# Characterizing the Repeatability and Reproducibility of Cardiovascular Responses to Hypoxic Apneas

**NCT06399575**

**June 26, 2024**

# *Characterizing the Repeatability and Reproducibility of Cardiovascular Responses to Hypoxic Apneas*

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Protocol/version #: **Pro00138947**
Current Version Date: **June 26, 2024**

# Table of Contents

# 1. Introduction/Significance

Reproducibility of findings is a critical scientific principle required to make clear interpretations from results. To date, no study has tested the reproducibility of cardiovascular (i.e., heart rate and blood pressure) responses to apneas. Notably, previous research studies employing apneas (e.g., Busch et al., 2018; 2020; 2021) base their conclusions on results from one apnea per person, or one apnea per condition per person when multiple conditions are included. These methodological decisions mean it remains unclear whether a second apnea would cause the same effect, necessitating an investigation to quantify the consistency of an individual's response to apneas. Apneas are commonly utilized in pre-clinical human research as an autonomic nervous system stressor. The frequency with which this stimulus is utilized in research justifies the need to determine the repeatability and reproducibility of apnea responses.

# 2 Study Objectives

## 2.1 Objectives

The primary objective of this study is to determine the degree of repeatability (i.e., consistency within a testing session) and reproducibility (i.e., consistency between testing sessions) of heart rate and blood pressure responses to apneas. This study also aims to determine the nature of inconsistency in these cardiovascular responses to apneas; we will ascertain whether inconsistency stems from random variability or systematic changes with repeated apneas.

## 2.2. Hypotheses

We hypothesize that heart rate responses to apneas are repeatable (within-subject standard deviation [WSSD] <5 bpm; see Section 4.2 below for further details) and that greater temporal space between apneas will increase the WSSD. We also hypothesize that blood pressure responses to apneas are repeatable (WSSD <4 mmHg). Additionally, we hypothesize that inconsistency between apneas will primarily stem from random variability.

# 3 Participants and Methods

## 3.1 Study Design

*General Design:*
We will evaluate the cardiovascular response to two maximal apneas and five repeated test apneas under standardized hypoxic conditions. Participants will complete two identical testing sessions 24 hours apart.

*Recruitment*:
Participant recruitment will follow non-probability quota sampling and will be facilitated by word of mouth by the study coordinator, as well as through posters or social media posts on designated accounts. All potential participants will be given a copy of the informed consent document to read through before agreeing to participate.

## 3.2 Subject Selection

We will recruit 20 healthy volunteers (male and female) between the ages of 18 and 70.

*Inclusion Criteria:*

- Males and females between the ages of 18-70.

*Exclusion Criteria:*

- Any individual having any known cardiovascular or nervous system disease.
- Anyone with low or high blood pressure (< 90/60 or >139/85, respectively).
- Anyone taking any prescribed medications (other than oral contraceptives) than may affect cardiovascular system function.
- We will exclude females who may be pregnant, as self reported.
- Anyone currently involved in any other studies.

## 3.3 Study Procedures

### *3.3.1 Pre-testing Instructions*

Participants will undergo two in-lab testing sessions beginning at the same time of day on consecutive days. Participants will arrive at the laboratory after fasting for two hours; they will also abstain from caffeine, alcohol, and strenuous physical activity 12 hours before each visit.

### *3.3.2 In-lab Testing*

*Questionnaire:*
Participants will review the consent form with a researcher and sign the form before participating in any testing. Participants will then be asked to fill out a Health History Questionnaire that asks about their medical history.

*Anthropometrics*:
We will measure the participant's height and weight.

*Baseline Assessments*:
Participants will perform a vital capacity maneuver to provide a measure of the maximum amount of air they can exhale; we will use this as a measure of functional lung volume. Participants will complete three vital capacity tests, and the highest of two values within 150mL will be that participant's vital capacity. We will then calculate 40% of this value as the amount of air participants will inhale, after a full breath out, immediately before each apnea (see *Apnea Procedure* below for further details).

