleen volume at each time point. Spleen images were saved and then read on DICOM software (Philips DICOM Viewer R3.0 L1 SP15, Philips North America, Corp.) on a later date. Spleen volume measurements were completed by covering the transducer with a thin clear waterproof plastic bag, with ultrasound gel placed between the bag and the transducer head to accommodate measurement in the water. For HOWI measurements, the sonographer then submersed the covered transducer in the water to obtain triaxial measurements of the spleen at the premarked locations.

During *visit 1*, hemoglobin mass (Hb_{mass}) was measured in the supine position using the 2-min carbon monoxide rebreathing technique described by Gore et al. (20). Participants laid supine for 15 min before commencing the rebreathing procedures, which involved participants breathing on a closed rebreathing device that contained 1 mL of carbon monoxide per kilogram of body weight, 5 L of 100% oxygen, and a CO₂ scrubber (soda lime) for 2 min. Finger-prick blood samples were collected before, at 6 min post, and at 8 min post carbon monoxide rebreathing. Following manufacturer's guidelines, we analyzed capillary blood samples for hemoglobin and carboxyhemoglobin (OSM3 Hemoximeter, Radiometer, Denmark).

During *visit 2*, an estimated change in Hb_{mass} from pre- to postapneas was derived using Hb_{mass} measured during *visit 1* and the hemoglobin concentrations measured during *visit 2*. Specifically, Hb_{mass} measured on *visit 1* was assumed to be constant, and preapnea hemoglobin concentration was used to estimate blood volume at each time point. This calculation assumes that Hb_{mass} is stable over the two testing days (21) and is stable across the menstrual cycle (22), allowing for the testing of women across the menstrual cycle, and does not change during HOWI, which allows for the quantification of changes in blood volume due to HOWI (13). The change in Hb_{mass} postapnea was estimated from the change in hemoglobin concentration from pre- to postapneas, assuming no appreciable change in blood volume from pre- to postapneas, except the increase in circulating red blood cells and, ultimately, Hb_{mass} due to spleen contraction. This assumption is predicated on the supposition that splenic hematocrit is ~97% (2, 23). These measures were only reported for 11 participants due to blood gas analyzer malfunction in one subject.

Visit 2: Experimental Trial Overview

Participants reported to a temperature-controlled laboratory (22 ± 1°C) for the experimental visit. Participants were instructed to refrain from performing strenuous exercise and consuming caffeine and alcohol for 12 h and to refrain from consuming food for 2 h before the experimental visit. Upon arrival to the laboratory, a small sample of urine was collected from all participants to ensure participants were well hydrated (i.e., urine specific gravity was ≤1.025) and to confirm a negative pregnancy test for female participants. Then, baseline nude body weight and oral temperature were measured. After the pretrial baseline measures, participants were instrumented for heart rate, blood pressure, and SpO₂.

Timeline of Visit 2

After 20 min of seated rest, participants had pre-HOWI spleen and hematological measures taken before commencing the first bout of apneas. Apnea bouts consisted of five maximal voluntary apneas, with a 2-min rest period between each successive apnea (Fig. 1). Apnea times were averaged over each bout to quantify average apneic duration for each bout. Before each apnea, subjects completed a short (<1 min) voluntary hyperventilation to decrease arterial carbon dioxide tension and thereby extend the apnea. Participants were instructed to hold their breath after a deep but not maximal inspiration. These methods were modified from Engan et al. (10). Before the first apnea and within 60 s after completion of the fifth apnea of each bout, spleen volume measures and finger-prick blood samples were collected. Participants completed a total of four bouts of five apneas over the course of the experimental trial.

After completing the first bout of apneas, participants continued sitting exposed to air for a 20-min washout period, which has been shown to be sufficient for spleen volume, hematocrit, and hemoglobin to return to preapnea values (18, 24). Spleen volume and hematological variables were measured again at the end of this washout period before participants were immersed in temperate (35.87 ± 0.72°C) water up to the xiphoid process and with their arms out of the water. Temperate temperature water was used to isolate the effect of HOWI, independent of changes in internal body temperature that often occur with immersion in warmer or cooler water. Spleen volume was measured 10, 20, and 30 min after the initiation of HOWI, along with finger-prick blood samples at 10 min and 30 min. After the 30-min measurement period, participants completed a bout of five maximal apneas, followed by spleen volume and finger-prick blood samples. Participants then continued temperate HOWI until total immersion time was 2.5 h before completing another bout of five maximal apneas with spleen volume measures and finger-prick blood samples being collected. Immersion-induced diuresis was measured by having all participants void their bladder at 60 and 135 min of HOWI via a urinal jug or portable toilet chair while being outside the water. Participants were asked not to void while immersed. These time points were chosen because previous work has shown that urine output is increased above baseline for the first 2 h of immersion but returns to preimmersion outputs at 180 and 240 min of immersion (25, 26). The total HOWI duration was 3 h, after which the participant towel dried and returned to a seated position in 22.6 ± 0.9°C air. Participants then sat for 20 min to allow post-HOWI fluid shifts to be completed. Following which, a 20-min air post-HOWI measure of spleen volume and a finger-prick blood sample were collected. Participants then completed a final bout of five maximal apneas, followed by collection of spleen volume measures and finger-prick blood samples. After which, a final nude body weight was obtained, and participants were cleared to leave the laboratory.

Data and Statistical Analysis

Before analyses, all data were checked for outliers and the necessary assumptions for the repeated-measures ANOVA and paired samples *t* test, and no corrections were necessary.

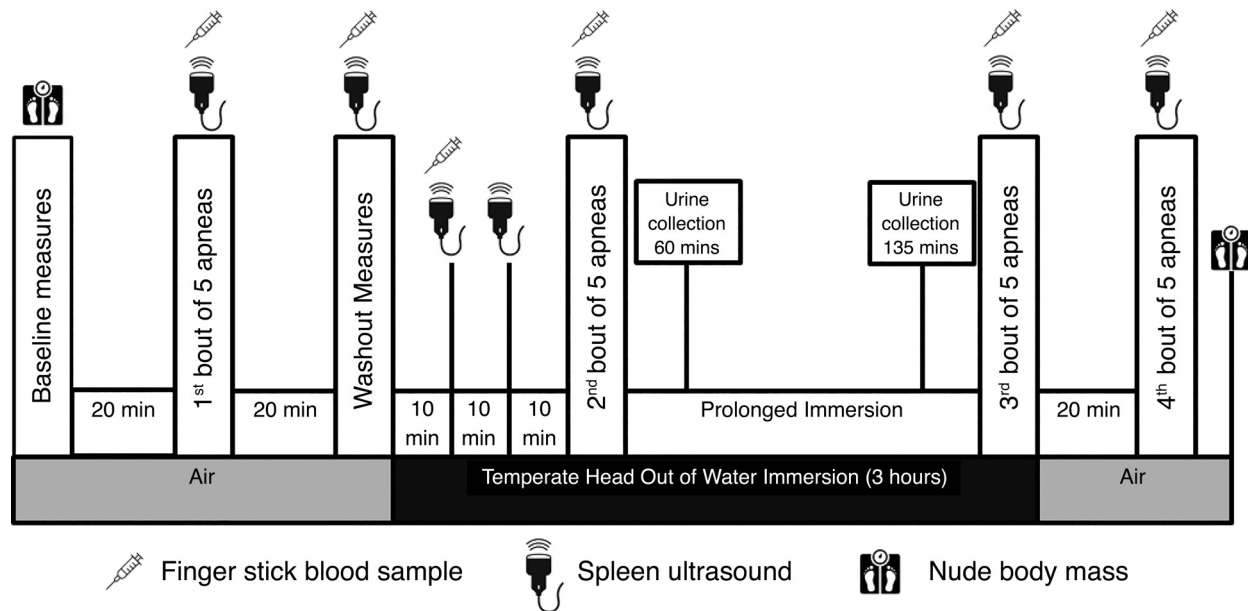


Figure 1. Visit 2: experimental day timeline. After baseline measures of nude body weight, participants were seated for 20 mins before the first bout of apneas. Apnea bouts consisted of five maximal apneas, with a 2-min rest period between successive apneas. Before the first apnea and after the fifth apnea of each bout, spleen volume measures and finger-prick blood samples were collected. At the completion of the first bout, participants continued sitting in air for 20-min washout period, which was confirmed with spleen volume measures. Participants were then moved into temperate water, with measures completed at 10, 20, and 30 min during immersion and before the second bout of apneas. Participants remained in the water until the third bout of apneas occurred at 150 min into immersion. After the third apnea bout, subjects were removed from the water, and they sat for 20 mins before the fourth apnea bout occurred. At the end of the fourth bout of apneas, participants completed a final nude body weight and were cleared to leave.

Consistent with the testing of our primary hypothesis, spleen volume, hematocrit, hemoglobin concentration, total blood volume, plasma volume, and red cell volume before, during, and after HOWI were analyzed using a one-way repeated-measures ANOVA (time: pre-HOWI, 10, 20, 30, and 150 min during HOWI; and post-HOWI), and when a significant effect was identified, post hoc analysis using Dunnett's multiple-comparisons test compared all conditions with pre-HOWI. Consistent with the testing of our secondary hypothesis, pre- and postapnea data were analyzed using a two-way repeated-measures ANOVA (condition: pre- or postapneas, time: pre-HOWI, 30 min of HOWI, 150 min of HOWI, or post-HOWI, and the interaction between time $\times$ condition). If a significant main effect or interaction was observed, post hoc tests using Sidak's method for multiple corrections were conducted. For the secondary hypothesis, data are also reported as a change from preapneas. These data were analyzed using a one-way repeated-measures ANOVA (condition: pre-HOWI, 30 and 150 min during HOWI, and post-HOWI). If a significant effect was identified, post hoc analysis using Dunnett's multiple-comparisons test compared all conditions with baseline. Mean apneic area under the curve (AUC) analyses were used to estimate cumulative hypoxemia (i.e., a function of both the absolute drop in Sp_{O_2} and apnea duration), which were averaged over the five apneas during each bout of apneas. Linear mixed models were used for incomplete datasets. Specifically, two individual hematocrit samples in one participant were excluded from analysis, as measurement error was noted by the study team poststudy completion. The use of linear mixed models is noted in the RESULTS section where appropriate. Notably, linear mixed

model assumptions were checked in all instances, and no corrections were necessary. A two-tailed paired sample t test was used to examine whether spleen volume, hematocrit, and hemoglobin concentration returned to baseline values during the washout period (i.e., before HOWI). The relation between mean apneic time and the reduction of spleen volume from pre- to postapneas at each time point was assessed using Pearson's product correlations. All data were analyzed using Prism software (version 9.1.2, GraphPad Software Inc, La Jolla, CA). A priori statistical significance was set at $P \leq 0.05$, and actual P values are reported where possible. Data are reported as means $\pm$ standard deviation and means with individual values. Pairwise comparisons are also reported as mean difference (diff.) and 95% confidence interval (CI).

RESULTS

Response to Head-Out-of-Water Immersion

Participants completed air and temperate HOWI exposure with no change in oral temperature at any time point ($P = 0.58$, Table 1). Post-HOWI body mass (88.1 ± 11.8 kg, $P < 0.01$, mean diff. = -1.1 , 95% CI = -1.4 to -0.8) was lower than baseline (89.2 ± 11.8 kg). Total urine output during trial was 816 ± 413 mL.

Spleen volume data at each time point are presented in Fig. 2. Pre-HOWI (276 ± 88 mL) and washout (278 ± 90 mL) spleen volumes did not differ ($P = 0.24$). Spleen volume increased during HOWI compared with pre-HOWI values ($P < 0.01$, Fig. 2, A and B). Spleen volume returned to pre-HOWI volume during post-HOWI ($P = 0.57$, Fig. 2A).

Table 1. Cardiovascular, oral temperature, and hematological responses compared with pre-HOWI during head-out-of-temperate water immersion

	Pre-HOWI	10 Min HOWI	30 Min HOWI	150 Min HOWI	Post-HOWI	P Values
Heart rate, beats/min	72 ± 11	62 ± 10*	63 ± 10*	68 ± 9	66 ± 17	0.03
Δ		10 (5, 15)	9 (2, 15)	4 (−4, 12)	6 (−8, 18)	
Oral temperature, °C	36.3 ± 0.3		36.4 ± 0.3	36.5 ± 0.3	36.4 ± 0.6	0.58
Δ			0 (−0.4, 0.3)	−0.2 (−0.5, 0.1)	−0.1 (−0.5, 0.4)	
Systolic blood pressure, mmHg	123 ± 12	110 ± 10*	105 ± 14*	107 ± 9*	115 ± 9	<0.01
Δ		13 (4, 21)	18 (9, 28)	15 (2, 29)	8 (−5, 20)	
Diastolic blood pressure, mmHg	77 ± 7	62 ± 8*	63 ± 10*	62 ± 7*	71 ± 12	<0.01
Δ		14 (8, 20)	14 (4, 23)	15 (9, 21)	6 (−5, 17)	
%Hematocrit‡	47 ± 3	46 ± 4	45 ± 4*	46 ± 4	48 ± 4*	<0.01
Δ		−1 (−2, 1)	−1 (−2, 0)	−1 (−1, 0)	2 (0, 3)	
Hemoglobin, mg·dL ^{−1}	15.1 ± 1.1	14.8 ± 1.1*	14.8 ± 1.3*	14.7 ± 1.1*	15.3 ± 0.9	<0.01
Δ		−0.4 (−0.6, −0.1)	−0.3 (−0.7, 0)	−0.5 (−0.8, −0.2)	0.1 (−0.1, 0.3)	
Total blood volume (n = 11), mL	6,491 ± 1,646	6,687 ± 1,722*	6,657 ± 1,614*	6,716 ± 1,744*	6,453 ± 1,730	<0.01
Δ		197 (72, 322)	167 (17, 317)	226 (71, 380)	38 (−145, 69)	
Plasma volume (n = 11), mL	3,408 ± 667	3,639 ± 932	3,648 ± 841*	3,584 ± 894	3,326 ± 968	<0.02
Δ		231 (−97, 560)	241 (−3, 484)	176 (−123, 475)	−82 (−431, 267)	
Red cell volume (n = 11), mL	3,083 ± 1,030	3,048 ± 854	3,009 ± 846	3,132 ± 933	3,127 ± 863	0.11
Δ		−34 (−312, 243)	−74 (−335, 187)	49 (−179, 278)	44 (−253, 340)	

Values are means ± SD; n = 12 participants. P values presented from F tests for the main effect from repeated-measures ANOVA. Dunnett's post hoc analyses were used for comparisons vs. pre-HOWI. Δ, change from pre-HOWI at each time point, presented as mean difference, 95% confidence interval (lower, upper); HOWI, head-out-of-water immersion. *Different from pre-HOWI ($P \leq 0.05$). ‡Linear mixed model used for analysis.

Hematological data at each time point are presented in Table 1. Hemoglobin concentration remained slightly above pre-HOWI (15.1 ± 1.1 mg·dL^{−1}) levels at washout (15.3 ± 1.0 mg·dL^{−1}, mean diff. = 0.2, 95% CI = 0.01–0.38, $P = 0.04$). Hemoglobin concentration decreased from pre-HOWI during 10 min of HOWI (mean diff. = −0.4, 95% CI = −0.65 to −0.12, $P < 0.01$), at 30 min of HOWI (mean diff. = −0.4, 95% CI = 0.01 to −0.67, $P = 0.05$), and at 150 min of HOWI (mean diff. = −0.5, 95% CI = −0.76 to −0.18, $P < 0.01$) but did not differ from pre-HOWI at post-HOWI (mean diff. = 0.1, 95% CI = −0.08 to 0.34, $P = 0.29$). Hematocrit remained at pre-HOWI ($47 \pm 3\%$) levels at washout ($47 \pm 3\%$, mean diff. = 0.2, 95% CI = −0.7 to 1.0, $P = 0.64$). Hematocrit did not differ from pre-HOWI ($P \geq 0.14$, Table 1) at any time point, except an increase was identified at post-HOWI (mean diff. = 1.7, 95% CI = 0.3–3.1, $P =$

0.05). Blood volume increased from pre-HOWI at 10 min of HOWI (mean diff. = 197 mL, 95% CI = 72–322, $P < 0.01$), 30 min of HOWI (mean diff. = 167 mL, 95% CI = 17–317, $P = 0.03$), and 150 min of HOWI (mean diff. = 226 mL, 95% CI = 71–380, $P < 0.01$) but was not different from pre-HOWI at post-HOWI (mean diff. = 38 mL, 95% CI = −145 to 69, $P = 0.70$). Elevations in plasma volume were observed at 30 min of HOWI (mean diff. = 241 mL, 95% CI = 3–484, $P = 0.05$) compared with pre-HOWI but did not differ from pre-HOWI at any other time point ($P > 0.19$). Red cell volume ($P \geq 0.80$) did not differ from pre-HOWI at any time point.

Response to Head-Out-of-Water Immersion and Apneas

Participants successfully completed each bout of apneas, with HOWI apneas producing longer apneic durations than at

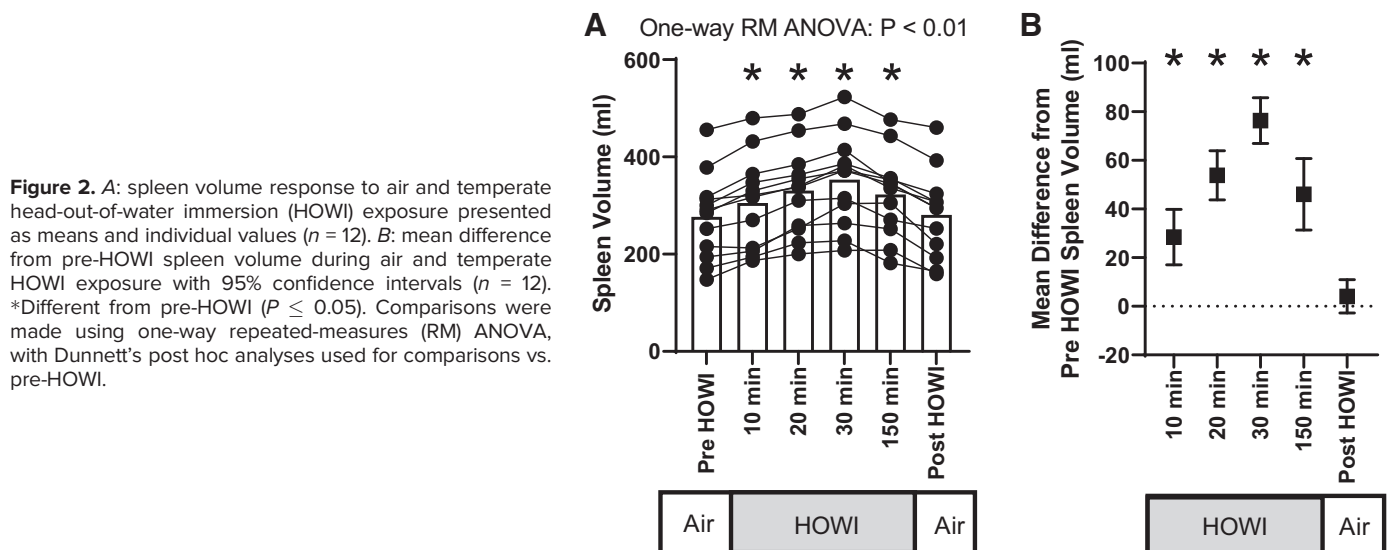


Table 2. Hematological responses pre- and postapneas compared with pre-HOWI during head-out-of-temperate water immersion

									P Values		
									Time	Condition	Time × Condition
	Pre-HOWI		30 Min HOWI		150 Min HOWI		Post-HOWI				
Mean apnea time, s	103 ± 30		116 ± 31*		123 ± 36*		112 ± 34		<0.01		
Δ to pre-HOWI (95% CI)	13 (3, 23)		20 (9, 30)		8 (−3, 20)						
SpO ₂ nadir, %	88 ± 5		87 ± 6		85 ± 8*		85 ± 7		0.01		
Δ to pre-HOWI (95% CI)	−1 (−4, 1)		−3 (−6, −1)		−3 (−7, −1)						
Mean apneic AUC, s-%	666 ± 381		845 ± 476*		1,026 ± 679*		907 ± 548		0.01		
Δ to pre-HOWI (95% CI)	179 (−5, 363)		360 (80, 640)		241 (−49, 531)						
Apnea time point	Pre	Post	Pre	Post	Pre	Post	Pre	Post			
%Hematocrit†	47 ± 4	48 ± 4†	45 ± 4	46 ± 4†	46 ± 4	47 ± 4†	48 ± 4	48 ± 4	<0.01	<0.01	0.52
Δ		1 (0, 2)		1 (0, 2)		1 (0, 2)		0 (−1, 2)		0.60	
Hemoglobin, mg·dL ^{−1}	15.1 ± 1.1	15.4 ± 1.1†	14.8 ± 1.3	15.0 ± 1.1	14.7 ± 1.1	15.0 ± 1.1†	15.3 ± 0.9	15.4 ± 1.0	<0.01	<0.01	0.63
Δ		0.3 (0.0, 0.6)		0.2 (−0.1, 0.5)		0.3 (0.0, 0.6)		0.2 (−0.1, 0.5)		0.58	
Δ Hb _{mass} (n = 11), g		20 ± 18		20 ± 52		34 ± 34		17 ± 37		0.51	

Values are means ± SD, $n = 12$ participants. P values presented from F tests for the main effects and interactions from repeated-measures ANOVA are reported. Dunnett's post hoc analyses were used for pre-HOWI comparisons, and Sidak's post hoc analyses were used for pre- and postapnea comparisons. Δ, mean difference from pre- to postapneas at each time point, with 95% confidence interval (CI; upper, lower); HOWI, head-out-of-water immersion. *Different from pre-HOWI ($P \leq 0.05$). †Different from preapneas ($P \leq 0.05$). ‡Linear mixed model used for analysis.

pre-HOWI ($P \leq 0.01$, Table 2). Apneic SpO₂ nadir was lower at 150 min of HOWI (mean diff. = 3%, 95% CI = 1–6, $P = 0.02$, Table 2) compared with pre-HOWI and did not differ from pre-HOWI at any other time point ($P \geq 0.09$). Mean apneic AUC was greater during both HOWI time points (30 min of HOWI: mean diff. = 179%·min, 95% CI = −5 to 363, $P = 0.05$; 150 min of HOWI: mean diff. = 360%·min, 95% CI = 80–640, $P = 0.05$) compared with pre-HOWI ($P \leq 0.02$, Table 2).

Spleen volume pre- to postapnea at each time point is presented in Fig. 3A. Spleen volume decreased from pre- to postapnea bouts at each time point ($P < 0.01$, Fig. 3A). Although spleen volumes decreased postapneas, spleen volume was larger during HOWI trials at both pre- and postapnea time points ($P < 0.01$). The magnitude of the reduction in spleen volume from pre- to postapneas was elevated at 30 min of HOWI compared with pre-HOWI (mean diff. = 17 mL, 95% CI = 0.4–33.1, $P = 0.04$, Fig. 3D) but did not differ at any other time point ($P \geq 0.86$). The reduction in spleen volume from pre- to postapneas was not correlated with mean apneic time over a five-apnea bout at any time point (pre-HOWI: $r = 0.01$, $P = 0.98$; 30 min of HOWI: $r = -0.08$, $P = 0.81$; 150 min of HOWI: $r = 0.03$, $P = 0.93$; post-HOWI: $r = 0.29$, $P = 0.36$).

Hematological data at each time point are presented in Table 2. During 30 and 180 min of HOWI, hemoglobin concentrations were decreased compared with pre-HOWI and postimmersion apnea bouts ($P < 0.02$, Table 2). Increases in hemoglobin concentration postapneas were evident at pre-HOWI (mean diff. = 0.3 g·dL^{−1}, 95% CI = 0.04–0.60, $P = 0.02$) and at 150 min of HOWI (mean diff. = 0.3 g·dL^{−1}, 95% CI = 0.04–0.60, $P = 0.02$). However, this did not translate to differences in the increase in hemoglobin mass pre- to postapneas at any time point ($P = 0.51$). There was an increase in hematocrit from pre- to postapneas at all time points ($P < 0.05$, Table 2) except post-HOWI ($P = 0.83$, Table 2). The changes in hematocrit from pre- to postapneas were not different at any time point compared with pre-HOWI ($P \geq 0.60$).

