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TITLE

Cerebral oxidative metabolism is decreased with extreme apnea in humans; impact of acidosis

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ABSTRACT

Prolonged apnea in humans is reflected in progressive hypoxemia and acidosis. Here, we explore the cerebral metabolic responses under extreme hypoxia and acidosis associated with prolonged apnea. We hypothesized that the cerebral metabolic rate for oxygen ($CMRO_2$) will be reduced near the termination of apnea, attributed in part to the acidosis. Fourteen elite apnea-divers performed a maximal apnea (range: 3:36 to 7:26 minutes) under dry laboratory-conditions. In a subset study with the same divers, the impact of acidosis on cerebral metabolism in the background of hypoxia was determined using varying levels of hypercapnic breathing. In both studies the $CMRO_2$ was calculated from the product of cerebral blood flow (ultrasound) and the radial artery-jugular venous oxygen content difference. Non-oxidative cerebral metabolism was calculated from the ratio of oxygen and carbohydrate (lactate and glucose) metabolism. The $CMRO_2$ was reduced by ~29% ($P<0.01$, Cohen's d = 1.18) near the termination of apnea when compared to baseline, but non-oxidative metabolism remained unaltered. In the subset study, in the background of hypoxia (arterial oxygen tension: ~38.4 mmHg), severe acidosis (arterial pH: ~7.30), but not mild-acidosis (arterial pH: 7.38), significantly depressed the $CMRO_2$ (~17%, $P=0.04$, Cohen's d = 0.87). Similarly to the apnea, there was no change in the non-oxidative metabolism. These data indicate that hypercapnic-induced acidosis can in part explain the reduction in $CMRO_2$ near apnea breakpoint. This acidosis-induced oxygen conservation may protect the brain against severe hypoxemia associated with prolonged apnea.

KEY POINTS

I. This report provides the first description of the cerebral oxidative and non-oxidative metabolism in man during a prolonged apnea (ranging from 3:36 to 7:26 minutes) that generates extremely low levels of blood oxygen and high levels of carbon dioxide.

II. We show that the cerebral oxidative metabolism, measured from the product of cerebral blood flow and the radial artery-jugular venous oxygen content difference, is reduced by ~29% at the termination of apnea, but there is no change in the non-oxidative metabolism.

III. A subset study indicates that elevated carbon dioxide concentrations can in part explain the cerebral metabolic reduction near apnea breakpoint.

IV. It is suggested that a hypercapnia-induced oxygen-conserving response may protect the brain against severe oxygen deprivation associated with prolonged apnea.

INTRODUCTION

Though it accounts for only 2% of total body mass, the human brain utilizes a disproportionate 20% of the body's basal oxygen consumption. The relatively high cerebral metabolic rate of oxygen ($CMRO_2$) is required to support a high rate of adenosine tri-phosphate (ATP) production and neuronal activity (Brown and Ransom, 2007). Constant oxygen supply is therefore obligatory for the maintenance of normal brain function. With respect to cerebral energy demands for oxygen, the current world record apnea duration in humans of 11:35 minutes is truly remarkable.

In terms of oxygen conservation, decreasing the cerebral oxidative metabolism and increasing non-oxidative metabolism is of teleological benefit. Indeed, a reduction in the $CMRO_2$ during deep hypothermia is readily utilized in medicine (e.g. during bypass surgery (Fukui and Takanashi, 2016)) and can in part explain extreme survival following prolonged anoxic (>15 min) cold-water immersion (Young *et al.*, 1980; Antretter *et al.*, 1994). It appears, however, that moderate hypoxia alone does not provide the stimulus of oxygen conservation. For example, no appreciable changes in the $CMRO_2$ have been reported during isocapnic or poikilocapnic hypoxic breathing (PaO_2 of ~40 – 55 mmHg) (Kety and Schmidt, 1948; Cohen *et al.*, 1967; Bailey *et al.*, 2009; Overgaard *et al.*, 2012; Ainslie *et al.*, 2014). In fact, using recent magnetic imaging techniques, some studies report significant elevations in $CMRO_2$ by 5-10% with acute poikilocapnic hypoxia (Xu *et al.*, 2012; Vestergaard *et al.*, 2015). Likewise, available data further indicate no

increases in cerebral non-oxidative metabolism (estimated via the oxidative carbohydrate index, OCI) with isocapnic hypoxic breathing (Ainslie *et al.*, 2014).

An increase in $CMRO_2$ with acute hypoxia shown by some studies (Vestergaard *et al.*, 2015), but not all (Ainslie *et al.*, 2014), may relate to the background $PaCO_2$, or pH. In one study (Ainslie *et al.*, 2014), subjects were kept at eucapnia, whereas in another (Vestergaard *et al.*, 2015) the hypoxia-induced increase in ventilation reduced $PaCO_2$ by an average 10 mmHg (absolute 31 mmHg). A large body of human and animal data indicates changing $PaCO_2$ may impact $CMRO_2$, with hypocapnia increasing, and hypercapnia decreasing it (see (Yablonskiy, 2011) for synopsis). Mechanistically, synaptic transmission (Dulla *et al.*, 2005; Thesen *et al.*, 2012) and phosphofructokinase (PFK) activity (Folbergrova *et al.*, 1975) are each dependent on pH. Because a prolonged breath-hold yields both extreme levels of hypoxia and hypercapnia (Willie *et al.*, 2014; Bain *et al.*, 2015a), it stands to reason that, in contrast to hypoxia alone, the $CMRO_2$ may in fact be reduced during apnea. Additionally, in contrast to hypoxic breathing (Ainslie *et al.*, 2014), a greater increase in sympathetic nerve activity during apnea, despite the same chemoreflex stimuli (Steinback *et al.*, 2010a), may promote a shift towards non-oxidative metabolic pathways. For example, infusion of high dose adrenaline (0.08 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 15 min) in humans significantly increases the percentage of non-oxidative cerebral metabolism (Seifert *et al.*, 2009), and adrenaline appears to underscore the increased non-oxidative metabolism during exercise (Larsen *et al.*, 2008). A shift towards non-oxidative cerebral metabolism, and a decrease in the $CMRO_2$, may in turn help describe how some elite breath-hold divers may hold their breath for over 10 minutes.