*Physiological Testing:*
Figure 1 shows a schematic of the in-lab protocol. Each test will begin with a 10-minute baseline period where participants breathe through a mouthpiece to determine resting values for heart rate, mean arterial pressure, and end-tidal partial pressures of oxygen and carbon dioxide. Participants will then complete a single warm-up apnea to familiarize themselves with the sensations of an apnea. Next, participants will breathe a hypoxic gas mixture for five minutes before completing a maximal apnea. A dynamic end-tidal forcing system will decrease end-tidal oxygen to 50 mmHg, corresponding to 80-85% peripheral oxygen saturation, and will maintain end-tidal carbon dioxide at baseline levels. Participants will complete two such apneas with a five-minute rest between the end of the first apnea and the beginning of the second hypoxic period. The longest of the two apnea durations will be used as the target time for all subsequent test apneas, rounded to the nearest second. Participants will be blinded to their apnea duration for the first maximal apnea to encourage a maximal effort without contributions from preconceived ideas about their apnea abilities. Participants will then be told their first maximal apnea time and be provided an opportunity to improve their maximal time with a live view of a stopwatch during their second apnea. Immediately following each apnea, participants will rate the difficulty of the apnea using a 10-point modified Borg Scale adapted from Foster et al. (2001) (Figure 2).

Finally, participants will perform five successive test apneas, each after a five-minute hypoxic stimulus. Apnea duration for all five test apneas will correspond to the same duration as the longest of the two maximal apneas. Participants will see a live stopwatch so they are aware of the remaining duration of their apneas. They will be instructed prior to the test apneas to hold their breath for the pre-specified duration to their best ability. Participants will get at least five minutes' rest between the end of the prior apnea and the beginning of the subsequent hypoxic period. Participants will only proceed to a hypoxic period once heart rate and mean arterial pressure return to within five units of their baseline values.

On the second testing day, the maximal apneas will be used only for a measure of improvement from the first day. The test apneas on the second testing day will use the same standardized time as determined on the first testing day. On the second day we will also set the end-tidal partial pressure of carbon dioxide to the baseline value from the first day; this will eliminate any differential effects of carbon dioxide on day-to-day reproducibility.

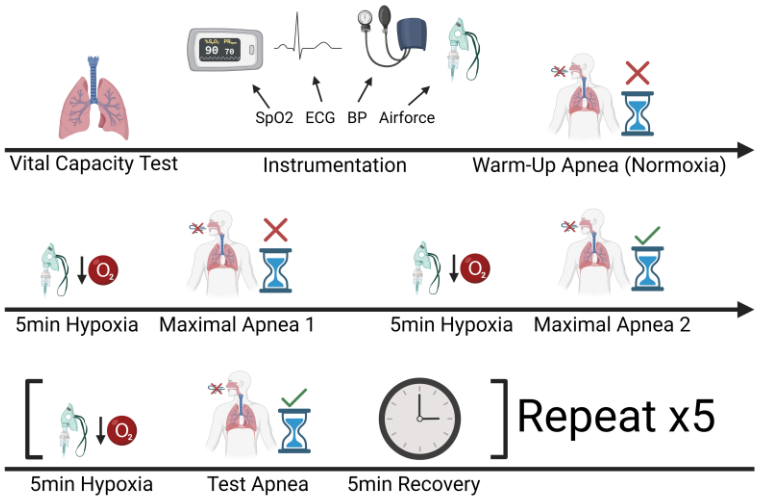


**Figure 1.** Protocol schematic. Participants will begin with a vital capacity test and instrumentation. They will then complete a warm-up apnea. The hourglass and symbol above refer to whether the participant will see their apnea duration in live time. Next, participants will complete two maximal apneas; the longest apnea duration will be the time used for the five test apneas. Participants will complete the same in-lab protocol on consecutive days. On the second day the protocol is the same except participants do not complete another vital capacity test, and the test apnea time is the same as the first day, regardless of the maximal apnea times. *SpO2* peripheral oxygen saturation, *ECG* electrocardiogram, *BP* blood pressure, *AirForce* refers to the dynamic end-tidal forcing system.

| Rating | Descriptor |
|---|---|
| 0 | Rest |
| 1 | Very, Very Easy |
| 2 | Easy |
| 3 | Moderate |
| 4 | Somewhat Hard |
| 5 | Hard |
| 6 | . |
| 7 | Very Hard |
| 8 | . |
| 9 | . |
| 10 | Maximal |