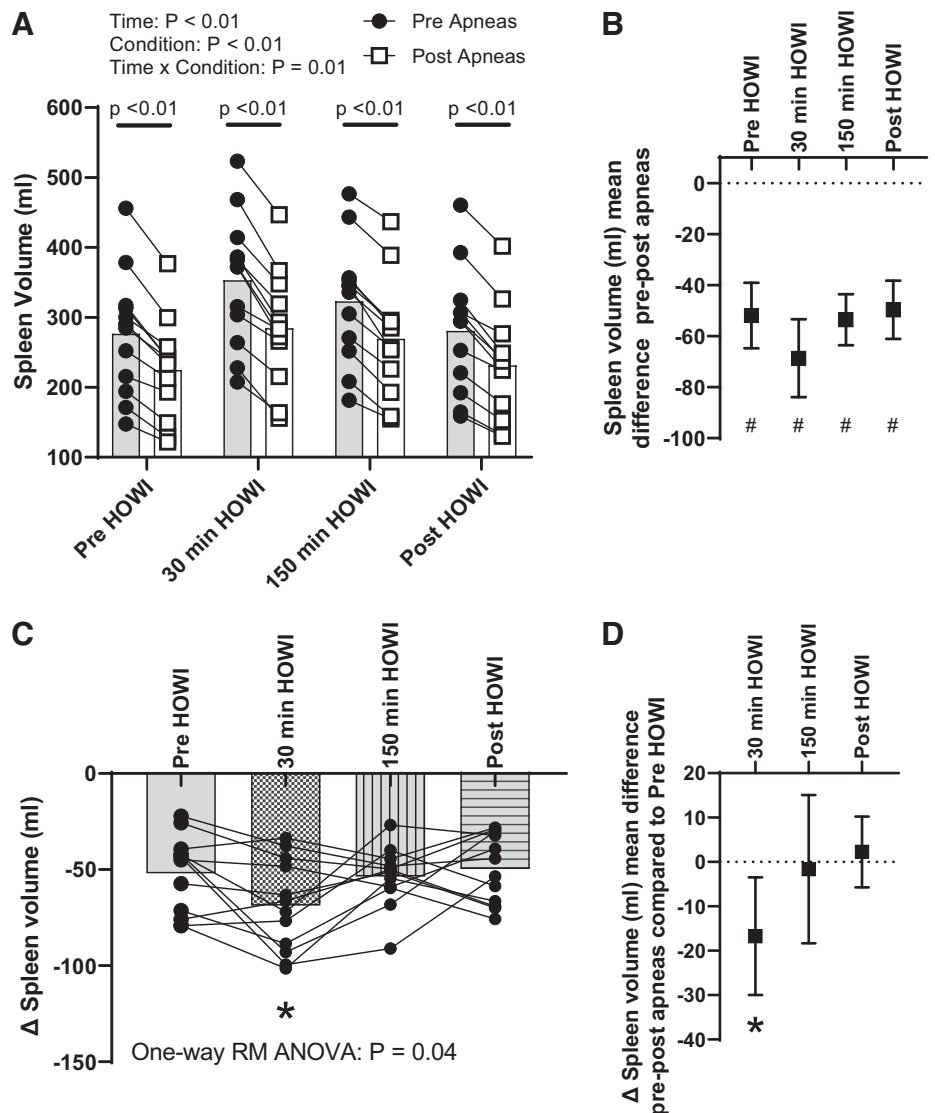
DISCUSSION

In partial support of our primary hypothesis, temperate HOWI acutely elevated spleen volume but did not return to pre-HOWI levels after more prolonged HOWI and subsequent diuresis. Specifically, spleen volume increased from pre-HOWI within the first 30 min and remained elevated through 150 min (Fig. 2). Moreover, and in partial support of our secondary hypothesis, spleen contraction induced by apneas was greater at 30 min of HOWI but returned to pre-HOWI levels at 150 min of HOWI and at post-HOWI (Fig. 3, A and B). Collectively, our findings indicate that during temperate HOWI, spleen volume is increased throughout, and spleen contraction induced by apneas is elevated with acute HOWI (up to 30 min) but not more prolonged HOWI.

Spleen Volume Changes Provoked by Temperate HOWI

In a study by Hajduczk et al. (14), conscious dogs underwent temperate HOWI, and it was found that visceral organ hyperperfusion was region, time, and cardiac output dependent. Specifically, acute immersion caused the translocation of blood to relocate to visceral organs at a rate proportional to the changes in cardiac output, except blood flow to the liver and spleen, which was disproportionately elevated (14). Comparatively, for the first time in humans, to the authors' knowledge, our study shows a similar response for spleen volume in that it increased with acute HOWI (Fig. 2). Unfortunately, we did not measure splenic arterial or venous blood flow. However, it is likely that this increase in spleen volume is the result of the translocation of blood to the thorax that caused an acute increase in splenic blood flow [as observed in dogs (14)] without a corresponding increase in venous outflow. This acute imbalance between splenic arterial inflow and venous outflow, when combined with a reduction in splenic smooth muscle tone, likely resulted in the observed acute elevation in spleen volume. We speculate that the mechanisms underlying these observations are largely mediated by the sympathetic nervous

Figure 3. Spleen contraction after apneas before, during, and after head-out-of-water immersion (HOWI). Data presented as means with individual responses ($n = 12$). **A:** spleen volume pre- and postapneas at each time point. P values presented from F tests for main effects and interaction from two-way repeated-measures (RM) ANOVA, and Sidak's post hoc analyses were used for multiple comparisons. **B:** mean difference in spleen volume from pre- to postapneas with 95% confidence intervals at each time point. #Different from preapnea ($P < 0.01$). **C:** change (Δ) in spleen volume from pre- to postapneas at each time point. *Different from pre-HOWI ($P \leq 0.05$). P values presented from F tests for the main effects from repeated-measures ANOVA. Dunnett's post hoc analyses were used for pre-HOWI comparisons.



system. For example, the spleen is directly innervated with sympathetic nerves, and activation of the sympathetic nervous system stimulates spleen contraction (2, 27, 28). Temperate HOWI decreases sympathetic activity, as demonstrated acutely by reductions in muscle sympathetic nerve activity (29, 30) and the suppression of circulating norepinephrine with more prolonged HOWI (31). These reductions in sympathetic activity are mostly caused by the loading of the baroreceptors occurring secondary to HOWI-induced central hypervolemia, the latter of which is caused by hydrostatic pressure-induced transcapillary fluid shifts from the intracellular to the extravascular compartment and decreases in venous capacity (13). Thus, the increase in spleen volume with acute HOWI is likely mediated by the withdrawal of sympathetic tone and the subsequent relaxation of splenic arteriolar and organ smooth muscle tone in the absence of corresponding increases in splenic venous outflow. This mechanism is at least partially supported by findings that increases in portal vein pressure, which likely occur with HOWI, stimulate increases in splenic blood flow and spleen volume that are mediated largely by the

sympathetic nervous system (32). That said, direct evidence of this phenomenon is required. It is also possible that the increase in spleen volume with HOWI may be the result (or cause) of splenic uptake of red blood cells. This is potentially supported by the observation that hemoglobin concentration was reduced throughout HOWI (Table 1). However, we think this is unlikely because the increase in hemoglobin concentration can be explained by HOWI-induced plasma volume expansion (Table 2) and subsequent hemodilution (to within $\sim 0.1 \text{ g} \cdot \text{dL}^{-1}$).

In contrast to our hypothesis, spleen volume did not return to pre-HOWI levels with prolonged HOWI, despite diuresis-induced restoration of plasma volume (Table 2), and presumably the attenuation of the magnitude of HOWI-induced central hypervolemia. Our findings might be interpreted to be in contrast with the data of Hajduczuk et al. (14) that indicated that spleen blood flow with prolonged HOWI was restored to pre-HOWI levels in dogs. However, organ volume is a function of arterial blood flow, venous outflow, and organ smooth muscle tone. Thus, it is possible that the maintenance of an elevated spleen

volume even after prolonged HOWI represents that any initial imbalance between splenic arterial blood flow and venous outflow and splenic smooth muscle tone reached a new steady state, such that spleen volume remained enlarged despite the restoration of spleen arterial blood flow. The mechanisms underlying this observation are unknown, but, again, we speculate that the sympathetic nervous system is involved. Specifically, after HOWI-induced diuresis and the reestablishment of a euvolemic state, it is likely that sympathetic activity is raised toward pre-HOWI levels, thereby establishing a new steady state for spleen blood flow but at a larger spleen volume. Indeed, a primary role of the sympathetic nervous system is indirectly supported by the findings that post-HOWI spleen volume returned to pre-HOWI levels (Fig. 2). For instance, upon leaving the water, central hypovolemia ensues (33), which is known to increase sympathetic activity as measured via muscle sympathetic nerve activity (30, 34). Thus, the activation of the sympathetic nervous system likely resulted in the restoration of spleen volume back to pre-HOWI levels by directly acting on splenic smooth muscle and/or by reducing arterial inflow without a concomitant acute reduction in venous outflow. Unfortunately, the role of the sympathetic nervous system as a modulator of HOWI-induced changes in spleen volume remains speculative. Thus, future studies should examine potential mechanisms by which spleen volume increases and is sustained with HOWI.

Apneas Induced Spleen Contraction during Head-Out-of-Water Immersion

Spleen contraction and subsequent increases in hemoglobin concentration and hematocrit have been demonstrated following a variety of sympathetic stimuli [apnea (10, 18), hypoxia (35, 36), and exercise (28, 37)]. Recently, splenic width has been demonstrated to decrease postapnea during HOWI with facial immersion (12). However, whether HOWI modifies the extent of spleen contraction volume was unknown until now. At each time point, spleen volume decreased from pre- to postapneas (Fig. 3A). These observations support previous suppositions that spleen contraction occurs during breath-hold diving (5, 6, 10, 18). A novel finding was that the magnitude of spleen contraction was increased at 30 min of HOWI (Fig. 3B) when spleen volume was increased above pre-HOWI levels (Fig. 2). However, despite preapnea spleen volume remaining elevated above baseline at 150 min of HOWI, apnea-induced spleen contraction fell back to pre-HOWI levels at this time point (Fig. 3B). The mechanisms for these temporal differences in apnea-induced spleen contraction are yet to be elucidated. However, we speculate that the increase in sympathetic activity caused by apneas was greater at 30 min of HOWI. This is supported by the observation that average apnea duration and mean apneic AUC were greater in HOWI than at pre-HOWI (Table 2). An argument against this, however, is that the Sp_{O_2} nadir postapneas did not differ from pre-HOWI until 150 min of HOWI, a time point in which the magnitude of splenic contraction did not differ from pre-HOWI. Thus, the most probable mechanism underlying the observation that splenic contraction was greater early in HOWI is centered on observations indicating

that apnea termination consistently occurs at the same absolute level of muscle sympathetic nerve activity, independent of apnea duration (38). As previously discussed, sympathetic activity was likely acutely reduced early in HOWI. Therefore, we speculate that the absolute, apnea-induced increase in sympathetic nerve activity was greater at 30 min of HOWI, which was translated to a greater magnitude of splenic contraction early in HOWI compared with both pre-HOWI and 150 min of HOWI. That said, formal evidence of such an arrangement is warranted.

Previous studies have shown that larger spleen volumes and contractions might improve dive performance in competitive athletes (6, 11) and native diving populations (1) by theoretically improving oxygen-carrying capacity and pH/ CO_2 buffering. During this investigation, hematological data showed very minor changes from pre- to postapneas (Table 2), which are similar to previous investigations. For example, hemoglobin concentration was increased postapneas by 0.2–0.3 mg·dL⁻¹ or 1–3%, which is in line with the findings of Schagatay et al. (11), Richardson et al. (39), Hurford et al. (40), and Bakovic et al. (41) in non-breath-hold diving populations. One intriguing finding was that hemoglobin concentration was unchanged at 30 min of HOWI from pre- to postapneas. We speculate that this was likely caused by a lowering of the hematocrit of the blood housed in the spleen, perhaps due to elevations in plasma volume at this time point. Therefore, increases in hemoglobin concentration from a given spleen contraction were likely smaller. Hematocrit slightly increased postapneas for all time points except during post-HOWI, which also aligns with previous studies in non-breath-hold diving population (11, 40). The lack of post-HOWI increases after apneas could be due to the hemoconcentration after prolonged HOWI, as the participants started with a higher hematocrit before apneas. A novel aspect of the present study was providing an estimate of apnea-induced changes in Hb_{mass} , which may provide some insights into the functional aspects of apnea-induced spleen contraction. Indeed, changes in estimated Hb_{mass} were not different from pre-HOWI at any time point, and although the measured change was positive, the small increases in Hb_{mass} only reflect a minor increase in oxygen-carrying capacity (~23–46 mL of O_2). Thus, whether the increases in Hb_{mass} postapneas represent a functional change in Hb_{mass} from the perspective of oxygen-carrying capacity to the whole body remains uncertain.

Experimental Considerations

There are a few experimental considerations to examine for the experimental design. The primary outcomes of this study were collected during one experimental visit. This was by design to reduce participant burden and was based on knowing that spleen volume and associated hematological responses return to baseline within 8–9 min after serial apneas (10, 18, 24). A return to baseline spleen volume after the first bout of apneas was confirmed during the washout spleen volume measurement period, which suggested that the effect of apneas on initial spleen volume during HOWI measures was minimal. Another consideration is the learning effect of conducting apneas over time, which could play a role in spleen contraction, as decreases in splenic width have been correlated with reductions in Sp_{O_2} and longer

apneic times (12). To better investigate this possibility, we conducted a post hoc linear mixed model analysis with repeated factors of apnea bout (pre-HOWI, 30 min HOWI, 150 min HOWI, and post-HOWI) and apnea number (one through five). These findings indicated that there was a within-apnea bout effect, supporting that the first apnea within each bout was consistently shorter than the other apneas. This analysis also confirmed the analyses reported in Table 2 such that apnea duration during HOWI was consistently higher than pre-HOWI and that apnea duration returned to pre-HOWI levels at post-HOWI. Thus, although there was a within-apnea bout effect at all time points, given that apnea duration did not differ between pre- and post-HOWI, it is unlikely that there was a meaningful learning effect between bouts of apneas. Therefore, we believe that a time control component to this investigation was not necessary and that a one-experimental-visit study design was acceptable.

Other considerations include the use of ultrasound, HOWI with apneas completed with the participant's head out of water, finger capillary samples for hematological variables, and our chosen participant population. First, the use of Doppler ultrasound over another form of imaging decreased subject burden while also allowing accurate measures of the spleen dimension that are comparable with those of computerized tomography (42), which is not possible during immersion. Numerous studies have used ultrasound-derived spleen volume measurements (1, 5, 10, 18). To be consistent with the breath-hold diving literature, we used the Pilström formula to estimate spleen volume (10, 18, 43). Therefore, we believe that ultrasound was a valid instrument to assess spleen volume in this study. Second, this study used temperate HOWI to test our hypotheses, and the breath holds were completed with the participant's head out of water. This was chosen to reduce the likelihood of anxiety stimulating the sympathetic nervous system, which is particularly notable given our use of non-breath-hold divers in this study. That said, stimulation of facial nerves by immersion enhances the cardiovascular response to the dive reflex in humans, but the effect of facial immersion during apneas on hematological and splenic contraction response is mixed (44, 45). Therefore, future studies are needed to better understand spleen volume changes during HOWI with facial immersion. Notably, Nordine et al. (12) recently reported that splenic width decreased during apneas conducted while the face was immersed in water under both dry and whole body water immersion conditions. Third, the use of finger capillary blood samples for our hematological measurements could have influenced our results due to vasoconstriction that accompanies apneas and the dive reflex (3). However, previous investigations of splenic contraction used finger capillary blood samples; therefore, finger capillary blood samples were used to be consistent with the literature (43, 46). That said, future studies should consider using venous or arterial samples to understand the possible vasoconstriction effects to the hematological values. Finally, our subjects were young healthy active individuals who were not breath-hold diving trained or currently competitive athletes. Therefore, these data may not

reflect how highly trained individuals respond to HOWI. Schagatay and colleagues (6, 46) have shown that elite breath-hold divers and endurance athletes have larger spleens with a greater ability to contract. Speculatively, highly trained individuals with larger spleens may have an attenuated response to HOWI due to being nearer to a maximum spleen volume, if a maximal organ volume exists. This also leads to the question of sex differences, as women typically have smaller spleens than men (47, 48). This investigation was not designed to look at sex differences and instead focused on changes over time within men and women. Future investigations of sex differences in spleen response to HOWI are necessary to understand if sex or the menstrual cycle plays a role in spleen volume changes.

Perspectives and Significance

A large spleen may be beneficial in the context of operating in the aquatic environment (1, 6). It is believed that this is because the jettison of red blood cells into the circulation is beneficial for diving operations. This study demonstrates that spleen volume increases at the start of temperate HOWI and remains elevated throughout 180 min of HOWI. This study also demonstrates that the spleen will contract during temperate HOWI in response to apneas, with a greater spleen contraction volume at 30 min of HOWI. This objective evidence supports previous research claiming that the spleen contracts during breath-hold diving (1, 6, 11). That said, the estimated increase in Hb_{mass} after apneas was small and not affected by HOWI. Future studies are required to examine whether spleen size causally impacts health, safety, and/or performance when operating in the marine environment (e.g., breath-hold diving, scuba diving).

Conclusions

Spleen volume is increased throughout 180 min of temperate head-out-of-water immersion. Apnea-induced spleen contraction is augmented after 30 min of head-out-of-water immersion but does not differ thereafter. Moreover, the apnea-induced increase in estimated hemoglobin mass is modest and not modified by head-out-of-water immersion.

ACKNOWLEDGMENTS

We thank the individuals who participated in this study.

GRANTS

This research was supported by an award from the Office of Naval Research (N00014-20-1-2593, to Z. J. Schlader).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

Zachary Schlader and Blair Johnson are editors of *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* and were not involved and did not have access to information regarding the peer-review process or final disposition

of this article. An alternate editor oversaw the peer-review and decision-making process for this article.

AUTHOR CONTRIBUTIONS

J.M.K., H.W.H., J.T.G., R.F.C., B.D.J., and Z.J.S. conceived and designed research; J.M.K., H.W.H., E.T., T.B.B., P.M.K., and J.T.G. performed experiments; J.M.K., T.B.B., P.M.K., and Z.J.S. analyzed data; J.M.K., H.W.H., E.T., J.T.G., R.F.C., B.D.J., and Z.J.S. interpreted results of experiments; J.M.K. prepared figures; J.M.K. and Z.J.S. drafted manuscript; J.M.K., H.W.H., E.T., T.B.B., P.M.K., J.T.G., R.F.C., B.D.J., and Z.J.S. edited and revised manuscript; J.M.K., H.W.H., E.T., T.B.B., P.M.K., J.T.G., R.F.C., B.D.J., and Z.J.S. approved final version of manuscript.

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Peer-reviewed abstract

Keeler JM, Tourula E, Baker TB, Logan R, Hess HW, Kerr PM, Greenshields JT, Chapman RF, Johnson BD, **Schlader ZJ**. Acute elevations in apnea-induced spleen contraction provoked by thermoneutral head out water immersion. Experimental Biology, Philadelphia PA, USA 2022.

Acute Elevations in Apnea-Induced Spleen Contraction Provoked by Thermoneutral Head Out Water Immersion

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INTRODUCTION: The spleen serves as a repository of erythrocytes that can be jettisoned into circulation following sympathetic stimulation, such as hypoxia or apnea. Evidence supports that there may be a functional benefit to spleen contraction during breath-hold diving, likely by increasing oxygen carrying and pH buffering capacities. However, the magnitude of apnea-induced spleen contraction during immersion, and the corresponding hematological changes, have not been quantified. We have recently identified that spleen volume increases after 30 min of thermoneutral head out water immersion (HOWI), likely due to initial hydrostatic pressure-induced increases in central blood volume, and remains elevated through 135 min, despite immersion-induced diuresis. Whether these HOWI-induced changes in spleen volume modify the magnitude of apnea-induced spleen contraction is unknown. Cross-sectional observations indicate that there is a positive relation between baseline spleen volume and the magnitude of apnea-induced spleen contraction.

PURPOSE: Test the hypothesis that the magnitude of apnea-induced spleen contraction increases during thermoneutral HOWI.

METHODS: Spleen volume was measured pre- and post- a set of five apneas in eight healthy adults (3 women; 27 ± 4 y) before, during (at 30 and 135 min) and 20 min following thermoneutral ($36 \pm 1^\circ\text{C}$) HOWI. At each timepoint, spleen length, width, and thickness were measured via ultrasound and spleen volume was calculated using the Pilström equation (test-retest CV: $2 \pm 2\%$). Hemoglobin concentration ([Hb]) and hematocrit were measured pre- and post- each set of apneas via capillary blood samples. Urine was collected at 60 min and 120 min of HOWI. Data are presented as mean $\pm$ SD.

RESULTS: Compared to pre-HOWI (97 ± 32 s), average apnea times were higher at 30 min (115 ± 36 s, $p=0.02$) and 135 min (120 ± 43 s, $p<0.01$) of HOWI, but returned to pre- levels at post-HOWI (103 ± 38 s, $p=0.70$). [Hb] increased from pre- to post- apneas (main effect: $p<0.01$), but the magnitude of the increase did not differ between timepoints (pre-HOWI: $+0.4 \pm 0.3$, 30 min of HOWI: $+0.3 \pm 0.5$, 135 min of HOWI: $+0.3 \pm 0.5$, post-HOWI: $+0.2 \pm 0.2$ g/dL, $p=0.68$). Hematocrit did not change from pre- to post- apneas (main effect: $p=0.72$), and the magnitude of changes from pre- to post- apneas did not differ between timepoints ($p=0.43$). Spleen volume decreased from pre- to post-apneas at each timepoint ($p<0.01$). The reduction in spleen volume was greater than pre-HOWI (-41 ± 18 mL) at 30 min of HOWI (-65 ± 26 mL, $p=0.03$), but did not differ from pre-HOWI at 135 min of HOWI (-50 ± 12 mL, $p=0.44$), despite marked diuresis (urine volume: 425 ± 193 mL), or at post-HOWI (-40 ± 13 mL, $p=0.99$).

CONCLUSION: Apnea-induced spleen contraction was acutely increased at 30 mins of HOWI. However, following immersion-induced diuresis the magnitude of spleen contraction at 130 min of HOWI returned to pre-HOWI levels. Increases in [Hb], which were presumably caused by spleen contraction, were not modified by HOWI, suggesting the hematological response to apnea-induced spleen contraction is not modified by immersion.

Supported by Office of Naval Research award N00014-20-1-2593.

Peer-reviewed abstract

Keeler JM, Tourula E, Baker TB, Logan R, Hess HW, Kerr PM, Greenshields JT, Chapman RF, Johnson BD, **Schlader ZJ**. Spleen volume is increased during thermoneutral head-out water immersion. American College of Sports Medicine Annual Meeting, San Diego, CA, USA, 2022.

Spleen Volume is Increased During Thermoneutral Head-out of Water

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PURPOSE: The spleen serves as a reservoir of red blood cells that can be released into circulation subsequent to sympathetic stimulation, such as hypoxia or apneas. Evidence supports that there may be a functional benefit to spleen contraction during breath-hold diving by increasing oxygen carrying capacity, which involves both a water immersion-induced expansion in blood volume and subsequent contraction during apnea. Splenic blood flow in dogs is transiently increased during head-out water immersion (HOWI) and is mediated by the hydrostatic pressure-induced increase in central blood volume as well as immersion-induced diuresis. However, the effects on spleen volume remain unknown. Therefore, we tested the hypothesis that spleen volume acutely increases with HOWI and returns to baseline levels following diuresis in humans.

METHODS: Spleen volume was measured in eight healthy adults (3 women; 27 ± 4 y) before, during (at 10, 20, 30, and 135 min) and 20 min following thermoneutral ($36 \pm 1^\circ\text{C}$) HOWI. At each timepoint, spleen length, width, and thickness were measured via ultrasound and spleen volume was calculated utilizing the Pilsröm equation (test-retest CV: $2 \pm 2\%$). Hemoglobin concentration and hematocrit were measured in capillary blood samples. Urine was collected at 60 and 120 mins of HOWI. Data are presented as mean $\pm$ SD.

RESULTS: Hemoglobin concentration and hematocrit decreased during HOWI ($p \leq 0.06$) and returned to baseline values post-HOWI ($p \geq 0.09$). During HOWI, spleen volume was increased above baseline (241 ± 75 mL) at all time points ($p < 0.01$), except 10 mins into HOWI (286 ± 108 mL, $p = 0.08$). Greatest spleen volumes were achieved at 20 (314 ± 102 mL) and 30 (325 ± 102 mL) mins of HOWI with no differences between time points ($p = 0.19$). Spleen volume remained elevated above baseline at 135 min of HOWI (295 ± 86 mL, $p < 0.01$), despite marked diuresis (urine volume: 425 ± 193 mL). Post-HOWI, spleen volume returned to baseline values (245 ± 79 mL, $p = 0.42$).

CONCLUSION: Spleen volume increases during HOWI and was sustained independent of immersion-induced diuresis. Spleen volume returned to baseline levels post-HOWI, suggesting that the increase in spleen volume during HOWI is due to sustained hydrostatic pressure and the translocation of blood from the limbs.

Supported by Office of Naval Research award N00014-20-1-2593

Is there a relation between spleen volume and apneas-induced elevations in hemoglobin mass?

Peer-reviewed manuscript published in Undersea and Hyperbaric Medicine Journal.

Keeler JM, Hess HW, Tourula E, Chapman RF, Johnson BD, **Schlader ZJ**. Relation between resting spleen volume and apnea-induced increases in hemoglobin mass. *Undersea and Hyperbaric Medicine Journal* 51: 59-69, 2024.



Relation between resting spleen volume and apnea-induced increases in hemoglobin mass

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ABSTRACT

Keeler JM, Hess HW, Tourula E, Chapman RF, Johnson BD, Schlader ZJ. Relation between resting spleen volume and apnea-induced increases in hemoglobin mass. Undersea Hyperb Med. 2024 First Quarter; 51(1):59-69.

Introduction: Indigenous populations renowned for apneic diving have comparatively large spleen volumes. It has been proposed that a larger spleen translates to heightened apnea-induced splenic contraction and elevations in circulating hemoglobin mass (Hb_{mass}), which, in theory, improves O_2 carrying and/or CO_2 /pH buffering capacities. However, the relation between resting spleen volume and apnea-induced increases in Hb_{mass} is unknown. Therefore, we tested the hypothesis that resting spleen volume is positively related to apnea-induced increases in total Hb_{mass} .

Methods: Fourteen healthy adults (six women; 29 ± 5 years) completed a two-minute carbon monoxide rebreathe procedure to measure pre-apneas Hb_{mass} and blood volume. Spleen length, width, and thickness were measured pre-and post-five maximal apneas via ultrasound. Spleen volume was calculated via the Pilsström equation (test-retest CV: $2 \pm 2\%$). Hemoglobin concentration ($[Hb]$; g/dl) and hematocrit (%) were measured pre- and post-apneas via capillary blood samples. Post-apneas Hb_{mass} was estimated as post-apnea $[Hb] \times$ pre-apnea blood volume. Data are presented as mean $\pm$ SD.

Results: Spleen volume decreased from pre- (247 ± 95 mL) to post- (200 ± 82 mL, $p < 0.01$) apneas. $[Hb]$ (14.6 ± 1.2 vs. 14.9 ± 1.2 g/dL, $p < 0.01$), hematocrit (44 ± 3 vs. $45 \pm 3\%$, $p = 0.04$), and Hb_{mass} (1025 ± 322 vs. 1046 ± 339 g, $p = 0.03$) increased from pre- to post-apneas. Pre-apneas spleen volume was unrelated to post-apneas increases in Hb_{mass} ($r = -0.02$, $p = 0.47$). O_2 ($+28 \pm 31$ mL, $p < 0.01$) and CO_2 ($+31 \pm 35$ mL, $p < 0.01$) carrying capacities increased post-apneas.

Conclusion: Larger spleen volume is not associated with a greater rise in apneas-induced increases in Hb_{mass} in non-apnea-trained healthy adults.

Keywords: apnea; breath-hold; hemoglobin mass; splenic contraction

INTRODUCTION

The spleen is believed to be important in extending apneic duration in humans. This is evidenced by apnea duration increasing during subsequent apneas when a splenic contraction occurs [1]. Indeed, during repeated apneas, splenectomized humans demonstrated smaller increases in apnea duration compared to subjects with spleens [2,3]. From this evi-

dence, it could be inferred that larger resting spleen volumes could enhance apnea durations, which is evidenced in the animal world as longer dive times in seals are correlated to spleen mass [4], indicating that absolute spleen size may be important in apneic diving performance in mammals. Furthermore, this is supported by human data, as better-competitive apneic divers had larger spleens than their

lower-performing counterparts [5]. Moreover, indigenous apneic diving populations renowned for long apneic dives are genetically endowed with larger spleens than nearby non-diving populations [6]. These findings underscore the spleen's role in apneic performance and suggest that resting spleen volume is important to extending apneic duration, which is believed to be facilitated by a greater release of stored red blood cells during an apnea via splenic contraction [1,5].

Splenic contraction, induced by sympathetic activation, releases stored red blood cells into circulation, as often demonstrated by increases in hemoglobin concentration and hematocrit. The release of stored red blood cells via splenic contraction was first observed in humans in the Japanese Ama freedivers [7]. This physiological response was subsequently confirmed when splenectomized patients displayed minimal or no differences in post-apneas hemoglobin concentrations and hematocrit changes following a sympathetic stimulus [2,3]. This physiologic response has been considered a component of the human diving response, including bradycardia and peripheral vasoconstriction [8]. The benefit of splenic contraction during apnea relies on the release of a portion of its approximately 200 to 250 mL of high hematocrit (~97%) blood [9], which may increase circulating hemoglobin concentrations on average by 2% to 4% and up to 10% [7,10,11]. In support of released red blood cells following apnea, our lab found a slight elevation in hemoglobin mass (Hb_{mass}) following apneas in non-apnea-trained healthy adults [1], thus theoretically improving oxygen-carrying capacity and carbon dioxide buffering capacity by increasing circulating hemoglobin mass, which subsequently would improve apneic performance.

The aforementioned observations assume that a larger resting spleen volume is related to the release of more red blood cells, which would increase circulating Hb_{mass} post-apnea. However, to our knowledge, no investigation has examined the relation between resting spleen volume and apnea-induced increases in Hb_{mass} . Therefore, we tested the hypothesis that resting spleen volume is positively related to apnea-induced increases in total Hb_{mass} in non-apnea-trained healthy adults. Secondarily, we quanti-

fied the theoretical maximum change in hemoglobin's oxygen and carbon dioxide carrying capacities based on changes in Hb_{mass} , which may provide insights into the functional benefits of splenic contraction during apnea.

METHODS

Experimental design

This study is a retrospective analysis of pooled data from two studies. Subjects underwent the same procedures, but the timing of the carbon monoxide (CO) rebreathing procedure differed between the two studies. One study completed the CO rebreath a day or more (6 ± 4 days) before an apnea experimental visit ($n=11$). The data from the first study ($n=11$) have been published previously, testing a unique hypothesis [1]. The other study completed the CO rebreath ~45 minutes before the set of apneas on the same day ($n=3$). Additionally, in this study, resting spleen volume measures were taken before the CO rebreath (153 ± 26 mL) and immediately prior to the set of apneas (155 ± 27 mL) to ensure there was no influence of the same-day CO rebreath on resting spleen volume prior to apneas. Since there was no difference (mean difference 2 mL, 95% CI -5, 9, $p = 0.32$) in spleen volume following CO rebreath, we determined that data could be pooled for this study.

Before any study activities commenced, both studies were approved by the Institutional Review Board at Indiana University (IRB#11855 and IRB#2001612727) and were subsequently performed by the standards set by the latest revision of the Declaration of Helsinki, except for the registration in a database. Prior to participation, all participants provided written informed consent after being fully informed of the experimental procedures and risks. From here on, the term study will refer to the combined study protocols unless otherwise noted.

This study utilized a within-subject design. Participants reported to the laboratory on two separate occasions. Visit one included consenting, screening, familiarization, and a CO rebreathing procedure (only for different day rebreath; $n=11$). Visit two was the experimental trial.

Participants

Fourteen healthy adults (29 ± 5 years, six women) without breath-hold diving or competitive apnea experience were recruited and completed the study. Participant characteristics were height: 177 ± 13 cm, weight: 84.9 ± 15.2 kg, total body surface 2.03 ± 0.23 m², and body mass index: 27.0 ± 3.4 kg·m⁻². Participants self-reported being moderately active, non-tobacco users, and free from splenic, hematological, cardiovascular, pulmonary, and autoimmune diseases. Females self-reported normal menstruation and were confirmed not to be pregnant via a urinary pregnancy test. Given the single-day experimental design, females were tested across the menstrual cycle (18 ± 14 days post menses).

Instrumentation and measurements

Body weight (kg) and height (cm) were measured using a stadiometer (Holtain Limited, Seritex, Wales, UK) and scale (Sauter, Balingen, Germany). A refractometer measured urine-specific gravity (Atago, Tokyo, Japan). Heart rate (bpm) and an estimate of arterial oxyhemoglobin saturation (SpO₂, %) were measured continually via pulse oximetry (Nellcor N600x, Medtronic Inc., U.S.) with the sensor placed on the forehead. The SpO₂ nadirs during or within 60 seconds post apneas were averaged over each set to quantify the hypoxemic minimum. Blood pressure was measured using an automated cuff (Suntech CT40, SunTech Medical, U.S.) on the left brachium. Following the warming of the hand via a heating pad, capillary blood samples were collected to assess hemoglobin concentration (HemoQue Hb 201+, DM System, Ängelholm, Sweden) and hematocrit (microcentrifugation). Hemoglobin concentration and hematocrit measurements were made in duplicate and averaged. Capillary blood samples were acceptable in this study despite the expectation of apnea-induced peripheral vasoconstriction because of the use of a heating pad to promote blood flow to the fingers.

Spleen volume was always measured with participants in a seated position on the dorsal side of the body via ultrasound (Philips Lumify, Philips North America Corp., U.S.) by a trained sonographer (JMK) with a spleen volume test-retest (within-subject)

coefficient of variation of $2 \pm 2\%$. Spleen volume was measured using a similar ultrasound imaging technique described by Schagatay and Holmstrom [12,13]. With participants seated, the best individual sites for spleen measurement were identified and marked with a permanent marker to allow repeatable measures. All spleen images were captured within 90 seconds of the measurement timepoint and 90 seconds before and after the set of apneas. Each spleen volume measurement consisted of a set of two frozen images, with one image containing maximal spleen length (L) and thickness (T), while the other image contained maximal width (W). Spleen volume was calculated using the Pilström Formula [$L\pi(WT-T^2)/3$] [13]. To minimize probe placement errors and pulsatile changes in spleen volume, a minimum of two sets of images were used to calculate the mean spleen volume at each time point. Spleen images were saved and then read on DICOM software (Philips DICOM Viewer R3.0 L1 SP15, Philips North America Corp., U.S.) at a later date.

Hemoglobin mass (Hb_{mass}) was measured at rest in the supine position using the two-minute CO rebreathing technique described by Gore et al. [14]. Participants laid supine for 15 minutes prior to commencing the rebreathing procedures, which involved participants breathing on a closed rebreathing device that contained 1 mL of carbon monoxide per kilogram of body weight, 5 liters of 100% oxygen, and a CO₂ scrubber (soda lime) for two minutes. Capillary blood samples were collected before, at six minutes post, and at eight minutes post carbon monoxide rebreathing. Following manufacturer guidelines, capillary blood samples were analyzed for hemoglobin and carboxyhemoglobin (OSM3 Hemoximeter, Radiometer, Denmark).

Estimated changes in Hb_{mass} from pre to post-apneas were derived utilizing Hb_{mass} measured at rest and the hemoglobin concentrations measured post-apneas. Specifically, Hb_{mass} measured at rest was assumed constant, and pre-apneas hemoglobin concentration was used to estimate blood volume. This calculation assumes Hb_{mass} is stable over the two testing days [15] and is stable across the menstrual cycle [16], allowing for the testing of women across the menstrual cycle. The change in Hb_{mass} post-ap-

neas was estimated from the change in hemoglobin concentration from pre- to post-apneas, assuming no appreciable change in blood volume from pre- to post-apneas, except the increase in circulating red blood cells and ultimately Hb_{mass} due to spleen contraction. This assumption is predicated on the supposition that splenic hematocrit is ~97% [9, 17].

Experimental trial overview

Participants reported to a temperature-controlled laboratory ($22 \pm 1^\circ\text{C}$) for the experimental visit. Participants were instructed to refrain from strenuous exercise, consuming caffeine and alcohol for 12 hours, and refrain from food for two hours prior to the experimental visit. Upon arrival to the laboratory, a small urine sample was collected from all participants to ensure participants were euhydrated (i.e., urine specific gravity was ≤ 1.025) and to confirm a negative pregnancy test for female participants. Then, baseline nude body weight was measured. Following pre-trial baseline measures, participants were instrumented for heart rate, blood pressure, and SpO_2 .

Timeline of Visit 2

After 20 minutes of seated rest, participants had resting (pre-apnea) spleen volume and hematological measures taken prior to commencing the set of apneas. The set of apneas consisted of five maximal voluntary apneas, with a two-minute rest period between each successive apnea. Apnea times were recorded to the nearest second. Prior to each apnea, subjects completed a short (less than one minute) voluntary hyperventilation to decrease arterial carbon dioxide tension and thereby extend the apnea. Participants were instructed to hold their breath after a deep but not maximal inspiration. These methods were modified by Engan et al. [18]. Prior to the first apnea and within 60 seconds following completion of the fifth apnea of the bout, spleen volume measures and capillary blood samples were collected, after which participants were cleared to leave the lab.

Data and statistical analysis

Prior to analyses, all data were checked for outliers, and the necessary assumptions for the repeated measures ANOVA and two-tailed paired samples t-test, and no corrections were necessary. Consistent with the testing of our primary hypothesis, the relation between resting spleen volume and the change in Hb_{mass} from pre- to post-apneas was assessed using Pearson Product Correlations, which were evaluated a weak ($r < 0.30$), moderate ($0.30 \leq r \leq 0.60$), strong ($r > 0.60$). As spleen size can be a function of sex [19,20], resting spleen volume and the change in Hb_{mass} correlations based on sex were performed. Spleen volume can also be dictated by body size; therefore, resting spleen volume and the change in Hb_{mass} correlations with resting spleen volume normalized to body surface area were utilized in additional analyses. Changes in spleen volume, hematocrit, hemoglobin concentration, Hb_{mass} , O_2 carrying capacity of hemoglobin, and CO_2 carrying capacity of hemoglobin before and following the set of apneas were analyzed using a paired samples t-test (pre-apnea and post-apnea). One-way repeated measures ANOVAs were used to analyze apneic time and nadir SpO_2 for each apnea. If a significant main effect was observed, post-hoc tests using Sidak's method for multiple corrections were conducted. Apneic area under the curve (AUC) analyses were used to estimate cumulative hypoxemia (i.e., a function of both the nadir SpO_2 and apnea duration) over an apnea.

Estimates of the maximal theoretical change in O_2 and CO_2 carrying capacity of hemoglobin were derived from changes in Hb_{mass} from pre- to post-apneas. Specifically, the O_2 carrying capacity of hemoglobin was estimated as the product of the oxygen-binding capacity of hemoglobin (1.34 ml O_2 per gram Hb, [21]) and measured Hb_{mass} . The CO_2 carrying capacity of hemoglobin was derived by converting the Hb_{mass} to molecules of Hb, multiplying that number by 4 to reflect the maximal number of binding sites for CO_2 . Then, using that value as our theoretical maximal number of CO_2 molecules that could be carried, the number of molecules of CO_2 was converted to moles, and then mL of CO_2 was used to find the theoretical maximal volume of

CO₂-carrying. While we estimate the functionality of the change in Hb_{mass} following apneas by deriving an O₂ and CO₂ carrying capacity of hemoglobin, it behooves us to note that other factors contribute to actual O₂ and CO₂ carrying capacities of hemoglobin. Specifically, the affinity of hemoglobin for O₂ or CO₂ is moderated by the Bohr effect [22] and the Haldane effect [23], respectively. Therefore, changes in temperature, pH, partial pressure of O₂, and CO₂ can all affect the functionality of the apneas-induced release of red blood cells and the subsequent increase in Hb_{mass}.

All data were analyzed using Prism software (Version 9.1.2, GraphPad Software Inc, La Jolla, CA, U.S.). A priori statistical significance was set at $p \leq 0.05$, and actual p-values were reported where possible. Data are reported as group mean $\pm$ standard deviation and mean with individual values on figures. Spleen volume, hemoglobin concentration, hematocrit, and Hb_{mass} are reported as group averages $\pm$ standard deviation and separately for men and women pre- and post-apneas. As this study was not designed to examine differences between men and women and, therefore, would be underpowered to do so, no comparisons were made between men and women for these variables.

RESULTS

Pre- to post-apneas changes in spleen and hematological data

Participants completed the set of apneas with longer apneic durations on subsequent apneas within each set ($p \leq 0.01$, Figure 1A). Apneic nadir SpO₂ was reduced with subsequent apneas ($p = 0.02$, Figure 1B). Apneic AUC increased with subsequent apneas ($p \leq 0.02$, Figure 1C). Spleen volume decreased from pre- (247 ± 95 mL) to post-apneas (199.5 ± 82 mL, mean difference -47 , 95%CI -58 , -36 , $p < 0.001$, Figure 2A). Spleen volume for men and women pre- and post-apneas were: men - pre: 310 ± 76 mL, post: 257 ± 58 mL; women - pre: 163 ± 24 mL, post: 123 ± 17 mL. Hemoglobin concentration increased from pre- (14.6 ± 1.2 g/dL) to post-apneas (14.9 ± 1.2 g/dL, mean difference 0.3 , 95%CI 0.1 , 0.5 , $p = 0.004$, Figure 2B). Hemoglobin concentration for men and women pre- and post-apnea were: men - 15.5 ± 0.5

g/dL, post: 15.8 ± 0.5 g/dL; women - pre: 13.4 ± 7 g/dL, post: 13.7 ± 0.9 g/dL. Hematocrit was increased from pre- ($44.3 \pm 2.9\%$) to post-apneas ($45.2 \pm 3.4\%$, mean difference 0.9 , 95%CI 0.04 , 1.8 , $p = 0.041$, Figure 2C). Hematocrit for men and women pre- and post-apneas were: men - pre: $46.3 \pm 1.2\%$, post: $47.5 \pm 2.3\%$; women - pre: $42.0 \pm 2.6\%$, post: $42.6 \pm 2.6\%$. Hb_{mass} increased from pre- (948.6 ± 281.4 g) to post-apneas (969.2 ± 283.5 g, mean difference 20.54 , 95%CI 7.1 , 33.9 , $p = 0.005$, Figure 2D-F). Hb_{mass} for men and women pre- and post-apnea were: men - pre: 1107 ± 242 g, post: 1128 ± 238 g; women - pre: 738 ± 173 g, post: 757 ± 189 g).

Spleen volume relations

Figure 3 shows strong correlations between resting spleen volume and pre-apneas Hb_{mass} ($r = 0.78$, $p < 0.001$, Figure 3A) and resting spleen volume and the change in spleen volume post-apneas ($r = -0.74$, $p = 0.001$, Figure 3B). However, there was no relation between resting spleen volume and apnea-induced changes in Hb_{mass} post-apneas, whether assessed as absolute resting spleen volume (Figure 3C) or when resting spleen volume was normalized to body surface area (Figure 3D). Indeed, there was no relation between resting spleen volume and the apnea-induced change in Hb_{mass} for men ($r = -0.02$, $p = 0.958$) or women ($r = 0.05$, $p = 0.931$). There was also no relation between the change in spleen volume and apnea-induced changes in Hb_{mass} post-apneas in absolute volume ($r = -0.09$, $p = 0.381$, Figure 3E) or when the change in spleen volume was normalized to body surface area ($r = -0.08$, $p = 0.795$, Figure 3F).

Theoretical O₂ and CO₂ carrying capacity of hemoglobin

The oxygen-carrying capacity of hemoglobin increased from pre- (1271 ± 377 mL) to post-apneas (1299 ± 380 mL, mean diff. 28 , 95%CI 10 , 45 , $p = 0.006$, Figure 4A-C). The CO₂ carrying capacity of hemoglobin increased from pre- (1439 ± 426.8 mL) to post-apneas (1470 ± 429.9 mL, mean diff. 31 , 95%CI 11 , 51 , $p = 0.006$, Figure 4D-F).

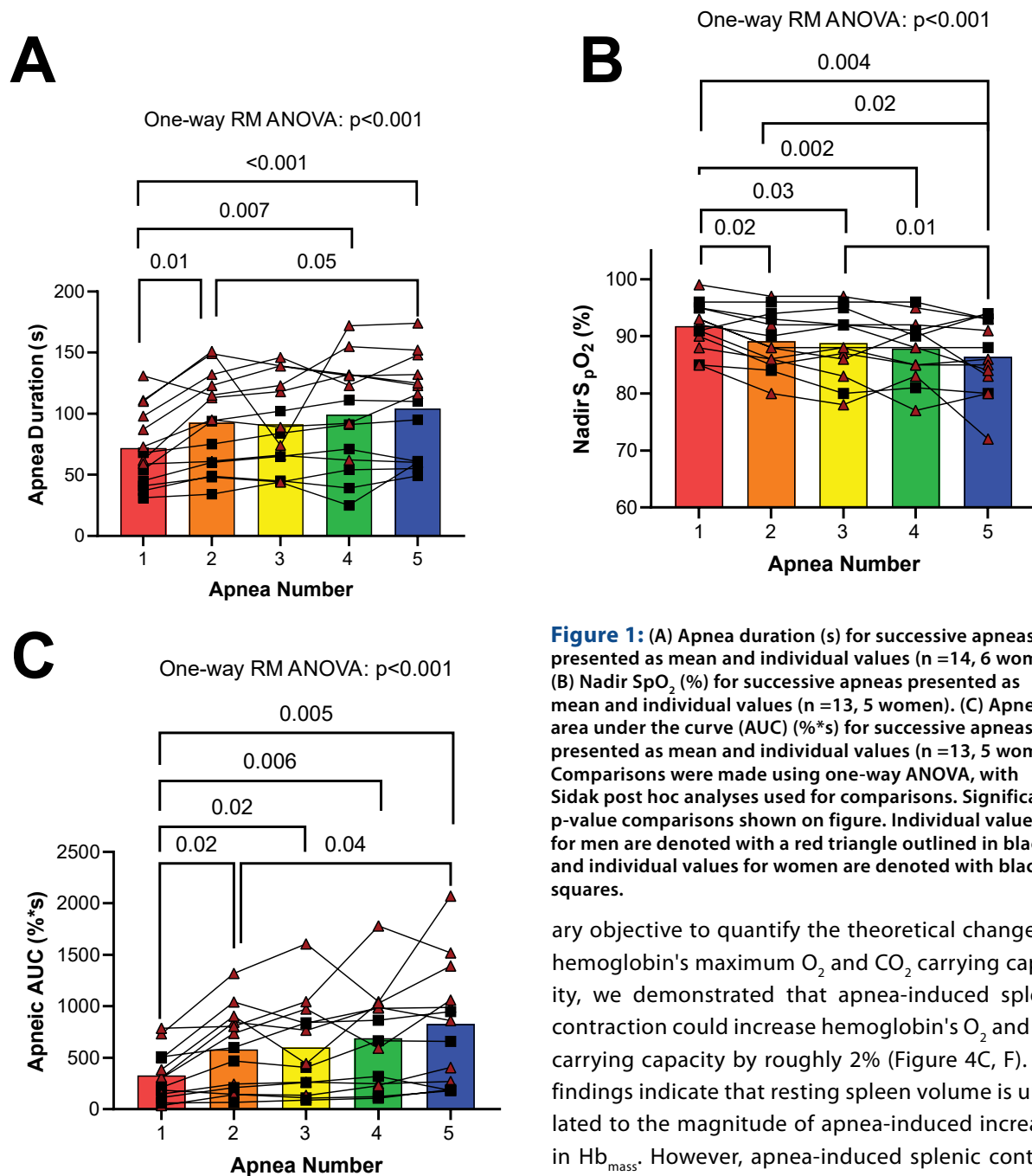


Figure 1: (A) Apnea duration (s) for successive apneas presented as mean and individual values ($n = 14$, 6 women). (B) Nadir SpO_2 (%) for successive apneas presented as mean and individual values ($n = 13$, 5 women). (C) Apneic area under the curve (AUC) (%*s) for successive apneas presented as mean and individual values ($n = 13$, 5 women). Comparisons were made using one-way ANOVA, with Sidak post hoc analyses used for comparisons. Significant p-value comparisons shown on figure. Individual values for men are denoted with a red triangle outlined in black and individual values for women are denoted with black squares.

ary objective to quantify the theoretical changes in hemoglobin's maximum O_2 and CO_2 carrying capacity, we demonstrated that apnea-induced splenic contraction could increase hemoglobin's O_2 and CO_2 carrying capacity by roughly 2% (Figure 4C, F). Our findings indicate that resting spleen volume is unrelated to the magnitude of apnea-induced increases in Hb_{mass} . However, apnea-induced splenic contraction can increase hemoglobin's O_2 and CO_2 carrying capacity by roughly 2% in non-apnea-trained healthy adults.

While larger resting spleen volumes have corresponded with improved apneic performances theoretically through greater augmentation of blood oxygen transport [5], here we did not observe a relation between resting spleen size and increases in Hb_{mass} post-apneas. This nonrelation persisted when looking at body surface area normalized

DISCUSSION

In opposition to our hypothesis, there was no relation between resting spleen volume and the magnitude of apneas-induced increases in Hb_{mass} in non-apnea-trained healthy adults. While Hb_{mass} increased following apneas (Figure 2D), the magnitude of the increase was not related to absolute pre-apneas spleen volume (Figure 3C). In pursuit of our second-

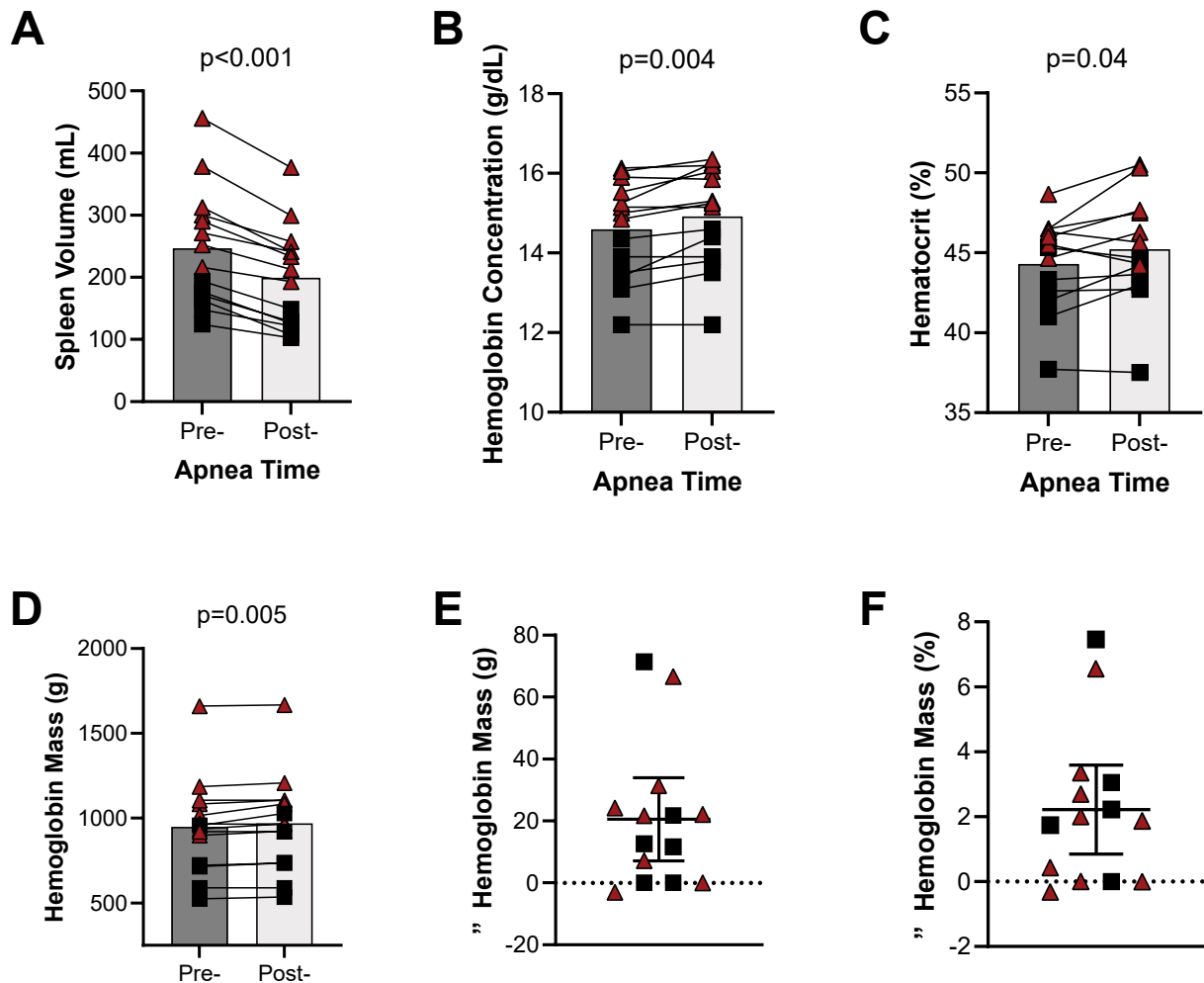


Figure 2: (A) Spleen volume response to apneas presented as mean and individual values ($n = 14$, 6 women). (B) Hemoglobin Concentration (g/dL) response to apneas presented as mean and individual values ($n = 14$, 6 women). (C) Hematocrit (%) response to apneas presented as mean and individual values ($n = 14$, 6 women). (D) Hemoglobin mass (Hb_{mass}) response to apneas presented as mean and individual values ($n = 14$, 6 women). (E) Absolute change in Hb_{mass} post-apneas. (F) Percent change in Hb_{mass} post-apneas. Comparisons were made using Paired T-Test, with p-values displayed. Individual values for men are denoted with a red triangle outlined in black and individual values for women are denoted with black squares.

resting spleen volume (Figure 3D), or resting spleen volume based on sex. Since there was a relation between resting spleen volume and the apnea-induced change in spleen volume post-apneas (Figure 3B), we explored the relation of apnea-induced change in spleen volume and apneas-induced increases in Hb_{mass} , which also showed no relation (Figure 3E, F). Thus, the current findings suggest that further investigation is needed to understand the mechanism by which spleen size influences apneic performance. To this point, the current study demonstrated a $\sim 2\%$ increase in Hb_{mass} , which coincided with a $\sim 0.6\%$ increase in red blood cell volume post apneas, calculated via CO rebreathing.