**Figure 2.** Modified Borg scale for rating of perceived exertion. Reproduced from Foster et al. (2001).

*Apnea Procedure*

Figure 3 shows a schematic of the mouthpiece setup. We will arrange the mouthpiece setup through which the participant will breathe as follows: the mouthpiece will connect to a filter, which will further attach to a pneumotachometer and a three-way non-rebreathing valve, all in series. The expired port of the non-rebreathing valve will be open to the room, and the inspired port will connect to a second three-way valve. Port 1 of the valve will attach to the mouthpiece setup, port 2 will attach to the dynamic end-tidal forcing system, and port 3 will attach to a standard three-liter calibration syringe (Model 5530, Hans Rudolph). The three-way valve has three positions during different phases of the protocol. Position 1 allows flow between ports 1 and 2; position 2 allows flow between all three ports; and position 3 allows flow between ports 1 and 3. Three researchers will work together during the apnea procedure: the first will control the dynamic end-tidal forcing system, the second will switch the positions of the three-way valve (valve operator), and the third will manage the volumes of a three-liter calibration syringe (syringe operator). The procedure begins with the valve in position 1 for the duration of the hypoxic step. Thirty seconds before the apnea, the syringe operator attaches an empty standard three-liter calibration syringe to port 3, and the valve operator subsequently switches the valve to position 2, allowing flow between all three ports. Next, the operator of the dynamic end-tidal forcing system adds gas corresponding to 40% of the participants' vital capacity (in addition to tidal volume) over one or two breaths, depending on tidal volume, and the syringe operator pulls gas into the syringe to the correct volume, rounded to the nearest 100 mL. Finally, the valve operator will instruct the participant to exhale fully; during the exhalation the valve operator will switch the valve to position 3 to isolate the syringe and the participant. The participant will then inhale from the syringe until an audible *clunk*, at which time the valve operator will assist in removing the mouthpiece, and the participant will hold their breath with their mouth closed. Since the dynamic end-tidal forcing system alters inspired gas concentrations on a breath-by-breath basis, the syringe system standardizes apnea lung volume while continuing to target the desired end-tidal partial pressures of oxygen and carbon dioxide on the final breath in a participant-specific manner.

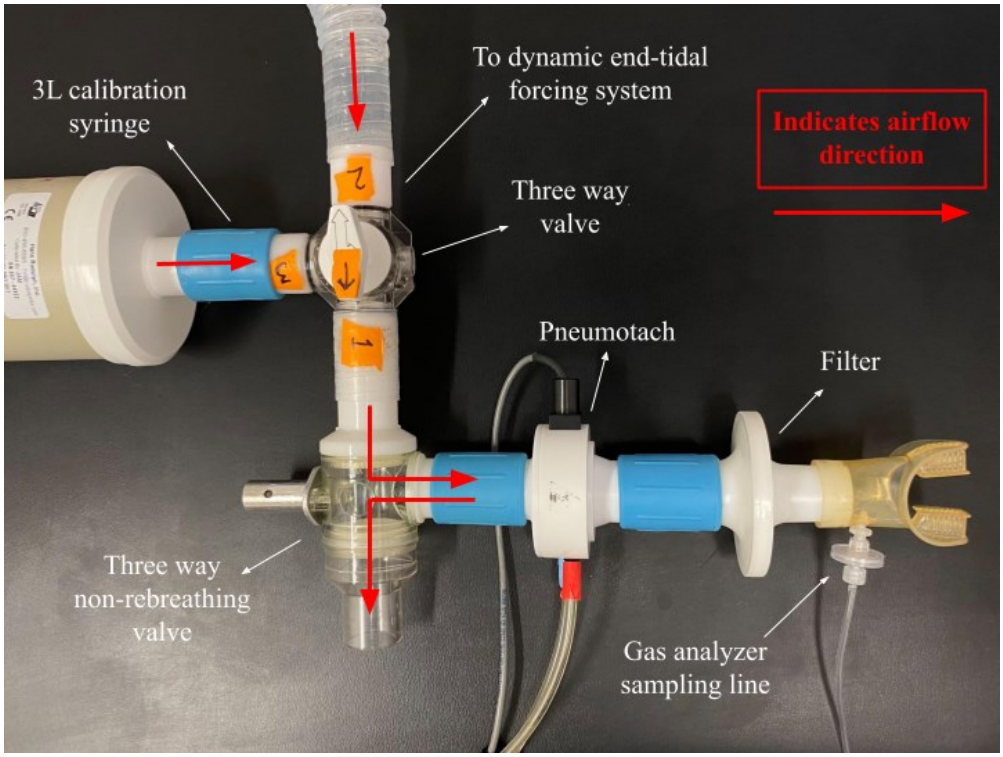


**Figure 3.** Mouthpiece setup during the hypoxic periods. The orange and black numbers on the three-way valve correspond to the port numbers, and the port to which the orange and black arrow points corresponds to the position of the valve (see *Apnea Procedure* for additional details).