Interestingly, Bakovic et al. showed that trained breath-hold divers had $\sim 5.7\%$ increase in red blood cell volume [24], almost tenfold higher than that identified in the current study. These differences could be attributed to stronger dive response and greater hypoxemic/hypercapnic tolerance in apnea-trained individuals compared to non-trained individuals [25]. Increases in hypoxemia and hypercapnia would induce increased sympathetic activation, potentially causing a stronger splenic contraction, which in theory increases red blood cell release and thus increases circulating Hb_{mass} . However, the present study also showed that the change in spleen

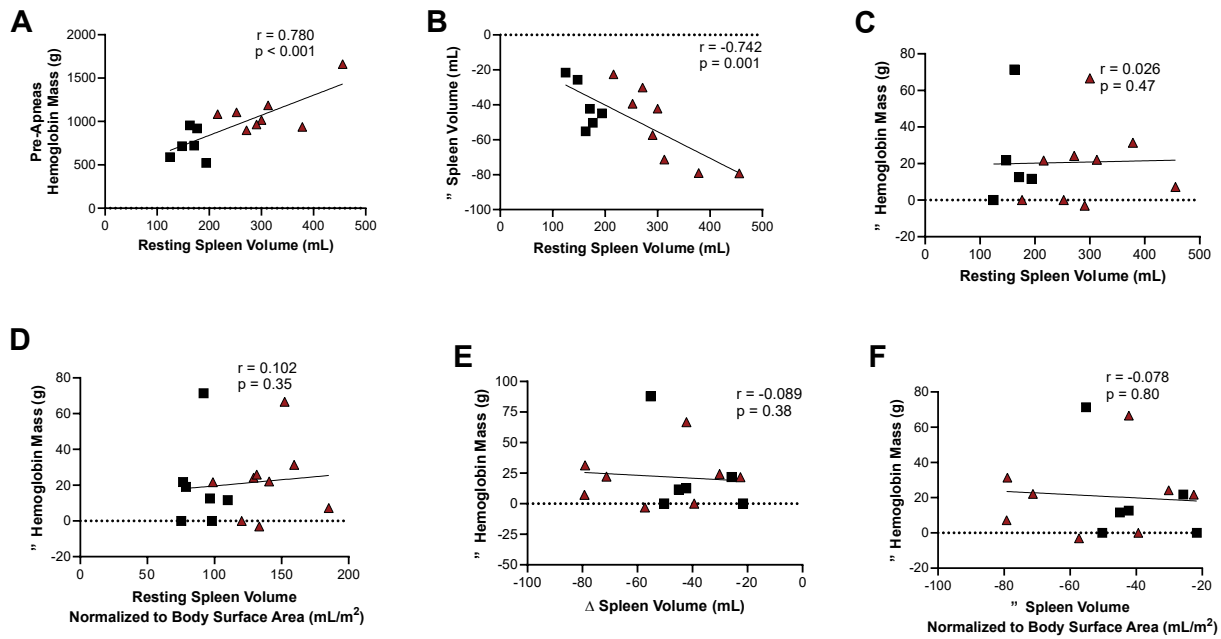


Figure 3. Pearson product moment correlations presented with correlation coefficients (r) and p -values. Grey triangles represent men and black squares represent women. (A) Correlation between resting spleen volume and pre-apnea hemoglobin mass (Hb_{mass}). (B) Correlation between resting spleen volume and the change (Δ) in spleen volume post-apneas. (C) Correlation between resting spleen volume and the change (Δ) in Hb_{mass} post-apneas. (D) Correlation between resting spleen volume normalized to body surface area and the change (Δ) in Hb_{mass} post-apneas. (E) Correlation between the change (Δ) in spleen volume post apneas and the change (Δ) in Hb_{mass} post-apneas. (F) Correlation between the change (Δ) in spleen volume post apneas normalized to body surface area and the change (Δ) in Hb_{mass} post-apneas. Individual values for men are denoted with a red triangle outlined in black and individual values for women are denoted with black squares.

volume was not correlated with increases in Hb_{mass} , so spleen volume and changes in spleen volume may improve performance, but likely in concert with other training adaptations, such as hematological changes and hypoxemic/hypercapnic tolerance. Additionally, larger spleen volumes could be related to factors other than increased red blood cells, such as illness (infectious mononucleosis) or being of a certain race [19].

Previous studies have shown that larger spleen volumes and contractions might improve dive performance in competitive athletes [3,5] and native diving populations [6] by theoretically improving oxygen-carrying capacity and pH/ CO_2 buffering. During this investigation, hematological data showed slight changes from pre- to post-apneas (Figures 2 and 3), similar to previous investigations. For example, hemoglobin concentration was increased post apneas by 0.3 mg·dL⁻¹ or ~2%, which aligns with previous work conducted in non-breath hold populations [3,10,7,24]. The present study's

secondary objective was to estimate theoretical maximal changes in hemoglobin's O_2 and CO_2 carrying capacities, which may provide insight into the functional aspects of apneas-induced spleen contraction. Apneas increased circulating Hb_{mass} (Figure 2D), which translated into increases in O_2 carrying capacity of hemoglobin (~28 mL, ~2%, Figure 4A-C) and CO_2 carrying capacity of hemoglobin (~31 mL, ~2%, Figure 4D-F). Previous work by Schagatay et al. estimated that breath-hold divers would utilize about 200 mL O_2 /minute, so a 28 mL increase in O_2 carrying capacity of hemoglobin would translate to about a nine-second increase in apnea duration [5]. Since three to five maximal apneas produce peak apnea-induced splenic contraction and an increase in hematocrit and hemoglobin concentration [2, 3], the time difference between apnea three and apneas four and five could represent the functional benefit of complete splenic dumping. Indeed, we observed a ~8-second increase from apnea 3 to 4 and a ~13-second increase from apnea three to five. Thus,

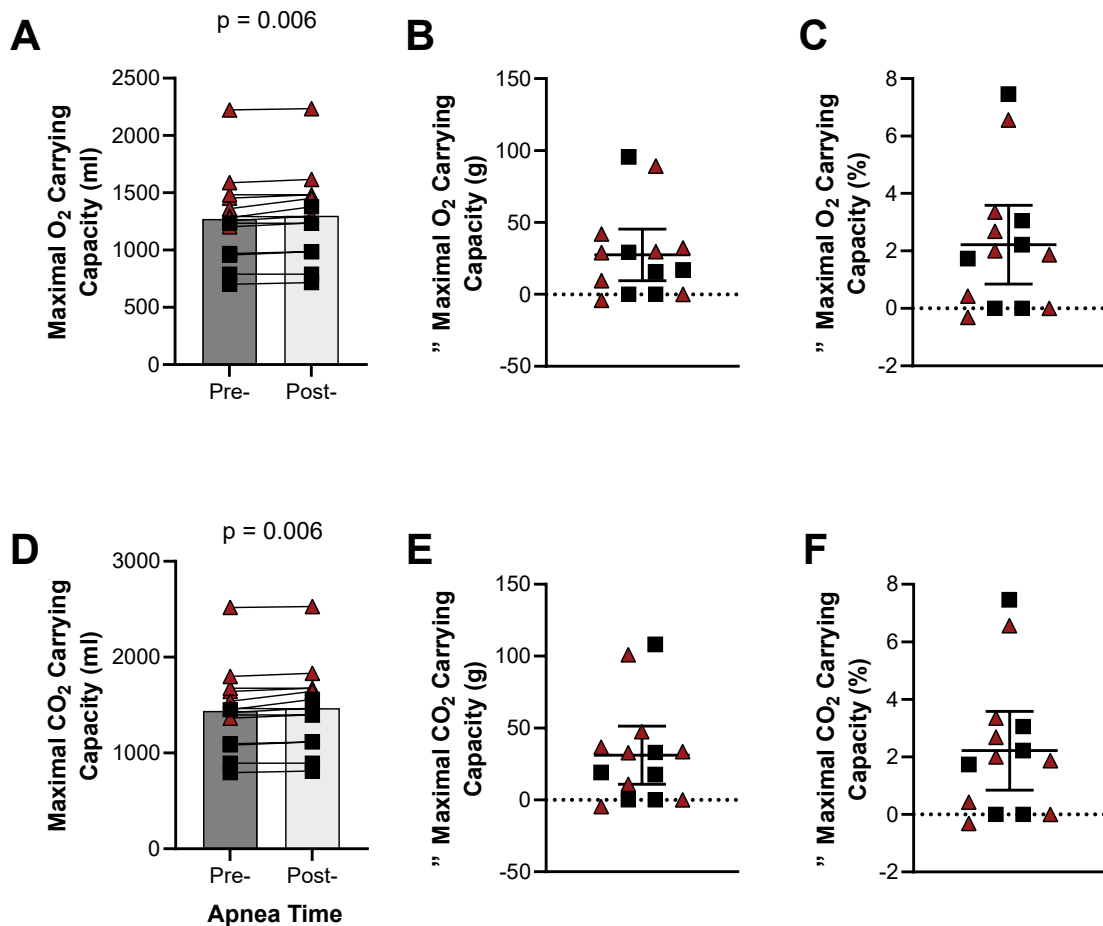


Figure 4. (A) Theoretical maximal O₂ carrying capacity response to apneas presented as mean and individual values (n = 14, 6 women). (B) Absolute change in O₂ carrying capacity post-apneas. (C) Percent change in O₂ carrying capacity post-apneas. (D) Theoretical maximal CO₂ carrying capacity response to apneas presented as mean and individual values (n = 14, 6 women). (E) Absolute change in CO₂ carrying capacity post-apneas. (F) Percent change in CO₂ carrying capacity post-apneas. Comparisons were made using Paired T-Test, with p-values displayed. Individual values for men are denoted with a red triangle outlined in black and individual values for women are denoted with black squares.

these data estimate for the functional benefits of splenic contraction to prolonging apnea duration.

Experimental considerations

There are a few experimental considerations to consider for the experimental design. The first experimental consideration is study design, as this study combines two studies with slightly different protocols. The primary difference between protocols was the timing of the CO rebreath, as 11 participants completed this procedure at least 24 hours before apneas, and three completed the CO rebreath about 45 minutes before. Participants who completed CO rebreathing at least 24 hours before apneas would have completed CO elimination before

starting the apneas. However, for the three participants who completed same-day CO rebreathing, there could have been a slight accumulation of CO still present in the blood, which could have compromised the participants' capacity to hold their breath. While this is a concern, we believe it was not a factor, as there were no differences in apnea duration (p = 0.20) or nadir SpO₂ (p = 0.28) between the protocols. At the same time, spleen volume returned to pre-CO rebreath levels prior to the apneas. Therefore, with this information, we believe combining the studies for this analysis was inconsequential to our findings. A second consideration is that the participant population could be a factor in the lack of correlation between spleen volume and apnea-induced changes

in Hb_{mass} . Trained apneist or elite athletes may have larger spleens, greater spleen contractions, different hematological profiles, and/or an increased hypoxic/hypercapnic tolerance. Future research should investigate how these factors may affect circulating Hb_{mass} changes post-apneas.

CONCLUSION

Larger spleen volumes have been proposed as beneficial in operating in the aquatic environment by enabling the jettisoning of red blood cells into the circulation [5,6]. However, this study demonstrates that resting spleen volume is unrelated to the post-apneas change in circulating Hb_{mass} in non-apneic trained healthy adults. Lastly, we quantified the

apnea-induced increases in the O_2 carrying capacity of hemoglobin (~28 mL, ~2%) and CO_2 carrying capacity of hemoglobin (~31 mL, ~2%), which may provide a functional benefit of increasing apnea time between eight and thirteen seconds.

Acknowledgments

We thank the individuals who participated in this study.

Funding

This research was supported by an award from the Office of Naval Research (N00014-20-1-2593).

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Peer-reviewed abstract:

Schlader ZJ, Hess HW, Baker TB, Logan R, Kerr P, Tourula E, Greenshields JT, Chapman RF, Johnson BD, Keeler JM. Preliminary evidence that resting spleen volume is related to the magnitude of apnea-induced increases in total hemoglobin mass in humans without a history of breath-hold diving. Undersea and Hyperbaric Medicine Annual Scientific Meeting, Reno, NV, USA, 2022.

Preliminary evidence that resting spleen volume is related to the magnitude of apnea-induced increases in total hemoglobin mass in humans without a history of breath-hold diving

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Introduction/Background: Indigenous populations renowned for breath-hold diving have comparatively large spleen volumes. It is speculated that a larger spleen translates to heightened apnea-induced splenic contraction and elevations in circulating hemoglobin mass (Hb_{mass}), thereby improving oxygen carrying and/or pH buffering capacity. This speculation assumes that pre-apnea spleen volume is associated with apnea-induced increases in total Hb_{mass} . Therefore, this study tested the hypothesis that pre-apnea spleen volume is positively related to apnea-induced increases in total Hb_{mass} .

Materials and Methods: Total Hb_{mass} (CO rebreathing) was measured in eight healthy adults without a history of breath-hold diving (27 ± 4 y, three women). 6 ± 4 days later, subjects completed five maximal apneas seated in air. Before and after the fifth apnea, spleen length, width and thickness were measured (ultrasound) and spleen volume was calculated (Pilström equation, test-retest CV: $2 \pm 2\%$). At these timepoints, hemoglobin concentration [Hb] and hematocrit were measured from capillary blood samples. Blood volume was calculated from total Hb_{mass} pre-apneas. Post-apneas, total Hb_{mass} was estimated from post-apnea [Hb] and pre-apnea blood volume. Data are mean $\pm$ SD.

Results: Apnea duration increased from the first (73 ± 24 s) to second (99 ± 36 s, $p=0.027$) apnea and was sustained thereafter ($p \geq 0.468$). Spleen volume decreased from pre- to post-apneas (241 ± 75 vs. 201 ± 64 cm³, $p < 0.001$). [Hb] (14.6 ± 0.9 vs. 15.0 ± 1.0 g/dL, $p=0.006$) and total Hb_{mass} (876 ± 204 vs. 899 ± 211 g, $p=0.018$) increased, but hematocrit did not change (pre: $46 \pm 4\%$, post: $46 \pm 3\%$, $p=0.532$) from pre- to post-apneas. There was a moderate, positive relation between pre-apnea spleen volume and increases in Hb_{mass} ($r=0.505$, $p=0.101$) and a weak, negative relation between the magnitude of splenic contraction and increases in Hb_{mass} ($r=-0.144$, $p=0.367$). Pre-apnea spleen volume explained more variance in Hb_{mass} than the magnitude of splenic contraction ($p=0.054$).

Summary/Conclusions: A larger spleen volume is associated with a greater rise in total Hb_{mass} following apneas.

Support: Office of Naval Research (N00014-20-1-2593).

Peer-reviewed abstract:

Keeler JM, Hess HW, Tourula E, Baker TB, Kerr PM, Greenshields JT, Chapman RF, Johnson BD, **Schlader ZJ**. Is there a relation between spleen volume and increases in hemoglobin mass following apneas in people without breath-hold diving experience? International Conference on Environmental Ergonomics, Niagara Falls, ON, Canada, 2022.

Is there a relation between spleen volume and increases in hemoglobin mass following apneas in people without breath-hold diving experience?

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INTRODUCTION: Indigenous populations renowned for breath-hold diving have comparatively large spleen volumes. It has been proposed that a larger spleen translates to heightened apnea-induced splenic contraction and elevations in circulating hemoglobin mass (Hb_{mass}), which in theory improves O_2 carrying and/or CO_2/pH buffering capacities. However, the relation between pre-apnea spleen volume and apnea-induced increases in Hb_{mass} is unknown. Therefore, we tested the hypothesis that pre-apnea spleen volume is positively related to apnea-induced increases in total Hb_{mass} .

METHODS: 16 healthy adults (6 women; 29 ± 6 y) completed a 2-min carbon monoxide rebreathe procedure for the measurement of pre-apneas Hb_{mass} and blood volume. Spleen length, width, and thickness were measured pre- and post- a set of five maximal apneas via ultrasound. Spleen volume was calculated via the Pilsström equation (test-retest CV: $2 \pm 2\%$). Hemoglobin concentration ([Hb]) and hematocrit were measured pre- and post-apneas via capillary blood samples. Hb_{mass} post-apneas was estimated as post-apnea [Hb] x pre-apnea blood volume. Data are presented as mean $\pm$ SD.

RESULTS: Spleen volume decreased from pre- (247 ± 90 ml) to post- (200 ± 80 ml, $P < 0.01$) apneas. [Hb] (14.7 ± 1.3 vs. 15.0 ± 1.3 g/dl, $P = 0.01$), hematocrit (45 ± 4 vs. $46 \pm 4\%$, $P = 0.05$) and Hb_{mass} (1025 ± 322 vs. 1046 ± 339 g, $P = 0.03$) increased from pre- to post-apneas. Pre-apneas spleen volume was not related to post-apneas increases in Hb_{mass} ($r = -0.07$, $P = 0.40$). O_2 ($+28 \pm 45$ ml, $P = 0.03$) and CO_2 ($+32 \pm 51$ ml, $P = 0.03$) carrying capacities increased post-apneas.

CONCLUSION: A larger spleen volume is not associated with a greater rise in apneas-induced increases in Hb_{mass} .

Supported by Office of Naval Research award N00014-20-1-2593.

Does hypoxia modify activation of the hypothalamus-pituitary-thyroid axis during cold water immersion?

Peer-reviewed manuscript in revision in Journal of Applied Physiology.

Keeler JM, Greenshields JT, Goss GS, Baker TB, Chapman RF, Johnson BD, **Schlader ZJ**. Acute moderate normobaric hypoxia does not modify circulating thyroid hormone concentrations induced by one hour of head out cold-water immersion. *Journal of Applied Physiology* In Revision.

Acute moderate normobaric hypoxia does not modify circulating thyroid hormone concentrations induced by one hour of head out cold-water immersion.

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Abstract word count: 240

Number of references: 34

Running header: Cold, hypoxia and thyroid hormones

ABSTRACT

This study tested the hypothesis that acute moderate normobaric hypoxia augments circulating thyroid hormone concentrations during and following one hour of cold head out water immersion (HOWI), compared to when cold HOWI is completed during normobaric normoxia. In a randomized crossover single blind design, 12 healthy adults (27 ± 2 y, 2 women) completed one hour of cold ($22.0 \pm 0.1^\circ\text{C}$) HOWI breathing either normobaric normoxia ($\text{FiO}_2 = 0.21$) or normobaric hypoxia ($\text{FiO}_2 = 0.14$). Free and total thyroxine (T3) and triiodothyronine (T4), and thyroid stimulating hormone (TSH) concentrations were measured in venous blood samples obtained before (baseline), during (15-, 30-, and 60-min), and 15 min following HOWI (post-), and were corrected for changes in plasma volume. Arterial oxyhemoglobin saturation and core (rectal) temperature were measured continuously. Arterial oxyhemoglobin saturation was lower during hypoxia ($90 \pm 3\%$) compared to normoxia ($98 \pm 1\%$, $p < 0.001$). Core temperature fell from baseline (normoxia: $37.2 \pm 0.4^\circ\text{C}$, hypoxia: $37.2 \pm 0.4^\circ\text{C}$) to post- cold HOWI (normoxia: $36.4 \pm 0.5^\circ\text{C}$, hypoxia: $36.3 \pm 0.5^\circ\text{C}$, $p < 0.001$) in both conditions but did not change differently between conditions (condition x time: $p = 0.552$). Circulating TSH, Total T3, Free T4, Total T3, and Free T4 concentrations demonstrated significant main effects of time (all $p \leq 0.024$), but these changes did not differ between normoxic and hypoxic conditions (condition x time: all $p \geq 0.163$). These data indicate that acute moderate normobaric hypoxia does not modify the circulating thyroid hormone response during one hour of cold HOWI.

Keywords: Cold stress, hypothermia, hypoxia, thermogenesis, erythropoiesis, diving

NEWS & NOTEWORTHY

One hour of head out cold (22°C) water immersion (HOWI) decreased core temperature and increased thermogenesis. This thermogenic response was paralleled by the activation of the hypothalamic-pituitary-thyroid axis, as evidenced by reductions in TSH and elevations in Total

41 and Free T4 and Free T3 during cold HOWI. However, cold HOWI in combination with exposure
42 to moderate normobaric hypoxia did not modify the thermogenic nor the circulating thyroid
43 hormone response. This finding suggests that hypoxia-induced alterations in thyroid hormone
44 concentrations are unlikely to acutely contribute to adaptations resulting from repeated cold-
45 water exposures.

INTRODUCTION

To improve autonomous operations in the aquatic environment there is a need to understand the adaptive responses to combinations of diving related stressors, such as immersion, cold exposure, apneas and hyperoxia or hypoxia, amongst others. Native apneic diving populations are exposed to repeated bouts of hypoxemia while diving in cold water (10-30°C), during working days 2-9 hours in duration (1, 2). It has recently been identified in the Bajau, a native diving population in Southeast Asia, that PDE10A expression is decreased and spleen size is larger compared to their nearest genetic relatives (3, 4). PDE10A is a gene that encodes an enzyme that hydrolyzes cAMP and cGMP to regulate cyclic nucleotide signaling, with reductions in PDE10A being associated with elevations in thyroid hormone production (3). Reduced expression of PDE10A has been shown to cause spleen growth (5) and stimulate erythropoiesis due to the release of thyroid-stimulating hormone (TSH) and its downstream effects (4), which has been presumed to be beneficial when operating in the aquatic environment (6). Thus, TSH and the resulting release of thyroxine (T4), and its conversion into the biologically active triiodothyronine (T3) by type 2 iodothyronine deiodinase, may not only be advantageous when operating in cold water by increasing metabolic rate, thereby aiding thermoregulation, but may also be beneficial by stimulating erythropoiesis via erythropoietin dependent (7) or independent (4) mechanisms.

Thyroid hormone associated genotypic and phenotypic adaptations have been mainly observed in native breath-hold diving populations who experience frequent bouts of hypoxemia while being exposed to cold water. It is possible, that hypoxemia elevates circulating thyroid hormone concentrations during exposure to cold water in non-native breath hold diving populations. Indeed, field-based studies provide evidence that hypoxia is associated with increases in circulating thyroid hormone concentrations in non-native breath hold diving populations (8–10). However, interpretation of data obtained from mountaineering-based studies is made difficult

72 because subjects were likely exposed to cold and hypoxia during the observation periods. For
73 example, data obtained before and after a mountaineering expedition indicate that a relatively
74 long duration exposure (14 days) to high altitude and cold reduces TSH, free and total T3, while
75 T4 concentrations (Free and Total) were unchanged (11). It remains unknown, therefore,
76 whether acute hypoxia exposure modifies circulating thyroid hormone concentrations during
77 shorter duration cold exposure, as would occur during diving. This knowledge gap limits the
78 potential to identify novel ways to optimally train divers to maximize performance when
79 operating in the aquatic environment. To this end, and primarily informed by data in native
80 diving populations (3) and short-term sojourns to high altitude (8, 9), the purpose of this study
81 was to test the hypothesis that acute moderate normobaric hypoxia augments circulating thyroid
82 hormone concentrations during and following one hour of cold head out water immersion
83 (HOWI), compared to when cold HOWI is completed during normobaric normoxia.

METHODS

Experimental Design

Before any study activities commenced, this study was approved by the Institutional Review Board at Indiana University (IRB# 2005869086) and was subsequently performed in accordance with the standards set by the latest revision of the Declaration of Helsinki, except for the registration in a database. This study utilized a single blind, block randomized, balanced, cross-over design. Participants reported to the laboratory on three separate occasions, with at least 5 days between experimental visits. Visit 1 included consenting, screening, and anthropometric measurements. Visits 2 and 3 were the experimental trials. Prior to participation, all participants provided written informed consent after being fully informed of the experimental procedures and risks.

Participants

A power analysis was conducted utilizing R version 4.0 (R Foundation for Statistical Computing, Vienna, Austria) and the pwr package version 1.3.(12). Previously published data from von Wolff et al. (2) showed that following ascent to basecamp free T3 increased from 4.57 ± 0.60 pmol/L to 5.26 ± 0.73 pmol/L, representing a Cohen's d of 1.03.(13). Using this effect size for a paired two-tail t-test (i.e., normoxia vs. hypoxia at any timepoint) with a pre-determined alpha value of 0.05, it was estimated that at least ten participants were necessary to achieve a power of 0.80. Therefore, twelve healthy adults (2 women) were recruited and completed the study to account for the possibility of missing data. Participant characteristics were age: 27 ± 4 y, height: 182 ± 10 cm, weight: 85 ± 10 kg, body mass index: 26 ± 1 kg·m⁻², body surface area: 2.1 ± 0.2 m², body surface area to mass ratio: 2.4 ± 0.1 cm²/kg, percent body fat: $20 \pm 6\%$, and sum of skin folds: 133 ± 37 . Participants self-reported being moderately active, nontobacco users, and free from any cancer, cardiovascular, colorectal, hematological, thyroid, and pulmonary diseases/disorders. Participants were free of any medications with direct effects on the

cardiovascular system and the thyroid. Women self-reported to be normal menstruating, not taking contraceptives, and were confirmed not to be pregnant via urine pregnancy test. Women were tested in the first 7 days post menstruation, a time frame in which progesterone and estrogen are at their lowest levels.