### *3.3.3 Measurements*

*Respiratory Measures:*
Figure 3 shows a schematic of the mouthpiece setup. Participants will wear a nose clip and breathe through a mouthpiece attached to a pneumotachometer (MLT3813H-V, ADInstruments) and a 3-way non-rebreathing valve (T-Shape Series, 2700, Hans Rudolph). The inspired port of the valve will connect to a dynamic end-tidal forcing system that will control end-tidal oxygen and carbon dioxide on a breath-by-breath basis using offline software (Labview 16.0, National Instruments). The mouthpiece will have a small sampling port connected to oxygen (S-3A/I Oxygen Analyzer & Model N-22M Sensor, AEI Technologies) and carbon dioxide (CD-3A Carbon Dioxide Analyzer & P-61B Sensor, AEI Technologies) gas analyzers run in parallel. The gas analyzer user manual claims accuracy within ±0.01% O2 and ±0.02% CO2 ("Respiratory Gas Analyzers," n.d.). The gas analyzer delays will be measured daily in accordance with the calibration procedures for the dynamic end-tidal forcing system. A respiratory strain gauge (Model TN1132/ST, ADInstruments) placed around the chest will measure respiratory movements during normal breathing and breath-holding and will assist in detecting the end of each apnea.

*Cardiovascular Measures:*
We will measure heart rate and rhythm using an electrocardiogram (lead II). We will measure blood pressure using finger photoplethysmography (Finometer Pro, Finapres Medical Systems) calibrated to manual blood pressure measurements taken in triplicate during the baseline. We will measure peripheral hemoglobin oxygen saturation using pulse oximetry (Nellcor N-600x, Medtronics).

*Borg Scale:*
We will ask participants to rate the effort of each test apnea on a scale of 1-10. We will also have a visual modified Borg scale diagram to assist participants in rating the effort of each apnea (Figure 2).

# 4 Statistical Analysis Plan

## 4.1 Data Analysis

We will collect all data at 1 kHz using LabChart software (Chart Pro, v8.1.3, ADInstruments). We will calculate mean arterial pressure as the arithmetic mean of the blood pressure waveform. We will also calculate minute ventilation as the product of breathing frequency and tidal volume; the latter is determined as the integral of the inspired portion of the respiratory pneumotach trace. We will then extract data into Microsoft Excel for offline analysis (Microsoft Inc., Washington, USA). We will extract free-breathing values as one-minute averages, and we will extract apnea values beat-by-beat.

We will determine four different apnea heart rates and blood pressures using four distinct methods. First, we will use the single slowest heartbeat (i.e., nadir beat [NADIR]) within the final 10 cardiac cycles of an apnea. Second, we will average the heart rates between the nadir beat and the preceding beat (AVG2). Third, we will average the nadir beat and the four beats surrounding it (AVG5). Fourth, we will average the final five cardiac cycles of each apnea (AVG_END). The different apnea values stem from an attempt to increase repeatability and reproducibility through analyzing more data rather than the standard technique of one cardiac cycle per apnea. Table 1 provides a visual representation of the methods to determine apnea heart rate and blood pressure.

We will calculate the change in heart rate as apnea heart rate minus free-breathing heart rate (a negative value would indicate a decrease in heart rate). We will analyze mean arterial pressure in the same manner as heart rate. We will calculate the minimum peripheral oxygen saturation as the lowest value during the apnea or within one minute following the termination of the apnea to account for the circulatory delay in detecting peripheral oxygenation; We will calculate the change in peripheral oxygen saturation as minimum minus free breathing. We will determine apnea duration using the airflow readings from the pneumotach at the start of apnea and the chest movement measured by the respiratory belt at the end of apnea. To identify arrhythmias, a trained individual (DY) will visually inspect the electrocardiograph during all apneas. All abnormal rhythms will be reviewed by a cardiologist who will verify the presence and type of arrhythmia.