Experimental Trials

The experimental timeline is presented in Figure 1. Participants reported to a temperature-controlled laboratory ($24.6 \pm 1.4^{\circ}\text{C}$) for the experimental visits. Participants were instructed to refrain from strenuous exercise, consuming caffeine, and alcohol for 12 hours and food for 2 hours prior to the experimental visit. Upon arrival to the laboratory, a small sample of urine was collected from all participants to confirm that participants were well hydrated (i.e., urine specific gravity <1.025) and to confirm a negative pregnancy test for female participants. Then participants were instrumented for core temperature, skin temperatures, heart rate, Arterial oxyhemoglobin saturation, and respiratory measurements, which were continually collected via LabChart software and PowerLab hardware (ADInstruments, Dunedin, New Zealand). Continuously collected data were extracted as a 60 s average at each experimental timepoint (Figure 1).

Following instrumentation, a 15 min out of water baseline was completed with the participants seated, while always breathing normoxic gas. The baseline blood draw was obtained at the end of the 15 mins. Participants were then exposed to either normobaric normoxia ($\text{FiO}_2 = 0.21$) or normobaric hypoxia ($\text{FiO}_2 = 0.14$) for 5 min, after which pre- cold HOWI data were collected. Participants remained exposed to the normoxic or and hypoxic inspire throughout the remainder of the experimental trial. The order of the normoxic and hypoxic trials was assigned in a block randomized manner and the participants were blinded to the experimental condition. An FiO_2 of 0.14 was chosen for the hypoxia condition to elicit an arterial oxyhemoglobin

saturation of ~90% but, for ethical reasons, no less than 80% at any given time. This hypoxic condition was chosen to simulate the cumulative effect of multiple apneas throughout a working day commonly encountered by indigenous diving populations, which could be up to 2 hours of underwater working time in a 2-9 hour work day (1). Following the pre- cold HOWI data collection period, participants then entered a continually circulated pool filled with 22°C water and were immersed such that the water was at the level of the nipple line. Indigenous diving populations, such as the Bajau, Ama and Haenyeo, dive in water temperature ranges of 10-30°C (2). A one hour exposure in water temperature of 22°C water was chosen to stimulate the drop in core temperature that might be expected when operating waters similar in temperature to those of indigenous diving populations. The left arm rested on a flotation device out of the water to maintain a clean and dry environment to obtain blood draws. Venous blood samples were obtained at 15, 30, and 60 mins during cold HOWI. Following the 60 min blood draw, participants were removed from the water, towel dried, and commenced a 15 min post-exposure period while sitting in a chair. A final blood draw was taken at the end of the 15 min post-exposure. Participants did not urinate throughout the experimental conditions. Following visit 2, participants returned to the laboratory after at least 5 days, with an average of 10 days and a range of 5-31 days between visits, to complete the opposite environmental exposure. Data were collected from October 2020 until August 2021.

Instrumentation and Measurements

Body weight and height were measured using a scale (Sauter, Balingen, Germany) and stadiometer (Holtain Limited, Wales, UK). Estimates of body composition (body density, fat free mass, and subcutaneous insulation) were performed by trained personnel (JTG) via seven site skinfold thickness measures (14). Urine specific gravity was measured using a refractometer (Atago, Tokyo, Japan). Heart rate was measured continually using a 3-lead electrocardiogram (Datex-Ohmeda, Helsinki, Finland). Estimated Arterial oxyhemoglobin saturation was measured

continually via a pulse oximetry sensor (Nellcor N600x, Medtronic Inc., USA) placed on the forehead. Core temperature was measured via single-use thermistor inserted 12 cm into the rectum. Skin temperatures of the supraclavicular space and over the trapezius muscle were measured on the participants right side via thermocouple probes (Omega Technologies, Inc., Westlake Village, CA, USA). Supraclavicular skin temperature was measured because it is superficial to a depot of brown adipose tissue, while trapezius skin temperature served as a control site because brown adipose tissue is not located under the trapezius muscle (15). The supraclavicular – trapezius skin temperature difference served as an index of brown adipose tissue activation, similar to other studies (16). Skin temperatures were only measured on the right side because the left arm was elevated to permit blood draws during cold HOWI, which prevented accurate measurement of supraclavicular skin temperature. Both skin temperature locations were not located within the water at any time.

Participants were instrumented with a one-way valve facemask (Hans Rudolph, Shawnee, KS, USA) that allowed delivery of the desired inspire from a Douglas bag containing an $\text{FiO}_2 = 0.14$ (hypoxia, equivalent to an altitude of 3048-3353 m) or $\text{FiO}_2 = 0.21$ (normoxia) and the analysis of expired gases. Normobaric hypoxia was delivered via nitrogen dilution using a hypoxic gas generator (Hypoxico Inc., New York, New York, USA). The rate of metabolic heat production was calculated from absolute oxygen uptake and the respiratory exchange ratio (17), which were determined via indirect calorimetry.

Venous blood samples were obtained after at least 15 min of rest in the same body position to control for plasma volume shifts associated with changes in body posture (18). Venous blood samples were obtained from an antecubital vein at baseline, at 15, 30, and 60 mins of cold HOWI, and 15 min post- cold HOWI (post). Hemoglobin concentration (HemoQue Hb 201+, DM System, Ängelholm, Sweden) and hematocrit (microcentrifugation) were analyzed in triplicate

immediately. Serum was then isolated and stored at -80°C for later analyses of total and free T3 and T4, and TSH concentrations, which were measured in duplicate via commercially available ELISA assay kits (Eagle Biosciences, Nashua, NH, USA). Cold HOWI reduces plasma volume (19). Thus, serum hormone concentrations were adjusted to plasma volume changes, as estimated from changes in hemoglobin and hematocrit (20), like previous studies (21). Venous blood samples were obtained from eleven subjects (2 women) Also, venous blood samples were unable to be obtained at all timepoints in three participants in the hypoxia trial and two participants in the normoxia trial. This is accounted for in our statistical approach (see below). The Free T4 to Free T3 ratio was calculated as an indirect measure of the impact of hypoxia on type 2 iodothyronine deiodinase, given that this enzyme is primarily responsible for the conversion of T4 to T3 (22).

Statistical Analysis

Prior to analyses, data were checked for the necessary assumptions for repeated measures linear mixed models. Data normality and the residuals of the linear mixed models were determined to be normally distributed in all cases. When the assumption of sphericity was violated, the Geisser-Greenhouse correction was applied. Data were analyzed using repeated-measures linear mixed models (time, condition, and time x condition). Measurement timepoints consisted of baseline during normoxic exposure each day (baseline), pre HOWI exposure following 5 mins of exposure to condition inspirate (pre, there was no blood draw for this timepoint), the three timepoints during HOWI and inspirate exposure at 15, 30, and 60 min (15, 30, 60), and 15 mins post HOWI with inspirate (post). Consistent with our hypothesis, if a significant main effect or interaction was observed post hoc tests using Sidak's methods for multiple comparisons were conducted. Importantly, the utilization of the linear mixed models ensure that missing data are accounted for, which was important given the difficulty associated with consistently obtaining venous blood samples during cold HOWI. Relations between

214 changes in core temperature (independent variable) and thyroid hormone concentrations
215 (dependent variables) during normoxia and hypoxia were also examined. Specifically, changes
216 from baseline in the plasma volume corrected hormone concentrations were plotted over
217 changes in core temperature for both the normoxia and hypoxia trials. These data were fitted
218 with a linear regression line and the slope of these relations were compared between hypoxia
219 and normoxia. All data were analyzed via GraphPad Prism Version 9.4.1 (GraphPad Software
220 Inc., La Jolla, CA). A priori statistical significance was set at $p \leq 0.05$, and actual p-values are
221 reported where possible. Data are reported as mean $\pm$ standard deviation and with individual
222 values. Effect sizes for main effects or interaction are reported as partial eta square (η^2_p).

RESULTS

Average heart rate was increased during hypoxia compared to normoxia at pre (normoxia: 71 ± 10 bpm, hypoxia: 77 ± 8 , $p = 0.029$), and remained elevated following inspire and immersion exposure (normoxia: 61 ± 9 bpm, hypoxia: 71 ± 14 , $p = 0.026$, main effect of condition: $p = 0.003$, $\eta^2_p = 0.570$). Average arterial oxyhemoglobin saturation was lower during hypoxia ($90 \pm 3\%$) compared to normoxia ($98 \pm 1\%$, $p < 0.001$, $\eta^2_p = 0.915$, Figure 2A). Core temperature fell from baseline (normoxia: $37.2 \pm 0.4^\circ\text{C}$, hypoxia: $37.2 \pm 0.4^\circ\text{C}$) to post cold HOWI (normoxia: $36.4 \pm 0.5^\circ\text{C}$, hypoxia: $36.3 \pm 0.5^\circ\text{C}$, main effect of time: $p < 0.001$, $\eta^2_p = 0.852$) in both conditions but did not change differently between conditions (condition x time: $p = 0.552$, $\eta^2_p = 0.048$ Figure 2B). End tidal O_2 tension was lower during hypoxia (69 ± 18 mmHg) compared to normoxia (108 ± 6 mmHg, $p < 0.001$, $\eta^2_p = 0.996$ Figure 2C). End tidal CO_2 tension increased from baseline (normoxia: 35 ± 2 mmHg, hypoxia 35 ± 3 mmHg) at 30 and 60 mins of HOWI (normoxia: 34 ± 3 mmHg, hypoxia 35 ± 2 mmHg, main effect of time: $p < 0.001$, $\eta^2_p = 0.466$), but did not change differently between conditions (condition x time: $p = 0.211$, $\eta^2_p = 0.129$, Figure 2D) Trapezius skin temperature decreased with cold HOWI (main effect of time: $p < 0.001$, $\eta^2_p = 0.710$) but did not change differently between conditions (condition x time: $p = 0.251$, $\eta^2_p = 0.193$, Figure 3A). Supraclavicular skin temperature (Figure 3B), the supraclavicular – trapezius skin temperature difference (Figure 3C), and the rate of metabolic heat production (Figure 3D) all increased with cold HOWI (main effect of time: all $p < 0.001$, $\eta^2_p \geq 0.365$) but did not change differently between conditions (condition x time: all $p \geq 0.146$, $\eta^2_p \leq 0.108$).

Plasma volume changed overtime throughout cold HOWI (main effect of time: $p < 0.001$, $\eta^2_p = 0.789$) but did not change differently between normoxia and hypoxia (condition x time: $p = 0.657$, $\eta^2_p = 0.052$, Figure 4A). Similarly, plasma volume adjusted TSH (Figure 4B), Total T4 (Figure 4C), Free T4 (Figure 4D), Total T3 (Figure 4E), and Free T3 (Figure 4F) demonstrated significant main effects of time (all $p \leq 0.024$, $\eta^2_p \geq 0.091$), but these changes did not differ

249 between conditions (condition x time: all $p \geq 0.163$, $\eta^2_p \leq 0.121$). The Free T4 to Free T3 ratio did
250 not change during cold HOWI (main effect of time: $p = 0.210$, $\eta^2_p = 0.208$), but a main effect of
251 condition was identified ($p = 0.029$, $\eta^2_p = 0.371$; hypoxia average: 1.76 ± 0.72 ; normoxia average:
252 1.90 ± 0.78). However, the Free T4 to Free T3 ratio did not change differently over time
253 between conditions (condition x time: $p = 0.177$, $\eta^2_p = 0.208$). Plasma volume adjusted changes in
254 hormone concentrations plotted over changes in core temperature revealed no differences
255 ($p \geq 0.127$, Figure 5A-E) in the slopes of the regression lines between hypoxia and normoxia.

DISCUSSION

In this study, we tested the hypothesis that acute moderate normobaric hypoxia would increase circulating thyroid hormone concentrations during and following cold HOWI in healthy adults, compared to a normobaric normoxic condition. We observed that cold HOWI, and subsequent drops in core temperature, stimulated thermogenesis (Figure 3D), which was at least partially contributed to by the activation of non-shivering thermogenesis (Figure 3C). This thermogenic response was paralleled by the activation of the hypothalamic-pituitary-thyroid axis, as evidenced by reductions in TSH (Figure 4B) and elevations in Total (Figure 4C) and Free T4 (Figure 4D) and Free T3 (normoxia only, Figure 4F) during cold HOWI. That said, moderate normobaric hypoxia did not modify the thermogenic (Figure 3) nor the circulating thyroid hormone (Figure 4) response. Thus, these results do not support our hypothesis.

Indigenous apneic diving populations are reported to spend more than half a working day operating in ocean waters with temperatures as low as 10°C (23). Cold exposure can trigger thyroid hormone release via activation of cold receptors causing activation of the hypothalamic-pituitary-thyroid axis to stimulate non-shivering and shivering thermogenesis (10). To date, investigations into acute cold stress and circulating thyroid hormone concentrations in humans have been relatively mixed in methodology and findings. For example, ice ingestion and acute cold air exposure, which decreased core temperature by ~0.5°C, did not acutely modify circulating TSH concentrations (24, 25). By contrast, TSH was up regulated during head out water immersion (25-28°C) that reduced core temp to 35°C (26), post ice swimming (27), and during a cold air test (21). To this end, the current work provides clarity, such that increases in Free and Total T4, as well as Free T3 were observed during and/or following 60 minutes of cold HOWI (Figure 4). Moreover, we observed a time effect for circulating TSH (Figure 4A), suggesting that the changes in circulating thyroid hormones was likely due to the activation of the hypothalamic-pituitary-thyroid axis. Further, the thyroid hormone changes paralleled

increases in the rate of metabolic heat production (Figure 3D). This increase in heat production was likely contributed to by non-shivering thermogenesis, as observed by increases in the supraclavicular - trapezius skin temperature difference (Figure 3C), and probably shivering thermogenesis, the latter of which was not quantified in the present study due to voluntary movement artifact (accelerometer data not reported) but was readily (yet informally) observed.

While breath-hold diving populations routinely experience cold exposure, these populations also experience repeated bouts of hypoxemia (28). Interestingly, a relative hypoxic state, as evidenced by stimulation of erythropoiesis, is common upon returning to the surface following self-contained underwater breathing apparatus (SCUBA) diving in which hyperoxia is often encountered when at depth (29). Thus, relative hypoxemia is common during and following many diving-related activities and is not limited to only instances of breath hold diving. As supported by the current study (Figures 2B & 3), previous studies indicate that moderate hypoxia has only a small (if any) effect on thermoregulation during prolonged cold exposure (30). That said, evidence in the Bajau demonstrating a genotype for reduced PDE10A expression suggests that repeated exposures to cold and hypoxemia over generations may augment thyroid hormone release (3). Thus, the present study aimed to determine whether moderate hypoxia during a single bout of cold HOWI acutely increases circulating thyroid hormone concentrations in people without breath-hold diving ancestry. This purpose is also congruent with studies employing field-based designs in which circulating thyroid hormone concentrations are elevated during altitude exposure (8, 9, 31), but this is not always the case (11). Our findings indicate that acute moderate normobaric hypoxia does not modify circulating TSH nor Total or Free T4 or T3 during cold head out water immersion (Figure 4). However, we did identify some indirect evidence that moderate hypoxia reduces the actions of type 2 iodothyronine deiodinase given the decrease in the Free T4 to Free T3 ratio in hypoxia (i.e., main effect of condition). This finding is corroborated by previous work indirectly indicating that

mountaineering (i.e., prolonged altitude and cold exposure) reduces T3 concentrations likely by modifying the iodothyronine deiodinases activity or concentrations (11). Therefore, future work should directly examine the combined effects of cold and hypoxia exposure on the iodothyronine deiodinases activity and/or concentrations. Nevertheless, our findings indicate that the co-occurrence of cold that is frequently encountered at high-altitude likely contributes to the observations of augmented thyroid hormone concentrations when at altitude.

It is possible that the magnitude of combined hypoxemia and hypothermia elicited by cold HOWI in the current study was not sufficient to modify hypothalamic-pituitary-thyroid axis stimulation. For example, the moderate hypoxia utilized herein may not have exerted a meaningful effect on the iodothyronine deiodinases (32). Indeed, type 3 iodothyronine deiodinase activity, a primary pathway that inactivates the physiological actions of Free T3 (22), is increased with hypoxia exposure and in a hypoxia-inducible factor dependent manner (33). It is also possible that the extent and duration of hypothermia, and therefore the magnitude of thyroid hormone release, was not sufficient to elicit the hypothesized effect for the utilized dose of hypoxia. Thus, future work should consider more extreme cold stress and/or hypoxic exposures that are longer in duration.

Experimental Considerations

In addition to those described previously, there are a few additional experimental considerations to consider. First, it is important to acknowledge that this study only addresses the acute effect of moderate hypoxia on thyroid hormone concentrations during cold HOWI. This study design was at least partially informed by native apneic diving populations who spend over half a working day diving in cold water. This is a larger stressor that could theoretically augment circulating thyroid hormone concentrations. Therefore, future work should consider examining thyroid hormone responses during and following more prolonged cold-water immersion and

include exposures that are repeated over several days. Second, we were only able to measure a select number of hormones associated with the hypothalamic-pituitary-thyroid axis. Future work should incorporate measures of thyrotropin releasing hormone gene expression (which can be detected in human blood) as well as circulating types 1-3 iodothyronine deiodinases and reverse T3 concentrations. Third, due to measurement error, we were unable to quantify shivering. Thus, we were unable to examine the potential contributions of shivering and non-shivering thermogenesis to the increase in metabolic heat production stimulated by cold HOWI. This is important because thyroid hormones play an important modulatory role in the activation of brown adipose tissue and non-shivering thermogenesis (34). Fourth, the current study was limited to HOWI, without facial immersion, and the resting position, which may have impacted our findings. Future studies should consider implementing whole body submersion (at various depths) and swimming exercise to elicit environmental conditions more closely related to those experienced by native diving populations. Lastly, our group of participants contained more men than women. Therefore, future studies should investigate the thyroid hormone response to acute cold HOWI exposure in women.

Perspectives

Improving occupational and military operations when operating at depth requires an understanding of the adaptive responses to the combinations of stressors encountered while diving and related stressors (e.g., relative hypoxia, cold, etc.). Data from native apneic diving populations, who regularly encounter cold water and hypoxemia, has introduced the potential advantage of a large spleen, which has been linked to thyroid hormone actions and the stimulation of erythropoiesis (3, 4). These observations provided the motivation for the current study, which aimed to determine whether acute moderate hypoxia modifies circulating thyroid hormone concentrations during cold HOWI. To this end, we have identified that acute moderate normobaric hypoxia had no impact on circulating thyroid hormones during and following cold

HOWI (Figure 3). This finding suggests that hypoxemia induced changes in thyroid hormone concentrations are unlikely to acutely contribute to adaptations resulting from repeated cold-water exposures. Future studies should consider examining thyroid hormone responses during and following more prolonged cold-water immersion and include exposures that are repeated over several days.

Conclusions

Cold head out water immersion in healthy adults stimulated thermogenesis, which was at least partially contributed to by the activation of non-shivering thermogenesis. This response occurred alongside activation of the hypothalamic-pituitary-thyroid axis, as evidenced by elevations in both Free T4 and Free T3. That said, moderate normobaric hypoxia did not modify the thermogenic nor the circulating thyroid hormone response during one hour of cold head out water immersion. This finding suggests that hypoxia-induced alterations in thyroid hormone concentrations are unlikely to acutely contribute to adaptations resulting from repeated cold-water exposures. Future work should consider examining thyroid hormone responses during and following more prolonged cold-water immersion and include exposures that are repeated over several days.

377 **Acknowledgements**

378 We thank the individuals who participated in this study.

379

380 **Disclosures**

381 No conflicts of interest, financial or otherwise, are declared by the authors.

382

383 **Funding**

384 This study was supported by awards from the Office of Naval Research (N00014-20-1-2593 and
385 N00014-21-1-2276).

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Figure legends

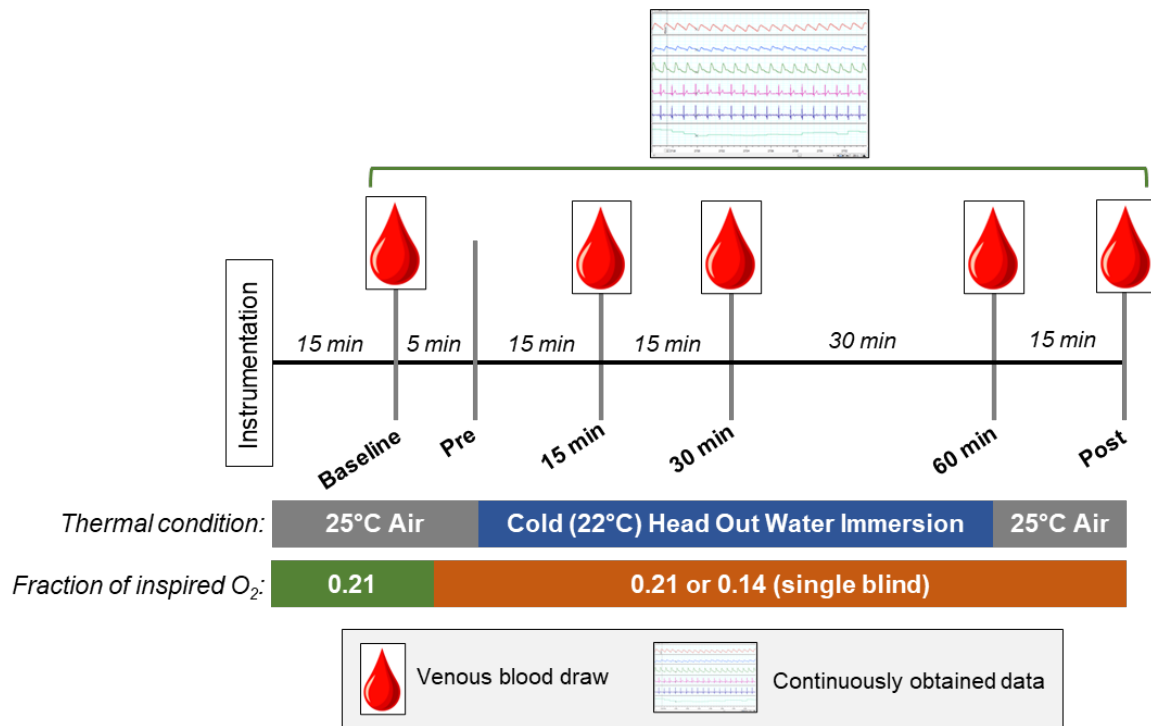
Figure 1: Study design.

Figure 2: Arterial oxyhemoglobin saturation (A), core temperature (B), end tidal O₂ tension (C), and end tidal CO₂ tension before and during cold head out water immersion (HOWI) in the normoxia (FiO₂: 0.21) and hypoxia (FiO₂: 0.14) trials. Data were analyzed using repeated measures linear mixed models, with Sidak's post hoc test conducted if a significant main effect of condition or interaction was observed. n=12 (2 women) in all instances. N represents timepoint is significantly different ($p \leq 0.028$) than Baseline during normoxia trials. H represents timepoint is significantly different ($p \leq 0.032$) than Baseline during hypoxia trials. Data are reported as mean $\pm$ standard deviation and with individual values. * Indicates different from Normoxia ($p \leq 0.001$). Effect sizes for significant main effects or interaction are report as partial eta square (η^2_p).

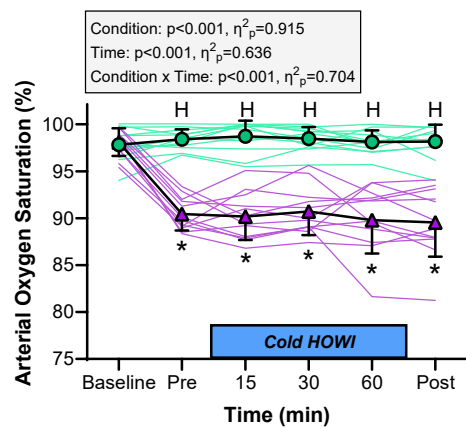
Figure 3: Trapezius skin temperature (A), supraclavicular skin temperature (B), the supraclavicular – trapezius skin temperature difference (C), and the rate of metabolic heat production (D) before and during cold head out water immersion (HOWI) in the normoxia (FiO₂: 0.21) and hypoxia (FiO₂: 0.14) trials. Data were analyzed using repeated measures linear mixed models. n=12 (2 women) in all instances. N represents timepoint is significantly different ($p \leq 0.025$) than Baseline during normoxia trials. H represents timepoint is significantly different ($p \leq 0.027$) than Baseline during hypoxia trials. Data are reported as mean $\pm$ standard deviation and with individual values. Effect sizes for significant main effects or interaction are report as partial eta square (η^2_p).

Figure 4: The change (Δ) in plasma volume (A), thyroid stimulating hormone (TSH) concentration (B), total thyroxine (T4) (C), free T4 (D), total triiodothyronine (T3) (E), and free T3 (F), Free T4 and T3 ratio (G) before and during cold head out water immersion (HOWI) in the normoxia (FiO_2 : 0.21) and hypoxia (FiO_2 : 0.14) trials. Hormone concentrations were corrected for changes in plasma volume. Data were analyzed using repeated measures linear mixed models. $n=11$ (2 women). N represents timepoint is significantly different ($p \leq 0.049$) than Baseline during normoxia trials. H represents timepoint is significantly different ($p \leq 0.054$) than Baseline during hypoxia trials. Blood draws were unable to be obtained at all timepoints in three participants in the hypoxia trial and two participants in the normoxia trial. Missing data is accounted for in the repeated measures linear mixed model. Data are reported as mean $\pm$ standard deviation and with individual values. Effect sizes for significant main effects or interaction are report as partial eta square (η^2_p).

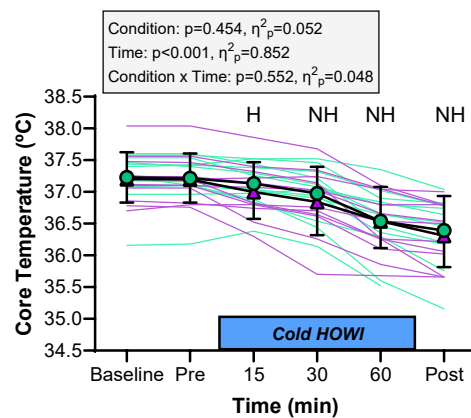
Figure 5: The change (Δ) in thyroid stimulating hormone (TSH) (A), total thyroxine (T4) (B), free T4 (C), total triiodothyronine (T3) (D) and free T3 (E) concentrations from baseline versus the Δ in core temperature during and post- cold head out water immersion (HOWI) in the normoxia (FiO_2 : 0.21) and hypoxia (FiO_2 : 0.14) trials. Hormone concentrations were corrected for changes in plasma volume. Data were analyzed using linear regression models. $n=11$ (2 women).



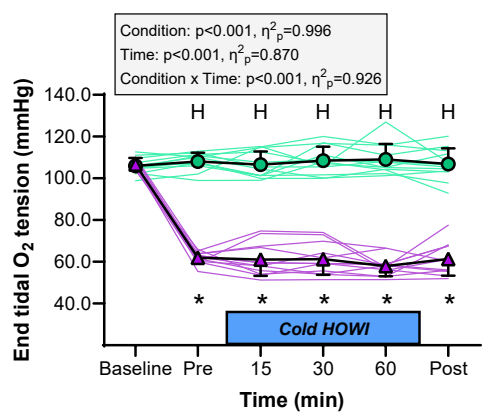
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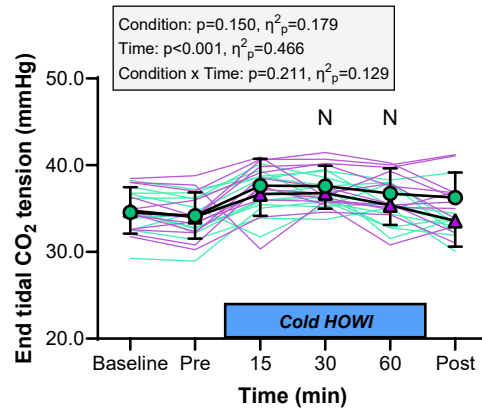
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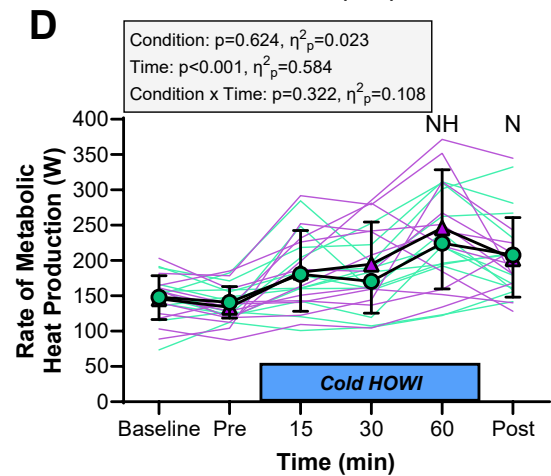
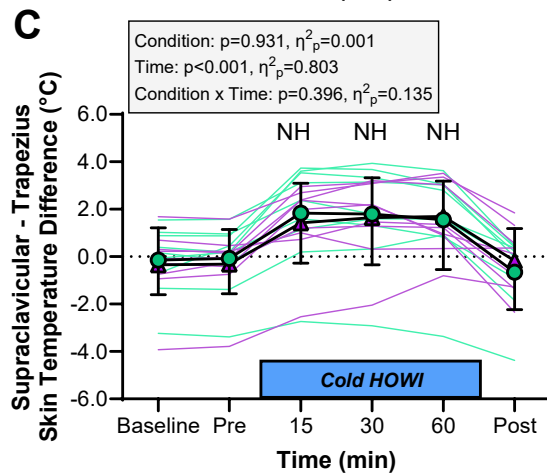
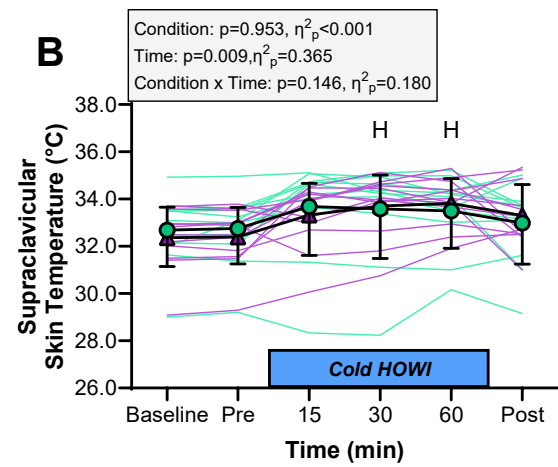
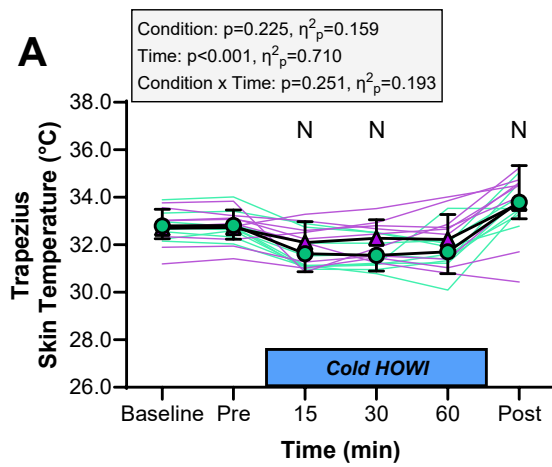
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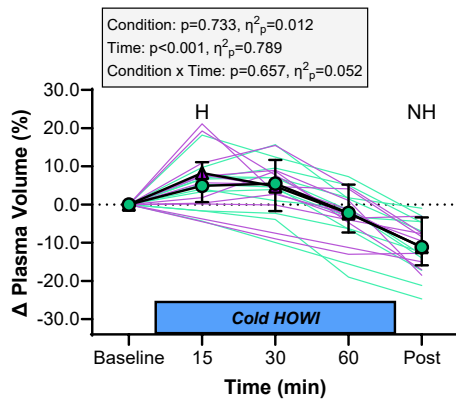
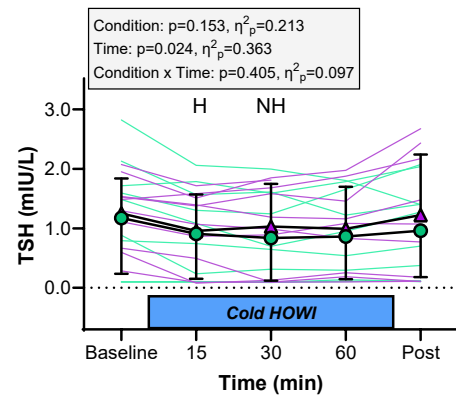
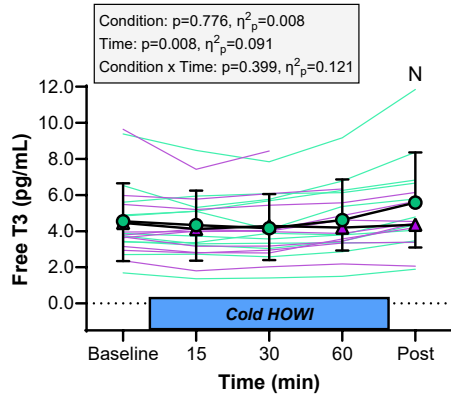
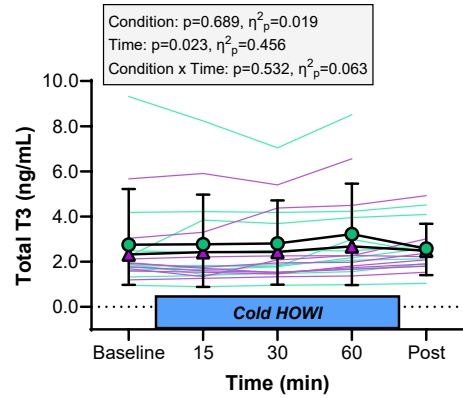
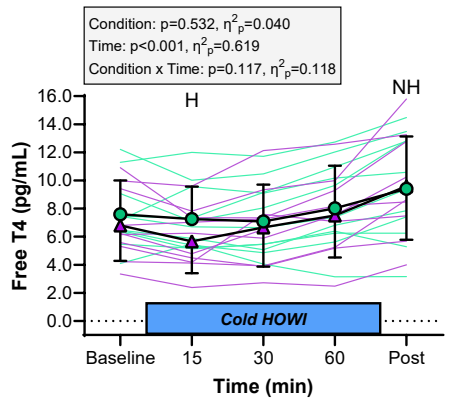
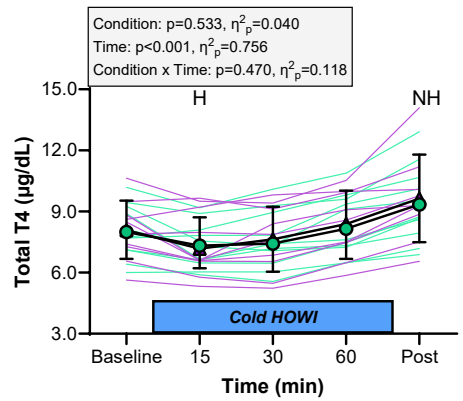
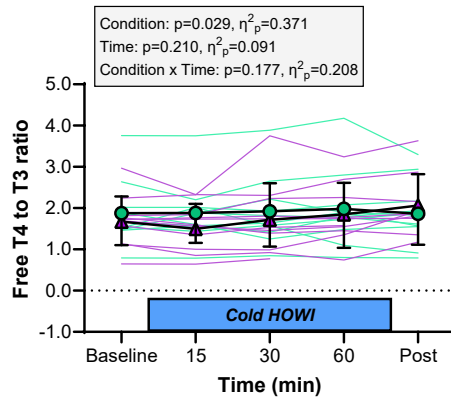
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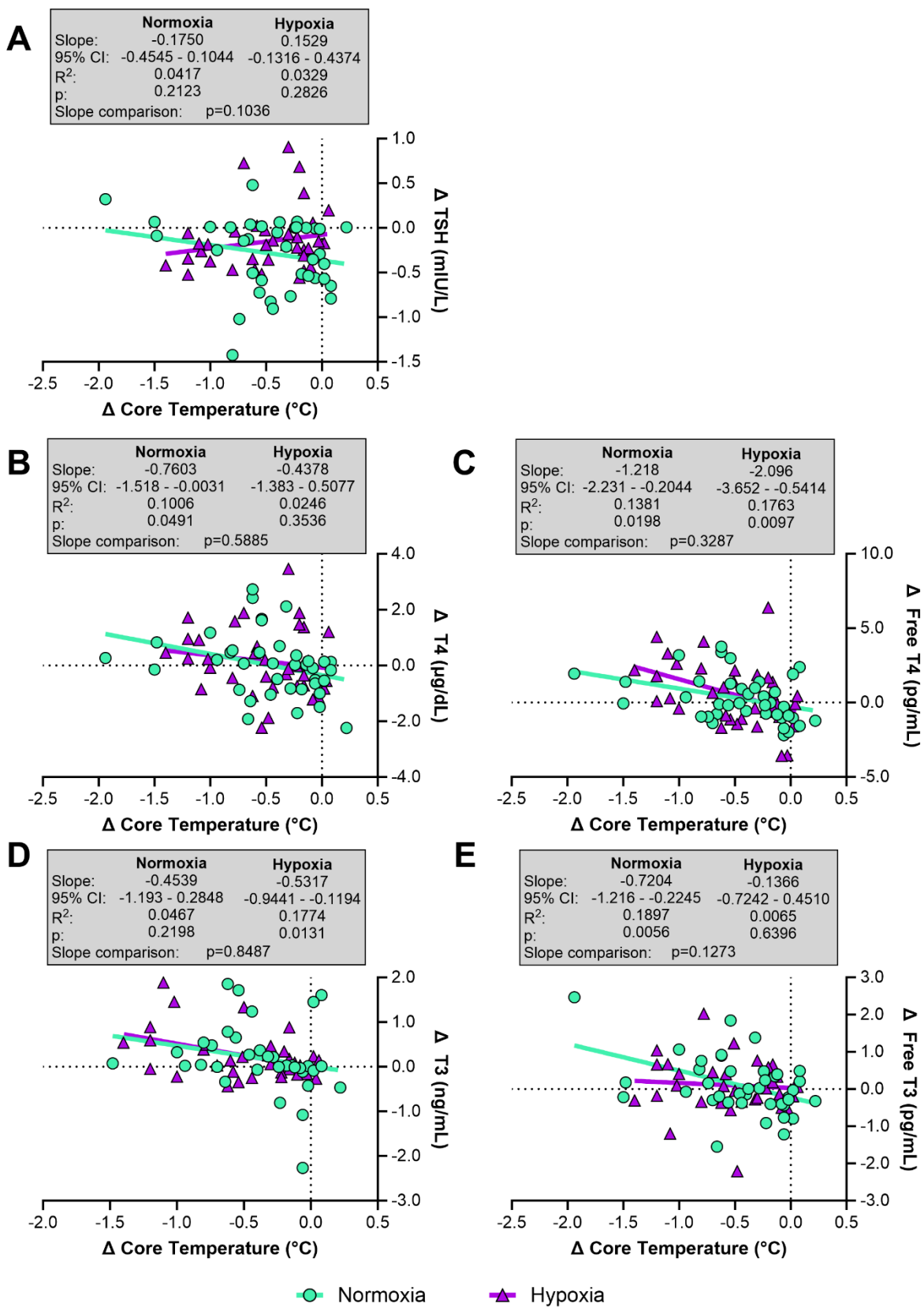
● Normoxia ▲ Hypoxia



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● Normoxia ▲ Hypoxia



Peer-reviewed abstract:

Keeler JM, Greenshields JT, Goss CS, Baker TB, Chapman RF, Johnson BD, **Schlader ZJ**. Modification of thermoregulation during cold water immersion by normobaric hypoxia. Undersea and Hyperbaric Medicine Annual Scientific Meeting, Virtual, 2021.

Modification of thermoregulation during cold water immersion by normobaric hypoxia

Jason M. Keeler, Joel T. Greenshields, Curtis S. Goss, Tyler B. Baker, Robert F. Chapman, Blair D. Johnson, Zachary J. Schlader

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Introduction

Breath-hold divers regularly develop hypoxia while diving in cold water. Hypoxia may delay and reduce the magnitude of shivering during cold exposure, which could reduce energy expenditure and the maintenance of core temperature during cold-water immersion. This study tested the hypothesis that normobaric hypoxia decreases total energy expenditure and causes a greater decrease in core temperature during head out cold-water immersion.

Materials and Methods

In a randomized crossover design, 7 healthy adults (27 ± 2 y, 1 woman) completed up to one hour of head out cold (22°C) water immersion breathing either normobaric normoxia ($\text{FiO}_2=0.21$) or hypoxia ($\text{FiO}_2=0.13$). Data were collected before (pre-), at 30 min (mid-), and at the end of water immersion (end-). Energy expenditure (EE) was estimated from indirect calorimetry. Arterial oxyhemoglobin saturation (SpO_2), rectal temperature, and bilateral (supraclavicular, trapezius) skin temperatures (T_{skin}) were measured. Supraclavicular T_{skin} provided an index of brown adipose tissue activation, while trapezius T_{skin} was a comparative control location. Data are presented as mean $\pm$ SD.

Results

SpO_2 was lower in hypoxia (90 ± 3 vs. $98 \pm 2\%$, $P < 0.01$). Rectal temperature did not differ at pre-immersion ($P = 0.56$) and decreased in both trials ($P < 0.01$) but was lower at mid- (36.8 ± 0.6 vs. $37.1 \pm 0.4^\circ\text{C}$, $P < 0.01$) and end- (36.4 ± 0.6 vs. $36.6 \pm 0.6^\circ\text{C}$, $P = 0.059$) immersion in hypoxia. EE did not differ at pre-immersion ($P = 0.80$) and increased in both trials ($P < 0.01$) but did not differ between hypoxia (end-: 12.8 ± 4.8 kJ) and normoxia (end-: 12.2 ± 2.7 kJ, $P = 0.98$). Neither supraclavicular ($P = 0.91$) nor trapezius ($P = 0.20$) T_{skin} differed at pre-immersion. Supraclavicular T_{skin} increased ($P < 0.01$) and trapezius T_{skin} decreased ($P = 0.061$) but did not differ between trials at mid- ($P \geq 0.53$) or end- ($P \geq 0.78$) immersion.

Conclusions

Normobaric hypoxia likely impairs the maintenance of core temperature during cold water immersion, which is unlikely related to differences in energy expenditure or brown adipose tissue activation.

Supported by an award from the Office of Naval Research (N00014-20-1-2593)

Peer-reviewed abstract:

Greenshields JT, Goss CS, Keeler JM, Baker TB, Logan RT, Chapman RF, Johnson BD, **Schlader ZJ**. HPT-axis activation during cold head out water immersion is not modified by moderate hypoxia. Experimental Biology, Philadelphia PA, USA 2022.

HPT- Axis Activation During Cold Head Out Water Immersion is Not Modified by Moderate Hypoxia

Joel T. Greenshields, Curtis S. Goss, Jason M. Keeler, Tyler B. Baker, Richara T. Logan, Robert F. Chapman, Blair D. Johnson, Zachary J. Schlader

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Purpose

The Bajau, an indigenous population renowned for breath-hold diving, have comparatively large spleen volumes. This adaptation is purportedly indicative of a greater capacity to store red blood cells, which may aid operations in the aquatic environment. Spleen size in the Bajau is associated with the PDE10A genotype that encodes an enzyme that catalyzes the release of thyroxine (T4) from the thyroid gland. T4 is readily released during cold stress as the terminus to activation of the hypothalamus-pituitary-thyroid (HPT) -axis to increase metabolic rate. T4, and downstream counterpart triiodothyronine (T3), can also stimulate erythropoiesis by increasing erythropoietin production, which can be further promoted with hypoxia. Thus, exposure to cold water may augment erythropoiesis as hypoxia develops during breath-hold diving via activation of the HPT-axis. However, interactions between cold exposure and hypoxia on HPT-axis activation have not been directly explored. Mountaineering expedition studies indirectly suggest that T4 release is elevated by hypoxia. Thus, this study tested the hypothesis that during cold head out water immersion (HOWI) acute hypoxia augments circulating thyroid hormone concentrations compared to normoxia.

Methods

In a randomized crossover single blind design, 12 healthy adults (27 ± 2 y, 2 women) completed one hour of cold ($22.0 \pm 0.1^\circ\text{C}$) HOWI breathing either normobaric normoxia ($\text{FiO}_2 = 0.21 \pm 0.00$, NORM) or normobaric hypoxia ($\text{FiO}_2 = 0.13 \pm 0.00$, HYP). Free (f) and total (t) T3 and T4, and thyroid stimulating hormone (TSH) were measured in venous blood samples obtained before (baseline), during (15-, 30-, and 60-min), and 15 min following immersion (post-). Arterial oxyhemoglobin saturation (SpO_2) and rectal temperature were measured continuously. Data are presented as mean $\pm$ SD.

Results

SpO_2 was lower in HYP ($90 \pm 3\%$) compared to NORM ($98 \pm 1\%$, $p < .001$). Rectal temperature did not differ between HYP and NORM ($p \geq .711$) but decreased over time in both trials (HYP - baseline: $37.2 \pm 0.4^\circ\text{C}$, post-: $36.4 \pm 0.5^\circ\text{C}$, $p < .001$, NORM - baseline: $37.2 \pm 0.4^\circ\text{C}$, post-: $36.3 \pm 0.5^\circ\text{C}$, $p < .001$). From baseline to post-HOWI, fT3 (HYP: $+0.3 \pm 0.4$ pg/mL, NORM: $+0.4 \pm 0.6$ pg/mL, $p < .001$), fT4 (HYP: $+1.6 \pm 1.7$ pg/mL, NORM: $+0.7 \pm 1.3$ pg/mL, $p < .001$), tT3 (HYP: $+0.3 \pm 0.5$ ng/mL, NORM: $+0.2 \pm 0.4$ ng/mL, $p = .053$), and tT4 (HYP: $+0.6 \pm 0.4$ $\mu\text{g}\%$, NORM: $+0.4 \pm 0.5$ $\mu\text{g}\%$, $p < .001$) increased, but the magnitude did not differ between inspirates ($p \geq .165$). Relative to baseline, tT4 at 15 min (HYP: -0.2 ± 0.3 $\mu\text{g}\%$, NORM: -0.3 ± 0.3 $\mu\text{g}\%$, $p = .025$), and TSH at 30 min (HYP: -0.2 ± 0.2 $\mu\text{IU/mL}$, NORM: -0.2 ± 0.3 $\mu\text{IU/mL}$, $p = .029$) and 60 min (HYP: -0.2 ± 0.2 $\mu\text{IU/mL}$, NORM: -0.3 ± 0.4 $\mu\text{IU/mL}$, $p = .008$) of HOWI were lower, but did not differ between inspirates ($p = .921$).

Conclusions

Increases in circulating thyroid hormone concentrations during one hour of cold HOWI are not modified by normobaric hypoxia. Thus, moderate hypoxia does not likely affect activation of the HPT-axis during short duration cold exposure. Further investigation is warranted to determine the possibility of circulating thyroid hormones to augment the erythropoietic response to acute hypoxia during cold exposure.

Supported by Office of Naval Research award N00014-20-1-2593.

Can hemoglobin mass be accurately measured non-invasively via carbon monoxide rebreathing?

Peer-reviewed abstract:

Tourula E, Keeler JM, Baker TB, Logan R, Hess HW, Carter SJ, Johnson BJ, Chapman RF, **Schlader ZJ**. Pulse oximetry carboxyhemoglobin saturation overestimates carbon monoxide rebreathing derived measures of total hemoglobin mass in health adults. American College of Sports Medicine Annual Meeting, San Diego, CA, USA, 2022.

Pulse oximetry carboxyhemoglobin saturation overestimates carbon monoxide rebreathing derived measures of total hemoglobin mass in healthy adults

Erica Tourula, Jason M. Keeler, Tyler B. Baker, Richara Logan, Hayden W. Hess, Stephen J. Carter, Blair D. Johnson FACSM, Robert F. Chapman FACSM, Zachary J. Schlader FACSM

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Purpose: Carbon monoxide (CO) rebreathing is commonly used to measure total hemoglobin mass (Hb_{mass}). This test requires measurement of carboxyhemoglobin saturation (COHb) from blood samples collected before and serially after the CO rebreathing procedures. However, this method is invasive and expensive. Pulse CO-oximetry may be a portable, cost-effective and non-invasive tool for estimating carboxyhemoglobin saturation (SpCO). Two studies have compared SpCO derived measurement of total Hb_{mass} against COHb-based analyses, with one study showing acceptable and the other showing poor agreement. This study tested the hypothesis that SpCO derived measurement of total Hb_{mass} has poor agreement with COHb-based analyses.

Methods: Following 20 min of supine rest, fourteen healthy adults (2 women, age: 28 ± 5 y, body surface area: 2.0 ± 0.3 m²) completed a modified 2 min CO rebreathing test that involved the delivery of 1.0 mL CO per kg body mass into a closed circuit spirometer connected to a 5 L anesthesia bag filled with 100% O₂ and a CO₂ scrubber. Capillary blood was taken pre- and 6 and 8 min post-CO rebreathing commencement and analyzed for COHb (OSM3, Radiometer, sensitive to the nearest tenth). SpCO was recorded for 1 min before and every 30 s during and following rebreathing using pulse CO-oximetry (Rad-57, Masimo Corp., sensitive to the nearest whole-number). Data are presented as mean $\pm$ SD.

Results: Following CO rebreathing the peak increase in COHb ($6.0 \pm 1.2\%$) was greater than the peak increase in SpCO ($5 \pm 1\%$, $P=0.008$), which was consistent when COHb was rounded to the nearest whole-number ($P=0.004$). Total Hb_{mass} was lower when calculated via COHb (951 ± 301 g) versus SpCO (1145 ± 320 g, $P=0.041$). Total Hb_{mass} calculated via COHb was moderately, positively correlated with SpCO derived values ($r=0.470$, $P=0.090$). Bland-Altman plot analyses indicated wide 95% limits of agreement (-821 to 434 g) and a bias towards overestimating total Hb_{mass} with SpCO (Bias: $+194 \pm 320$ g), although 13 of 14 (93%) individual differences fell within the limits of agreement.

Conclusion: Pulse oximetry derived SpCO has poor agreement and overestimates CO rebreathing measures of total Hb_{mass} in healthy adults compared to when COHb is measured using blood gas analyses.

Supported by Office of Naval Research Award N00014-20-1-2593.

Peer-reviewed abstract:

Lignier MJ, Tourula E, Keeler JM, Hite MJ, Regester A, Carter SJ, Johnson BD, Chapman RF, **Schlader ZJ**.
Determination of total hemoglobin mass by pulse CO-oximetry in health men and women. American College of Sports Medicine Annual Meeting, Boston, MA, USA, 2024.

Determination of Total Hemoglobin Mass by Pulse CO-Oximetry in Healthy Men and Women

Maxime Jeanovitch Lignier, Erica Tourula, Jason M. Keeler, M. Jo Hite, Amanda Regester, Stephen J. Carter, Blair D. Johnson FACSM, Robert F. Chapman FACSM, Zachary J. Schlader FACSM

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Purpose: Carbon monoxide (CO) rebreathing is commonly used to determine total hemoglobin mass (Hb_{mass}), which requires measurement of carboxyhemoglobin saturation (COHb). Pulse CO-oximetry may be an alternative to estimate total Hb_{mass} via measurement of carboxyhemoglobin saturation (SpCO). Previous studies have compared SpCO derived measurement of total Hb_{mass} against COHb-based analyses and agreement results remain equivocal. However, 85% of the subjects in those studies were men. We tested the hypothesis that SpCO derived measurement of total Hb_{mass} has poor agreement with COHb-based analyses in both men and women.