**Table 1**. A visual representation of the four methods of determining apnea heart rate (HR) in beats per minute and mean arterial pressure (MAP) in mmHg. The single nadir beat (NADIR for HR) or peak beat (PEAK for MAP) is enlarged and bolded. The average of two beats (AVG2) includes the NADIR/PEAK beat and the preceding beat, underlined. The average of five beats (AVG5) includes the NADIR/PEAK beat and the four closest beats, shown in red text. The average of the five final beats (AVG_END) is shown highlighted in yellow.

| | Cardiac Cycle | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | -10 | -9 | -8 | -7 | -6 | -5 | -4 | -3 | -2 | -1 | 0 |
| HR | 71 | 70 | 66 | 64 | 59 | 54 | **51** | 53 | 52 | 55 | 54 |
| MAP | 86 | 85 | 88 | 87 | 86 | 90 | 89* | 92* | 96 | 94 | **97** |

HR: NADIR=51, AVG2=52.5, AVG5=53.8 AVG_END=53
MAP: PEAK=97, AVG2=95.5, AVG5=93.6, AVG_END=93.6
*Note: in any instance where the NADIR/PEAK beat occurs at beat 0, -1, -9 or -10 the AVG2 and/or AVG5 calculations are shifted such that only beats -10 to 0 are included in any calculations. If two beats in the final 10 cardiac cycles of an apnea are tied for NADIR/PEAK, the beat closest to the end of the apnea will be used.

## 4.2 Statistical Methods

We will report all continuous data as mean ± standard deviation; modified Borg ratings will be reported as median (interquartile range). We will complete all statistical analyses in R Statistical Software (v4.3.1 R Core Team 2023). We will use summary statistics alongside boxplots and histograms to confirm the normality of continuous data. To assess absolute repeatability, we will compute within-subject standard deviations (WSSD) for heart rate, mean arterial pressure, peripheral oxygen saturation, and minute ventilation. The WSSD is the average amount we can expect an individual's future score to deviate from their current score. It is computed using a two-way ANOVA with the participant and apnea number (i.e., the identity of the apnea in a sequence of repeated measurements) as factors. The WSSD is the square root of the mean square of residuals (Hopkins, 2000; Weir, 2005). We will also calculate two-way mixed effects, absolute agreement, single measurement intraclass correlations (ICC) for heart rate, mean arterial pressure, peripheral oxygen saturation, and minute ventilation (Koo & Li, 2016). We will present all arrhythmia data as summary statistics and average percent incidence within individuals. We will present modified Borg data as summary statistics. Because none of our analyses will utilize p values or rely on the outcome of a null hypothesis test *per se*, we do not have an *a priori* alpha for statistical significance.

## 4.3 Sample Size Determination

We will recruit 20 participants to ensure we obtain 16 participants who reach their target apnea time on at least 4 of the 5 test apneas on both test days. McAlinden et al. (2015) presented a method for calculating sample size for a repeatability and reproducibility study:

$$1.96\frac{WSSD}{\sqrt{2n(m-1)}} = WSSD \times LC$$

where $n$ is the sample size, $m$ is the number of repeated measurements, and $LC$ is the desired level of uncertainty between the sample WSSD and the population parameter. Note that WSSD appears on both sides of the equation and therefore divides to one; in other words, the magnitude of the WSSD does not affect the sample size calculation. Rearranging the equation to isolate $n$, using 20% uncertainty between the sample estimate and the population parameter ($LC$ = 0.20), and four repeated trials ($m$ = 4), we require 16 participants. Therefore, to account for instrumental challenges and incomplete trials we will recruit up to 20 participants to obtain a complete sample of eight males and eight females who will complete five apneas per trial. If all apneas go well then we will analyze the first four and discard the final apnea; we decided *a priori* that we will only use the final apnea for analysis if an apnea duration deviates more than two seconds from the target time, or if the heart rhythm is uninterpretable during analysis from excessive participant movement or any other cause. We will record and report every instance that we use the final apnea for analysis, and the reason for its use.

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