Methods: Following 20 min of supine rest, 45 healthy adults (20 women) completed a modified 2 min CO rebreathing test involving the delivery of 1.0 mL CO per kg body mass into a closed-circuit spirometer connected to a 5 L anesthesia bag filled with 100% O_2 and a CO_2 scrubber. Capillary blood was taken pre- and 6- and 8-min post-CO rebreathing commencement and analyzed for COHb (OSM3, Radiometer). SpCO was recorded for 1 min before and every 30 s during and following rebreathing using pulse CO-oximetry (Rad-57, Masimo Corp.).

Results: Among all subjects, total Hb_{mass} calculated via COHb (890 ± 244 g) vs. SpCO (957 ± 347 g) did not differ ($p=0.100$), and COHb and SpCO derived values were positively correlated ($r=0.639$, $p<0.001$). Bland-Altman plot analyses indicated wide 95% limits of agreement (592 to -458 g) and a bias towards overestimating total Hb_{mass} with SpCO (by 67 ± 268 g), which was greater at higher levels of Hb_{mass} (slope: $+0.425$, $p=0.004$). Similar trends were observed between genders, where total Hb_{mass} calculated via COHb vs. SpCO did not differ ($p \geq 0.150$). COHb and SpCO derived Hb_{mass} were correlated in men ($r=0.531$, $p<0.009$) but not women ($r=0.290$, $p=0.191$). Wide 95% limits of agreement and a bias towards overestimating total Hb_{mass} with SpCO (by 88 ± 284 g & 45 ± 255 g for men and women) were also observed. The difference between COHb and SpCO derived total Hb_{mass} did not differ between men (-40 ± 136 g/m²) and women (-21 ± 158 g/m², $p=0.662$).

Conclusion: In comparison to COHb measured via blood gas analyses, pulse CO-oximetry overestimates CO rebreathing measures of total Hb_{mass} in healthy adults. The magnitude of such differences persists independent of gender.

Support: R01OH011528 & N00014-20-1-2593

Appendix 2

The following abstracts have been presented or accepted for presentation at national/international conferences. These abstracts are derived from the primary data collection under this project.

- Keeler JM, Hess HW, Tourula E, Hite MJ, Chapman RF, Johnson BD, **Schlader ZJ**. The combined effect of 14 days of hypoxia and apnea training on hemoglobin mass and reticulocyte counts. American Physiology Summit, Long Beach, CA, USA, 2023
- Walker C, Keeler JM, Tourula E, Hite MJ, Chapman RF, Johnson BD, **Schlader ZJ**. Fourteen days of hypoxia and apnea training does not modify pulmonary gas exchange efficiency. American College of Sports Medicine Annual Meeting, Denver, CO, USA, 2023
- Keeler JM, Hite MJ, Hess HW, Tourula E, Chapman RF, Johnson BD, **Schlader ZJ**. The effects of hypoxia and apnea training on hypoxemic and hypercapnic tolerance. American College of Sports Medicine Annual Meeting, Denver, CO, USA, 2023
- **Schlader ZJ**, Keeler JM, Hite MJ, Hess HW, Tourula E, Chapman RF, Johnson BD, Thom SR. Preliminary evidence that fourteen days of apnea and hypoxia exposures reduces circulating pro-inflammatory microparticles. Undersea and Hyperbaric Medicine Annual Scientific Meeting, San Diego, CA, USA, 2023
- Skutnik BC, Keeler JM, Hite MJ, Hess HW, Tourula E, Walker C, Chapman RF, Johnson BD, **Schlader ZJ**. Fourteen days of cool water immersion does not modify an indirect index of brown adipose tissue activation. American Physiology Summit. Long Beach, CA, USA, 2024
- Keeler JM, Hite MJ, Hess HW, Tourula E, Chapman RF, Johnson BD, Ilardo M, Thom SR, **Schlader ZJ**. No relation between increases of spleen volume and reductions in circulating pro-inflammatory microparticles following fourteen days of apnea and hypoxia exposures. American Physiology Summit. Long Beach, CA, USA, 2024
- Keeler JM, Hite MJ, Hess HW, Tourula E, Chapman RF, Johnson BD, Ilardo M, Thom SR, **Schlader ZJ**. Hypoxia and apnea training during water immersion (but not air) increases spleen volume. American College of Sports Medicine Annual Meeting, Boston, MA, USA, 2024.
- Bobbitt G, Keeler JM, Hite MJ, Hess HW, Tourula E, Thom S, Ilardo M, Chapman RF, Johnson BD, **Schlader ZJ**. No effect of water immersion on training-induced improvements in apnea duration. American College of Sports Medicine Annual Meeting, Boston, MA, USA, 2024.
- **Schlader ZJ**, Keeler JM, Hite MJ, Hess HW, Tourula E, Chapman RF, Ilardo M, Johnson BD, Thom SR. No correlation between increases in spleen volume and change in circulating pro-inflammatory microparticles following fourteen days of apneas and hypoxia exposures. Undersea and Hyperbaric Medicine Annual Scientific Meeting, New Orleans, LA, USA, 2024.

The combined effects of 14 days of hypoxia and apnea training on hemoglobin mass and reticulocyte counts

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INTRODUCTION: Indigenous diving populations like the Ama and Bajau have extraordinary breath-hold abilities, which includes tolerating low levels of oxygen and high levels of carbon dioxide. While these populations have genetic adaptations that improve their hypoxemic and hypercapnic tolerances, other populations can improve these tolerances through training adaptations that allow for increases in oxygen carrying and pH buffering capacities. For example, two weeks of residence at altitude stimulates increase in hemoglobin mass (Hb_{mass}) via erythropoiesis. While completing 10 maximal apneas daily for 14 days elevates reticulocyte count. However, it is unclear whether relatively brief (<4 h) daily exposures to moderate hypoxia and maximal apneas, such as may occur with diving, meaningfully stimulates erythropoiesis in people without breath hold diving experience or ancestry.

PURPOSE: Test the hypothesis that 14 days of daily 3 h of moderate hypoxia and 10 maximal apneas augments circulating Hb_{mass} and reticulocyte count.

METHODS: Eighteen healthy adults (11 women; 27 ± 6 y) completed 14 days of training within a 16-day period. Training days consisted of 10 maximal apneas and 3 h of exposure to normobaric hypoxia ($FiO_2=0.14$). Average apnea duration for each day was recorded and presented as mean $\pm$ SD. On Days 1, 7, and 14 subjects completed a carbon monoxide rebreath procedure pre and post training to estimate Hb_{mass} ($n=11$) and provided a venous sample of blood for reticulocyte count ($n=14$) pre, post and 2-hr post training. Repeated measures linear mixed models were used to assess change in Hb_{mass} and reticulocytes from Day 1. These data are presented as mean difference (diff.) and 95% confidence intervals.

RESULTS: Average apnea duration increased from Day 1 (69 ± 29 s) to Day 7 (83 ± 30 s, $p < 0.01$) and to Day 14 (90 ± 29 s), but there was no change from Day 7 to Day 14 ($p = 0.26$). There was no interaction ($p = 0.79$) or effects of Day ($p = 0.54$) or pre/post training ($p = 0.94$) on Hb_{mass} . Specifically, pre training Hb_{mass} did not differ from Day 1 on Day 7 (mean diff. 23 (–131, 178) g, $p = 0.91$) or Day 14 (mean diff. 5 (–154, 164) g, $p = 0.99$) and post training Hb_{mass} did not differ Day 1 on Day 7 (mean diff. 29 (–152, 210) g, $p = 0.90$) or Day 14 (mean diff. 5 (–154, 164) g, $p = 0.99$). There was an interaction effect ($p = 0.02$) for reticulocyte count, which showed a mean increase from pre to post training on Day 14 (mean diff. 9 (3, 15) $\# \mu L^{-1}$, $p < 0.01$). However, there was no change in reticulocyte count between Days ($p = 0.53$). The pre training Day 1 reticulocyte count was not different from Day 7 (mean diff. -3 (–9, 4) $\# \mu L^{-1}$, $p = 0.48$) or Day 14 (mean diff. -2 (–9, 6) $\# \mu L^{-1}$, $p = 0.79$).

CONCLUSION: Fourteen days of brief daily exposures to moderate hypoxia and maximal apnea training did not augment circulating Hb_{mass} or reticulocytes.

Supported by Office of Naval Research award N00014-20-1-2593.

Fourteen days of hypoxia and apnea training does not modify pulmonary gas exchange efficiency

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Breath hold divers have extraordinary tolerance to low levels of oxygen and high levels of carbon dioxide. Training induced improvements in apnea performance are speculated to be, at least partially, due to increases in oxygen carrying and pH buffering capacities, which may improve pulmonary gas exchange. However, it is unclear whether relatively brief (<4 h) daily exposures to moderate hypoxia and maximal apneas, such as may occur with diving, modifies pulmonary gas exchange efficiency (GEE) and function in people without breath hold diving experience.

Purpose: Test the hypothesis that 14 days of daily 3 h of moderate hypoxia and 10 maximal apneas augments pulmonary GEE and function.

Methods: 17 healthy adults (27 ± 6 y; 11 women) completed 14 days of training within a 16-day period. Training days consisted of 10 maximal apneas and 3 h of exposure to normobaric hypoxia ($\text{FiO}_2 = 0.14$). On Days 1, 7, 14 subjects completed pulmonary function testing, which included measurements of forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), FEV_1/FVC , force expiratory flow at 25-75% ($\text{FEF}_{25-75\%}$) and peak expiratory flow (PEF), and a noninvasive assessment of pulmonary GEE in which the primary outcome variable was the alveolar-arterial O_2 difference (O_2 debt). Data are presented as mean $\pm$ SD.

Results: Apnea duration increased from Day 1 (69 ± 29 s) to Day 7 (83 ± 30 s, $p < 0.01$) and to Day 14 (90 ± 29 s, $p < 0.01$), but there was no change from Day 7 to Day 14 ($p = 0.26$). There were no changes in FVC (main effect: $p = 0.14$), FEV_1 (main effect: $p = 0.29$), FEV_1/FVC (main effect: $p = 0.29$), or $\text{FEF}_{25-75\%}$ (main effect: $p = 0.21$). PEF increased from Day 1 (7.5 ± 1.9 L/min) to Day 7 (8.0 ± 1.9 L/min, $p = 0.03$) and Day 14 (8.4 ± 1.5 L/min, $p = 0.01$), but was not changed from Day 7 to Day 14 ($p = 0.13$). O_2 debt did not differ (main effect: $p = 0.25$) between Days 1 (-0.7 ± 6.1 mmHg), 7 (2.3 ± 7.0 mmHg) and 14 (0.2 ± 6.5 mmHg).

Conclusion: PEF increased with as little as 7 days of moderate hypoxia and maximal apnea training. However, no other changes in pulmonary function or gas exchange efficiency were observed. Further research is required to better understand how relatively brief daily exposures to moderate hypoxia and maximal apneas improves breath-hold capabilities

Funding: Office of Naval Research (N00014-20-1-2593)

The effects of hypoxia and apnea training on hypoxemic and hypercapnic tolerance

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Breath-hold divers have extraordinary apneic abilities due to an enhanced ability to suppress ventilatory drive as the arterial partial pressures of carbon dioxide rise and oxygen falls. Indeed, increased hypercapnic and hypoxemic tolerance is associated with longer apnea durations. Previous work demonstrated that 12 days of maximal apnea training increases apnea duration. However, it is unclear whether relatively brief daily exposures to moderate hypoxia and maximal apneas, such as may occur with diving, stimulates adaptations to improve hypercapnic and hypoxemic tolerances in people without breath hold diving experience or ancestry.

PURPOSE: Test the hypothesis that 14 days of moderate hypoxia for 3 hours per day and 10 maximal apneas per day augments the immediately post-apnea partial pressure of end tidal (P_{ET}) of CO_2 and reduces $P_{ET}O_2$.

METHODS: Eleven healthy adults (27 ± 6 y; 7 women) completed 14 days of training within a 16-day period. Training days consisted of 10 maximal apneas with 5 apneas prior to 3 hours of exposure to normobaric hypoxia ($FiO_2=0.14$), and 5 maximal apneas post exposure. Average apnea duration, heart rate (HR) nadir, arterial oxyhemoglobin saturation (SpO_2) nadir and the immediately post-apnea peak $P_{ET} CO_2$ and $P_{ET} O_2$ nadir were recorded. Data are presented as the average of the last four apneas during the first set of 5 apneas on Days 1 and 14 to ensure no confounding effect of prior hypoxia. Data are presented as mean $\pm$ SD.

RESULTS: Average apnea duration increased from Day 1 (56 ± 14 s) to Day 14 (89 ± 36 s, $p < 0.01$). There was a decrease in HR nadir from Day 1 (60 ± 13 bpm) to Day 14 (52 ± 11 bpm, $p = 0.04$). Subject's SpO_2 nadir was lower on Day 14 ($90 \pm 5\%$) compared to Day 1 ($94 \pm 2\%$, $p = 0.03$). $P_{ET}CO_2$ did not differ between Day 1 (52 ± 4 mmHg) and Day 14 (51 ± 6 mmHg, $p = 0.65$). $P_{ET}O_2$ did not differ between Day 1 (89 ± 11 mmHg) and Day 14 (87 ± 17 mmHg, $p = 0.81$).

CONCLUSION: Fourteen days of brief daily exposures to moderate hypoxia and maximal apneas increased apnea duration in which subjects tolerated greater drops in SpO_2 . There was no change in post-apnea $P_{ET}CO_2$ and $P_{ET}O_2$, which suggests that the rate of CO_2 accumulation during the apneas was reduced.

Supported by Office of Naval Research award N00014-20-1-2593.

Preliminary evidence that fourteen days of apnea and hypoxia exposures reduces circulating pro-inflammatory microparticles

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Introduction/Background: Indigenous populations renowned for breath-hold diving have comparatively large spleen volumes, suggesting a functional benefit of a larger spleen in the context of diving. The spleen plays a role in clearing bloodborne microparticles (MPs). Pro-inflammatory MPs are increased with diving and have been implicated in the etiology of decompression sickness. Therefore, one potential benefit of a larger spleen may be enhanced splenic clearance of MPs. Repeated apneas and hypoxia exposure increases spleen volume in people without breath-hold diving ancestry. This study tested the hypothesis that repeated apnea and hypoxia exposures reduce blood-borne pro-inflammatory MPs.

Materials and Methods: 17 healthy adults completed 10 maximal apneas and 3 h of exposure to normobaric hypoxia ($F_{iO_2}=0.14$) for 14 days within a 16-day period. On Days 1 and 14, venous blood was collected before training, and analyzed for MPs via flow cytometry. Data are presented as mean $\pm$ SD.

Results: Average apnea duration increased from Day 1 (72 ± 29 seconds) to Day 14 (89 ± 30 seconds, $p<0.01$, Cohen's $d_z:1.1$). Total MPs decreased from Day 1 (1415 ± 838) to Day 14 (1102 ± 577 , $p=0.06$, $d_z:0.39$). MPs expressing neutrophil-specific CD66b (248 ± 252 vs. 158 ± 73 , $p=0.02$, $d_z:0.52$) and endothelium-specific CD146 (341 ± 202 vs. 253 ± 118 , $p=0.04$, $d_z:0.44$) were lower on Day 14, but MPs expressing platelet-specific CD41a did not differ between Days 1 (83 ± 80) and 14 (55 ± 39 , $p=0.17$, $d_z:0.24$). The proportion of CD66b+ neutrophils ($n=15$) co-expressing myeloperoxidase (Day 1: $5.4\pm 6.2\%$; Day 14: $4.3\pm 3.9\%$, $p=0.10$, $d_z:0.26$) or CD18 (Day 1: $7.6\pm 9.8\%$; Day 14: $7.8\pm 9.9\%$, $p=0.48$, $d_z:0.01$) did not differ between Days.

Summary/Conclusions: These findings provide preliminary evidence of a moderate effect of repeated apnea and hypoxia exposures on reductions in blood-borne pro-inflammatory MPs. Further work is needed to understand the role of the spleen in these observations.

Support: Office of Naval Research (N00014-20-1-2593)

Fourteen days of cool water immersion does not modify an indirect index of brown adipose tissue activation.

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Military divers often operate in cold water eliciting reductions in skin and core temperatures despite the use of thermal protection. Decreases in body temperatures triggers shivering and non-shivering thermogenesis, the latter of which is largely due to the activation of brown adipose tissue (BAT). Ten days of cool air exposure (15-16°C) and seven days of cold-water exposure (14°C) augments non-shivering thermogenesis. However, it is unknown if repeated exposure to cool head out water immersion (CWI) modifies non-shivering thermogenesis. This study tested the hypothesis that fourteen days of CWI for 4 h/d will increase BAT activation.

Healthy adults were randomly assigned to either CWI (n=10, 5 women) or thermoneutral head out water immersion (35°C, TNW, n=9, 5 women). CWI and TNW exposures were 4 h in duration and were completed once daily for 14 d in a 16-d period. CWI consisted of the initial immersion in 28°C water and following a 0.5°C reduction in rectal temperature, the water temperature was increased to up to 30°C so that a mild hypothermic state was maintained during the 4 h. Rectal temperature, oxygen uptake (indirect calorimetry), and supraclavicular and trapezius skin temperatures (thermocouples) were measured preexposure and during the middle 3 h of immersion. This analysis was a part of a larger study. Therefore, during the 3 h period, all participants were exposed to hypoxia (FiO₂: 0.14). The supraclavicular-trapezius skin temperature difference (T_{sk} diff) provided an index of BAT activation. Data collected on Day 1 and Day 14 are presented (mean ± SD).

Peak decreases in rectal temperature from preexposure were greater in CWI (-0.7±0.3°C) vs. TNW (-0.2±0.4°C, p<0.001) and did not differ between Day 1 and Day 14 in either group (p=0.923). Average oxygen uptake was higher in CWI (0.54±0.09 L/min) vs. TNW (0.38±0.12 L/min, p<0.001) and did not differ between Day 1 and Day 14 in either group (p=0.443). Average T_{sk} diff did not differ between groups (CWI: 2.2±0.8°C, TNW: 1.6±0.1°C, p=0.090) and did not differ between Day 1 and Day 14 in either group (p=0.111). Peak decreases in rectal temperature were significantly correlated with average oxygen uptake (r=-0.552, p<0.001) but not average T_{sk} diff (r=-0.152, p=0.362).

Fourteen days of CWI did not modify thermogenesis (oxygen uptake) nor BAT activation, as estimated from T_{sk} diff. However, a potential interaction between moderate hypoxia and BAT activation during CWI cannot be excluded. Future work is required to determine the effect of mild hypothermia, such as that experienced by divers operating in cold water even with thermal protection, on more direct markers of BAT activation.

Funding: Office of Naval Research (N00014-20-1-2593 & N00014-21-1-2276).

No relation between increases of spleen volume and reductions in circulating pro-inflammatory microparticles following fourteen days of apnea and hypoxia exposures

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INTRODUCTION: Indigenous breath-hold diving populations have comparatively large spleen volumes, suggesting a functional benefit of a larger spleen in the context of diving. The spleen plays a role in clearing blood-borne microparticles (MPs). Pro-inflammatory MPs are increased with diving and have been associated with the etiology of decompression sickness. Thus, a potential benefit of a larger spleen may be enhanced splenic clearance of MPs. Repeated apneas and hypoxia exposure increases spleen volume in people without breath-hold diving ancestry.

PURPOSE: Test the hypothesis that repeated apnea and hypoxia exposures reduce blood-borne pro-inflammatory MPs and the reduction is correlated with changes in spleen size.

METHODS: 20 healthy adults (10 women) completed 10 maximal apneas and 3 h of exposure to normobaric hypoxia ($F_{iO_2}=0.14$) for 14 days within a 16-day period. On Days 1 and 14, pre-exposure spleen volume was measured via ultrasound using the Pilström equation and venous blood was collected for blood-borne pro-inflammatory MPs analysis via flow cytometry. MPs data were log transformed for analyses. Data are presented as mean $\pm$ SD.

RESULTS: Average apnea duration increased from Day 1 (76 ± 31 s) to Day 14 (91 ± 30 s, $p=0.004$, $d_z=0.48$). Pre-exposure spleen volume increased from Day 1 (169 ± 36 ml) to Day 14 (182 ± 39 ml, $p=0.002$, $d_z=0.33$). Total MPs decreased from Day 1 (1407 ± 814 #/ μ l) to Day 14 (1092 ± 561 #/ μ l, $p=0.047$, $d_z=0.45$). MPs expressing CD66b+ (neutrophil origin; 194 ± 89 vs. 156 ± 72 #/ μ l, $p=0.014$, $d_z=0.47$) and CD146 (endothelial origin; 337 ± 197 vs. 250 ± 116 #/ μ l, $p=0.037$, $d_z=0.53$) were lower on Day 14, but MPs expressing CD41a (platelet origin) did not differ between Days 1 (82 ± 78 #/ μ l) and 14 (55 ± 38 #/ μ l, $p=0.166$, $d_z=0.45$). Changes in MP counts from Day 1 to Day 14 were not correlated with changes in spleen volume for Total MPs ($r=0.02$, $p=0.93$) nor MPs expressing CD66b+ ($r=0.30$, $p=0.23$), CD146 ($r=-0.04$, $p=0.86$), or CD41a ($r=0.31$, $p=0.20$).

CONCLUSION: Repeated apnea and hypoxia exposures increase spleen volume and reduce blood-borne pro-inflammatory MPs. However, there was no relation between exposure-induced changes in spleen size and MP counts. Thus, spleen size may not directly impact MP clearance. Rather, alterations in the MP phagocytic properties found within the spleen or another systemic change likely contribute to the observed reductions in MPs. Further work is needed to understand the role of splenic function in these observations.

Support: Office of Naval Research (N00014-20-1-2593)

Hypoxia and Apnea Training During Water Immersion (But Not Air) Increases Spleen Volume

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Indigenous breath-hold divers have comparatively large spleen volumes (SV), likely due to natural selection. SV is positively associated with apnea duration in breath-hold divers. Thus, increasing SV may be an adaptation to diving related stressors, such as apneas, relative hypoxemia, water immersion, and thermal stress. Twelve days of apnea training increases apnea duration and SV. Moreover, SV is positively associated with thyroid hormone bioavailability, which is increased with cold exposure, suggesting that cold water may augment training-induced splenic growth. However, it is unknown whether repeated exposure to diving related stressors modifies SV in people without breath hold diving experience or ancestry.

PURPOSE: Test the hypothesis that 14 d of moderate hypoxia for 3 h and 10 maximal apneas increases SV, and that increases in SV will be greater with cold water immersion.

METHODS: 27 healthy adults completed 14 days of training within a 16-day period. Training consisted of 5 apneas prior to and following 3 h of exposure to normobaric hypoxia ($\text{FiO}_2=0.14$). Subjects underwent one of three conditions for the entirety of the training: thermoneutral air (Air, $n=8$, 5 women), thermoneutral (TN) head out water immersion (HOWI) ($\sim 35.5^\circ\text{C}$, $n=9$, 5 women), or cool (CW) HOWI ($\sim 28^\circ\text{C}$, $n=10$, 5 women). On Days 1 and 14, SV was measured (ultrasound) while seated in air and calculated using the Pilström equation.

RESULTS: Average apnea duration increased from Day 1 (79 ± 33 s) to Day 14 (98 ± 36 s, $p<0.01$), but no condition or interaction ($p\geq 0.41$) effects were observed. SV increased from Day 1 (163 ± 37 ml) to Day 14 (180 ± 39 ml, main effect of time: $p<0.01$). Post hoc analysis indicated that SV increased with HOWI (TN HOWI: mean diff. 15 ml, $p=0.02$, CW HOWI: mean diff. 26 ml, $p<0.01$), but not Air (mean diff: 9 ml, $p=0.39$). The change in SV from Day 1 to Day 14 was not different between Air and TN HOWI ($p=0.68$) or TN and CW HOWI ($p=0.26$) but was higher in CW HOWI compared to Air ($p=0.05$). Changes in SV were not correlated with changes in apnea duration ($r = -0.29$, $p = 0.15$).

CONCLUSION: SV increased when 14 days of exposure to moderate hypoxia and apneas took place in HOWI. Further research is needed to better understand the diving relevant functional benefits underlying the observed splenic growth.

Support: Office of Naval Research (N00014-20-1-2593)

No effect of water immersion on training-induced improvements in apnea duration

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Apnea training increases tolerance to hypercapnia and hypoxemia. Apnea training often occurs out of water or when submerged in water, the latter of which increases thoracic pressure and causes mild CO₂ retention. We have previously demonstrated that apnea time is acutely elevated during head-out water immersion (HOWI). However, it is unknown whether training improvements in apnea duration are modified when completed during HOWI compared to air.

Purpose: Test the hypothesis that training-induced improvements in apnea duration are heightened during HOWI.

Methods: Nineteen healthy adults completed 14 days of training within a 16-day period. Training consisted of five maximal apneas prior to 3 h of moderate normobaric hypoxia (FiO₂: 0.14) and five maximal apneas post-hypoxia. Apneas were separated by 2.5 min of rest. The HOWI group (n=9, 5 women) were immersed (mid-sternum) while seated in the upright position in thermoneutral water (~35.5°C). The air (AIR) group (n=10, 5 women) sat in the upright seated position in moderate temperature air (~23°C). During training, apnea duration, heart rate nadir and arterial oxyhemoglobin saturation (SpO₂) nadir were recorded. In this analysis, pre-hypoxia apneas on Days 1, 7 and 14 are included and data were averaged over apneas 3-5 (to control for splenic contraction). Data are presented as mean±SD.

Results: On Day 1, apnea duration (HOWI: 80±32 s, AIR: 71±19 s, p=0.430), heart rate nadir (HOWI: 56±7 bpm, AIR: 54±17 bpm, p=0.728) and SpO₂ nadir (HOWI: 88±8%, AIR: 93±2%, p=0.114) did not differ between groups. Compared to Day 1, apnea duration increased by 21±5 s on Day 7 and 23±12 s on Day 14 (main effect of time: p=0.002) but did not change differently between groups (group x time: p=0.324). Neither heart rate nadir nor SpO₂ nadir changed with training (main effect of time: p≥0.264) and did not change differently between groups (group x time: p≥0.541).

Conclusions: Training-induced improvements in apnea duration were not modified by water immersion. Furthermore, indices of the activation of the dive reflex (e.g., heart rate and SpO₂ nadirs) during apneas were not impacted by 14 days of apnea training.

Support: Office of Naval Research (N00014-20-1-2593)

No correlation between increases of spleen volume and changes in circulating pro-inflammatory microparticles following fourteen days of apnea and hypoxia exposures

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Introduction/Background: Breath-hold divers have comparatively large spleen volumes (SpVol). The spleen plays a role in clearing bloodborne microparticles (MPs). Pro-inflammatory MPs are associated with decompression sickness. One potential benefit of a larger SpVol may be enhanced clearance of MPs. Repeated apneas and hypoxia exposure increases SpVol, with the magnitude of adaptation modified by thermal stress. We tested the hypothesis that repeated apnea and hypoxia exposure-induced increases in SpVol are correlated with changes in pro-inflammatory MPs.

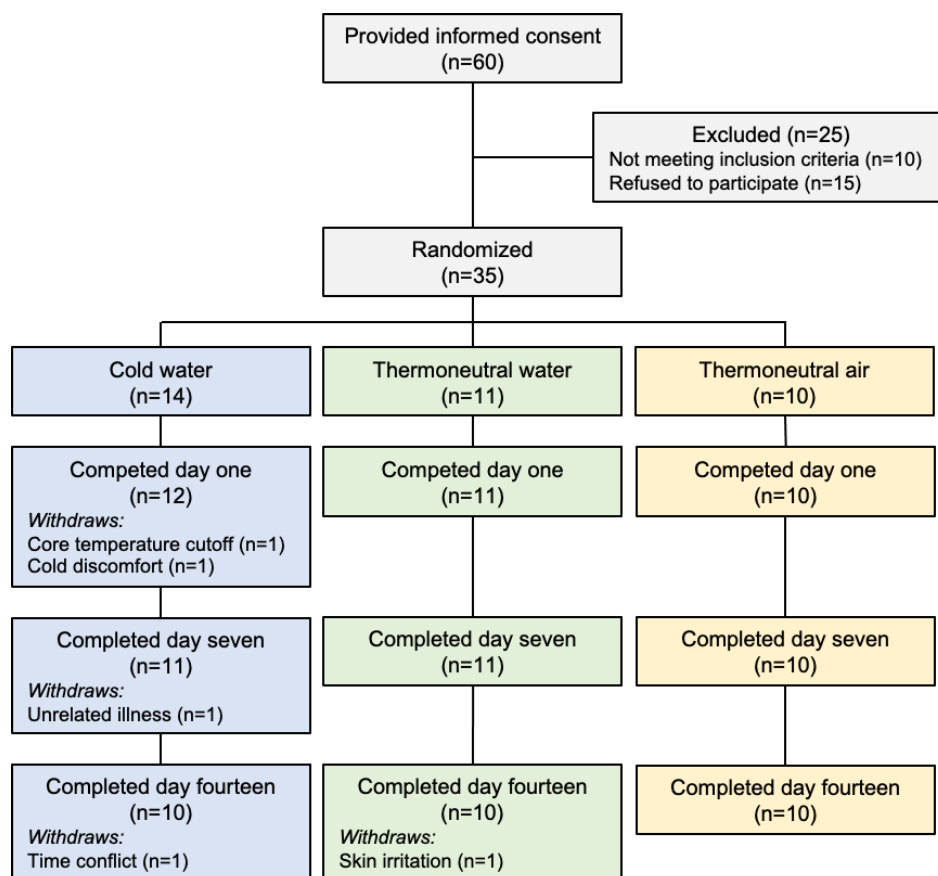
Materials and Methods: 30 healthy adults completed 10 maximal apneas and 3 h of exposure to normobaric hypoxia ($F_{iO_2}=0.14$) for 14 days within a 16-day period during exposure to cool (CW) head out water immersion (HOWI) ($\sim 28.0^{\circ}\text{C}$, $n=10$, 5 women), thermoneutral (TN) HOWI ($\sim 35.5^{\circ}\text{C}$, $n=10$, 6 women) or TN air ($n=10$, 5 women). On Days 1 and 14, pre-exposure SpVol was measured (ultrasound, Pilström equation), normalized to body surface area, and venous blood was collected for pro-inflammatory MPs analysis via flow cytometry ($n=26$).

Results: SpVol increased with TN (mean diff. 8 ml/m^2 , $p<0.01$) and CW (mean diff. 13 ml/m^2 , $p<0.01$) HOWI but not air (mean diff: 3 ml/m^2 , $p=0.16$). MPs expressing neutrophil-specific CD66b decreased from Days 1 ($248\pm 252\text{ \#/\mu L}$) to 14 ($158\pm 73\text{ \#/\mu L}$, day effect: $p=0.02$). MPs expressing platelet-specific CD41a did not differ between Days 1 ($79\pm 14\text{ \#/\mu L}$) and 14 ($58\pm 10\text{ \#/\mu L}$, day effect: $p=0.19$). Endothelium-specific CD146 MPs were lower on Day 14 in air (mean diff: $-153\text{ \#/\mu L}$, $p<0.01$) but not TN (mean diff: $-11\text{ \#/\mu L}$, $p=0.76$) or CW (mean diff: $0\text{ \#/\mu L}$, $p=0.79$) HOWI. Changes in SpVol were not correlated with changes in MPs expressing CD66b+ ($r=0.22$, $p=0.28$), CD146 ($r=0.05$, $p=0.79$), or CD41a ($r=0.21$, $p=0.31$).

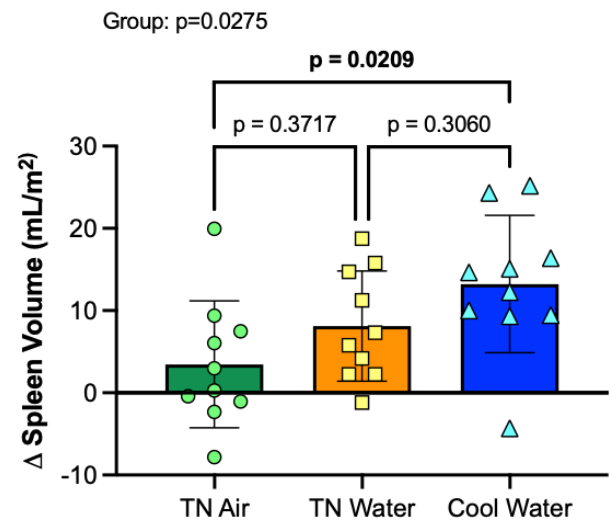
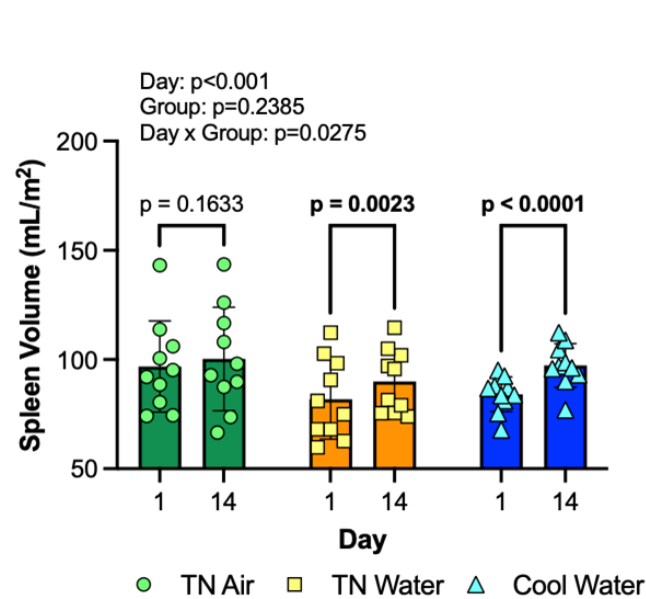
Summary/Conclusions: Repeated apnea and hypoxia exposure-induced changes in SpVol were not correlated with pro-inflammatory MPs. SpVol may not directly impact MP clearance.

Support: Office of Naval Research (N00014-20-1-2593)

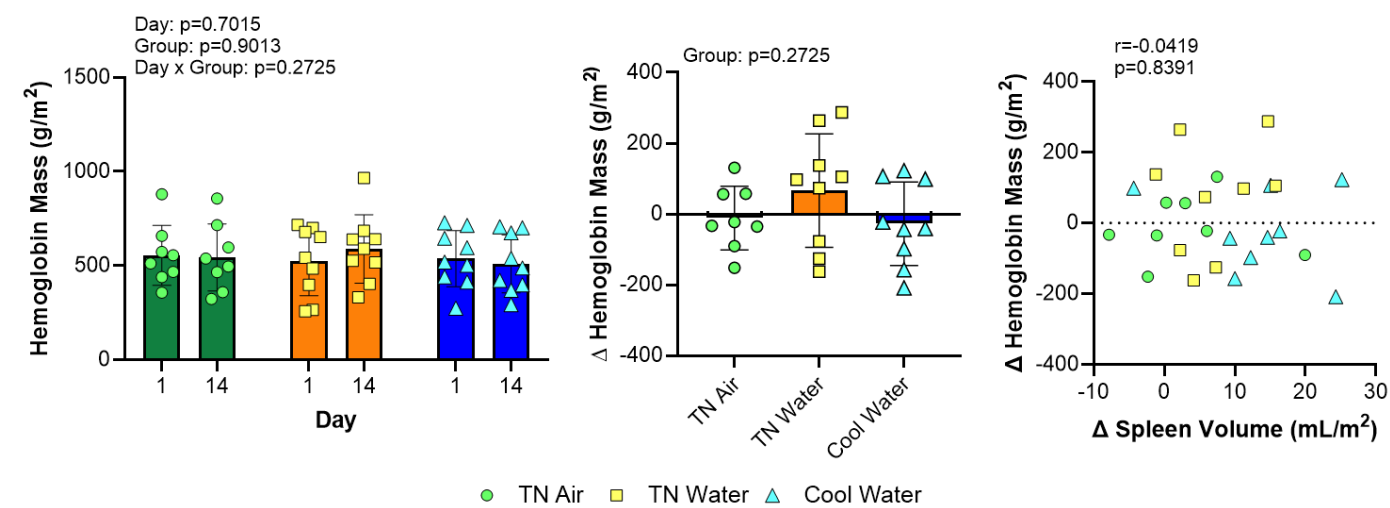
Appendix 3



Spleen size:

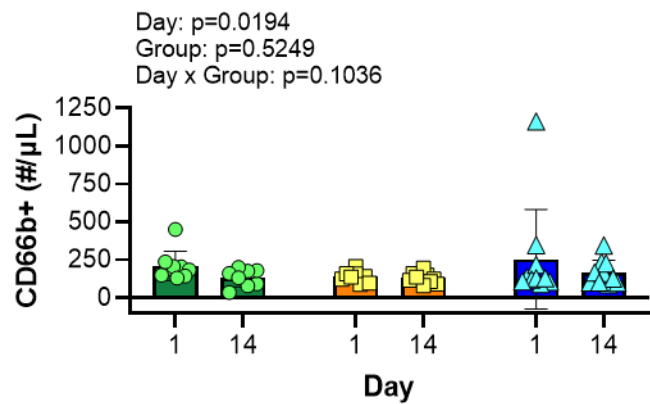


Hemoglobin mass:

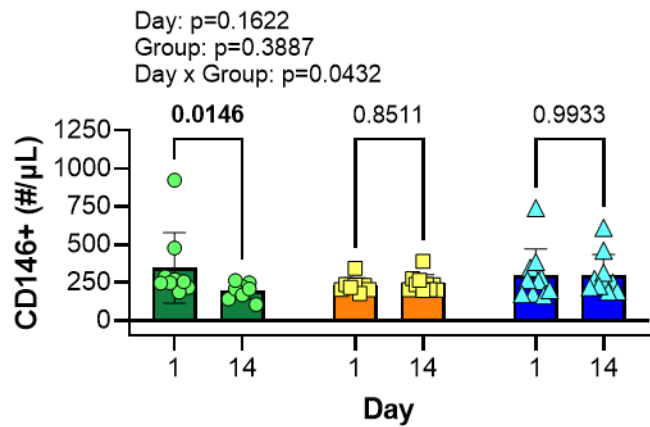


Pro-inflammatory microparticles:

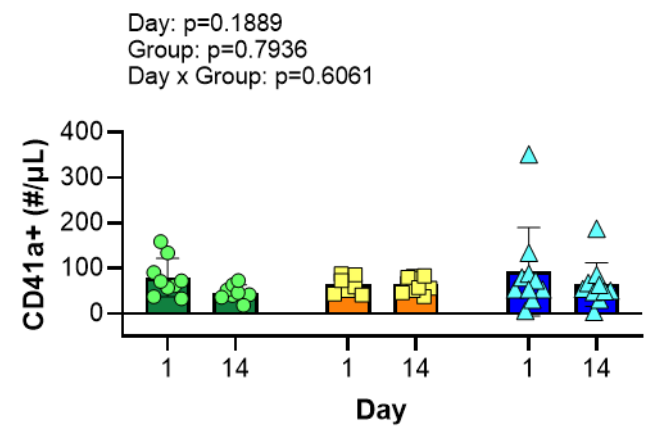
Neutrophil-specific



Endothelium-specific



Platelet-specific

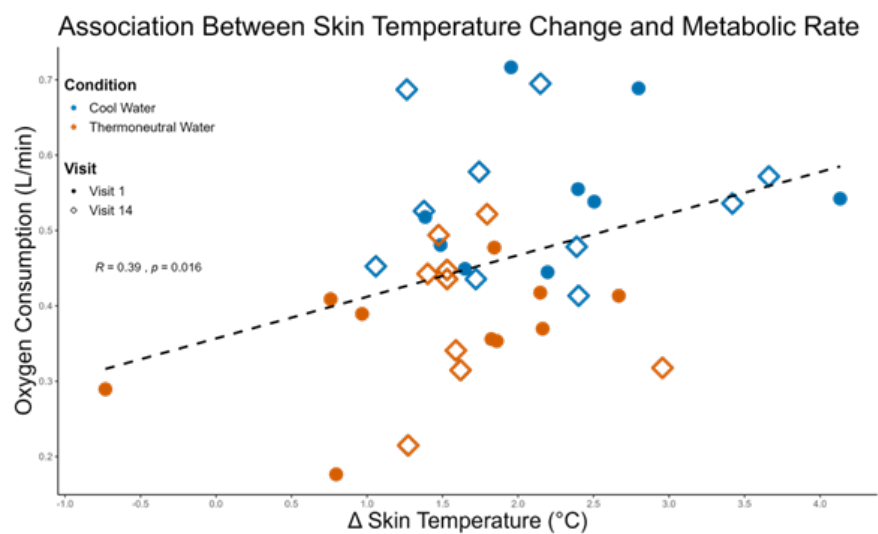
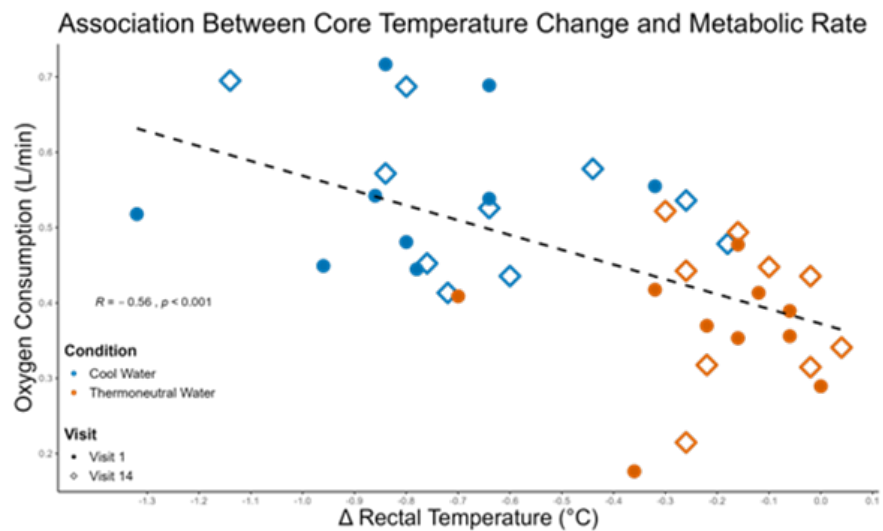


- TN Air
- TN Water
- ▲ Cool Water

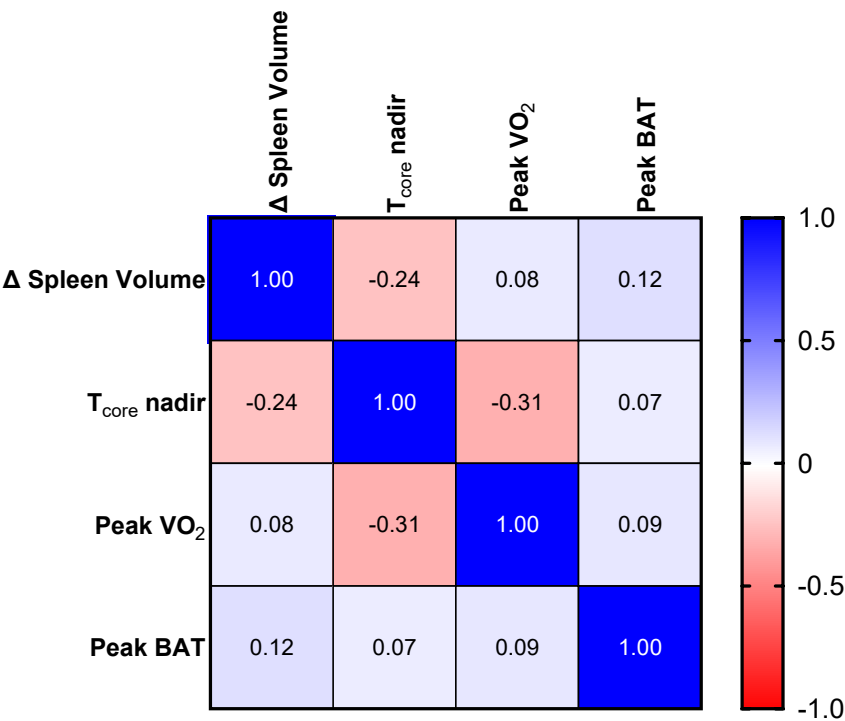
Correlations between changes in spleen size and changes in pro-inflammatory microparticles:

	Δ Spleen volume	Δ CD66b+	Δ CD146+	Δ CD41a+
Δ Spleen volume	--	r= 0.22 p= 0.288	r= 0.05 p= 0.276	r= 0.21 p= 0.307
Δ CD66b+		--	r= 0.44 p= 0.023	r= 0.58 p= 0.002
Δ CD146+			--	r= 0.05 p= 0.807
Δ CD41a+				--

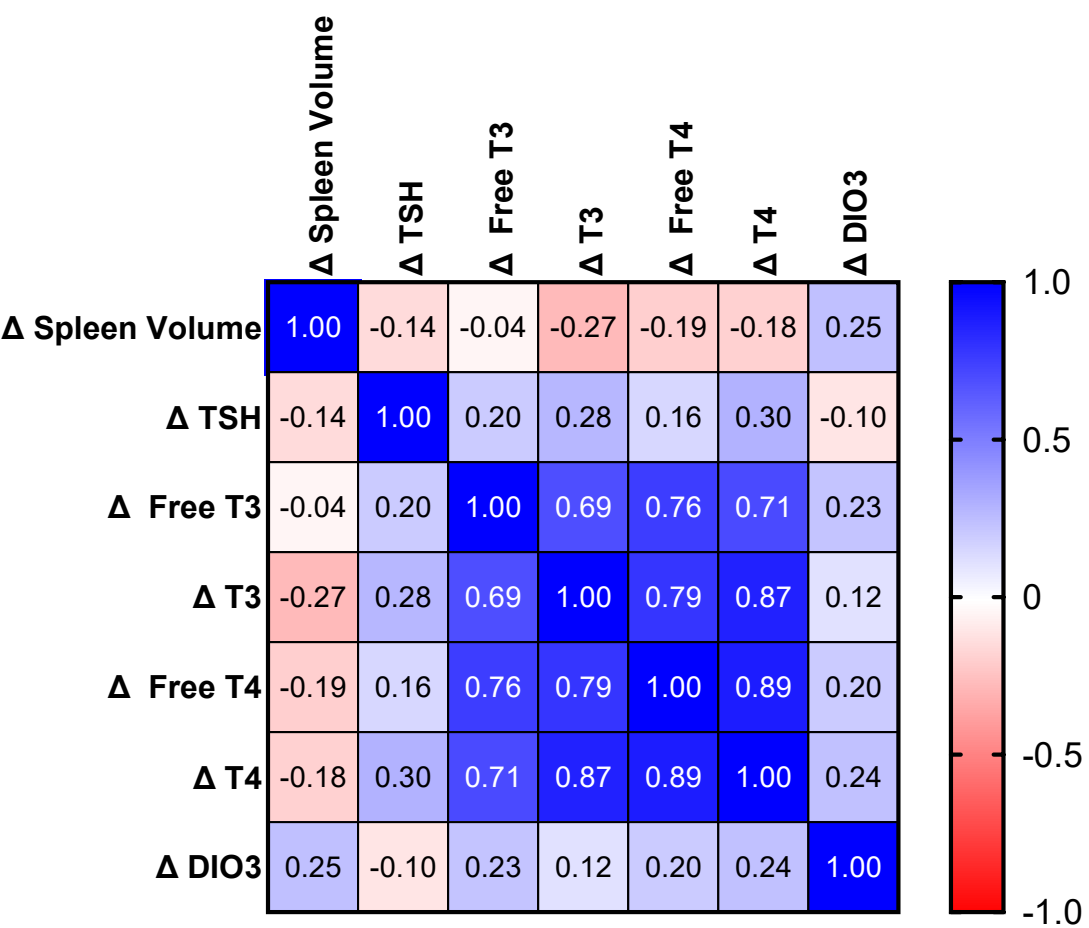
Thermogenic responses to cool and thermoneutral water immersion:



Correlations between changes in spleen size and thermogenic responses:



Correlations between changes in spleen size and thyroid hormones:



REPORT DOCUMENTATION PAGE

1. REPORT DATE 04/10/2024	2. REPORT TYPE Final report	3. DATES COVERED	
		START DATE 06/22/2020	END DATE 12/22/2023
4. TITLE AND SUBTITLE Harnessing splenic adaptations to improve diving capabilities			
5a. CONTRACT NUMBER	5b. GRANT NUMBER N00014-20-1-2593	5c. PROGRAM ELEMENT NUMBER	
5d. PROJECT NUMBER	5e. TASK NUMBER	5f. WORK UNIT NUMBER	
6. AUTHOR(S) Zachary J. Schlader			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Trustees of Indiana University 509 E 3rd St Bloomington, IN 47401 Phone: 317-278-3473			8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Office of Naval Research 875 N Randolph Street, Suite 1425 Arlington, VA 22203-1995		10. SPONSOR/MONITOR'S ACRONYM(S) ONR	11. SPONSOR/MONITOR'S REPORT NUMBER(S)
12. DISTRIBUTION/AVAILABILITY STATEMENT DISTRIBUTION A. Approved for public release: distribution unlimited.			
13. SUPPLEMENTARY NOTES			

14. ABSTRACT

Current evidence indicates that efforts to optimize splenic function are important for Navy and Special Warfare divers given the potential to improve O₂ carrying capacity, augment pH buffering, and/or enhance pro-inflammatory microparticle clearance. The resulting diver health and performance benefits of harnessing such splenic adaptations are wide-ranging and include improvements in gas exchange, which could improve physical and mental performance, a reduced risk of CO₂ (and/or O₂) toxicity, and/or a lower risk of decompression sickness. Thus, this project aimed to experimentally determine if spleen size can be increased and/or spleen function can be improved with repeated breath-hold diving related exposures in people without breath-hold diving ancestry (Aim 1). We also determined the mechanistic (Aim 2) and environmental (Aim 3) modulators underlying the observed phenotypic adaptations. This was accomplished by exposing men and women without a history of breath-hold diving to hypoxic challenges that simulate the demands of breath-hold diving over a 4 h period on each day for 14 consecutive days. The breath-hold diving exposures started with five maximal breath-holds that will be equally spaced over the first 30 min. This was followed by 3 h of breathing 14% oxygen. During the final 30 min, the participants again completed five maximal breath-holds. The simulated breath-hold diving exposures took place during i) head out cool water (28-30°C) immersion, ii) head out thermoneutral water (35°C) immersion, and iii) exposure to thermoneutral air (24-26°C), which were randomly assigned to a given participant (n=10 in each group). Primary dependent variables included spleen volume and spleen function, which was operationally defined as the capacity to constrict the spleen and mobilize red blood cells during breath-holds and clear circulating pro-inflammatory microparticles. These variables were measured before, during, and following simulated breath-hold diving exposures on the 1st, 7th, and 14th consecutive days of exposure. The findings indicate that spleen volume can be increased with repeated breath-hold diving related exposures, and the magnitude of this increase is greatest when conducted in cool water. However, this did not translate to increases in hemoglobin mass. During resting conditions, neutrophil-specific microparticles were modestly reduced but this was not affected by condition/group, nor were these changes correlated with changes in spleen volume. Finally, despite that spleen size increased to the greatest extent in the cool water condition, the change in spleen size was unrelated to any thermal, thermogenetic or indices of hypothalamus-pituitary-thyroid axis activation. Ongoing work will further characterize apneas-induced splenic contraction, HPT-axis activation, and erythropoiesis pathways. Collectively, this work provides the basis for future work aiming to directly exploit splenic adaptations to augment autonomous diving operations.

15. SUBJECT TERMS

Diving, water immersion, cold stress, spleen, hypoxia, apneas

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