Despite obvious implications in neuropathology, describing the cerebral metabolic profile under severe apneic stress in humans has remained a methodological and ethical challenge. Elite breath-hold divers provide a unique population to test the limits of hypoxemia tolerance beyond possible in otherwise healthy humans. As such, the primary purpose of this study was to examine the oxidative and non-oxidative cerebral metabolism during a prolonged dry-land apnea in elite human breath-hold competitors. In a subset study, we garnered mechanistic information by quantifying the impact of hypercapnia / pH on cerebral metabolism in the background of hypoxia. We examined the novel hypotheses that 1) compared to a resting baseline, $CMRO_2$ will be reduced and indices of cerebral non-oxidative metabolism will be increased during a prolonged apnea, and 2) the reductions in $CMRO_2$ will in part be a function of a pH-mechanism.

METHODS

Participants

Fourteen competitive and elite breath-hold divers (2 female; age 29.5 ± 7.3 years; BMI 23.5 ± 2.5 kg/m^2) were recruited from the Croatian national apnea team, and provided informed written consent for participation. All participants were actively competing, and had been practicing competitive breath hold diving for 1.5 to 14.0 years (mean 5.2 ± 3.7 years). The majority of subjects were competitive in the discipline of dynamic apnea (maximal swimming distance underwater). Some were also involved in various depth

disciplines (with regular exposure to high pressure). Seven of the subjects were world-class free diving competitors, having placed top-ten within the last three years in international competition in at least one event. Three subjects had recently set new official world records. All subjects were normotensive and free from cardiovascular and respiratory disease. The ethical committees of the University of Split School of Medicine, the University of British Columbia, and the University of South Wales approved the experimental procedures. All experimentation conformed to the standards set by the *Declaration of Helsinki.*

Experimental Design

Experimentation was completed on a single day, following strict adherence to pre-testing protocol, including abstinence from vigorous exercise and alcohol at least 48 hours, and from caffeine at least 12 hours before arriving to the laboratory. All testing was performed at the University of Split, School of Medicine, Department of Integrative Physiology. Upon arrival, a medical history and standard anthropometric and pulmonary functioning metrics were assessed. After, a 20-gauge arterial catheter (Arrow, Markham, Ontario, Canada) was placed in the right radial artery, and a central venous catheter (Edwards PediaSat Oximetry Catheter) was placed in the right internal jugular vein and directed cephalad to the jugular bulb. Using the identical technique and performed by the same anesthesiologist, correct placement of the jugular bulb catheter has previously been verified by lateral skull X-ray (Ainslie *et al.*, 2014). Arterial and jugular cannulations were completed under ultrasound guidance with local anesthesia (1% lidocaine), and

under sterile conditions. Following cannulation, the catheters were attached to an in-line waste-less sampling system and a pressure transducer located at the height of the right atrium (Edwards Lifesciences VAMP), and TruWave transducer (Irvine, CA, USA). Subjects were then instrumented with the remaining measurements (see measurements).

Apnea: Baseline measures were acquired following a minimum of 30 minutes supine rest, and prior to preparatory apneas. After baseline measures were acquired, each participant completed two preparatory (non-experimental) apneas. The first preparatory apnea was performed after a normal end-expiration until seven involuntary breathing movements were attained. Two minutes later the second preparatory apnea was performed at total lung capacity until ten involuntary breathing movements were attained. These preparatory apneas were performed to generate the longest possible time for the experimental maximal apnea, chosen by the national Croatian apnea team coach (co-author, Ivan Drvis) based on a best-suited standardized preparatory phase for all subjects. Following the preparatory phase, subjects rested for six minutes before commencing the maximal apnea. Subjects were allowed to lung pack (glossopharyngeal insufflation) prior to the experimental apnea, based on individual preference to attain the longest apnea possible. Data were collected throughout the maximal breath-hold until breathing was resumed. Arterial and jugular venous blood draws were attained prior to the preparatory apneas (baseline), every 30 seconds throughout the maximal apnea, and immediately upon termination (100% of apnea).

Subset Study - end-tidal forcing: Following the maximal apnea, participants' rested supine for a minimum of 30 minutes before starting the hypercapnic-hypoxic breathing subset trials. The mild-hypercapnic hypoxic (mild-HH) and severe-hypercapnic hypoxia (severe-HH) breathing was performed using a custom built end-tidal (P_{ET}) forcing system. Subjects were equipped with a mouthpiece and nose clip, and were instructed to breath normally. Gases were sampled at the mouthpiece and analyzed by a calibrated gas analyzer (ML206 ADInstruments, Colorado Springs, CO, USA). Respiratory flows were measured by pneumotachography (HR 800L, Hans Rudolph, Shawnee, KS, USA). Custom written software (Labview, Austen, TX, USA) determined the breath-by-breath tidal volumes and end-tidal partial pressures of oxygen and carbon dioxide ($P_{ET}O_2$ and $P_{ET}CO_2$). The end-tidal forcing system prospectively delivered inspired gases to clamp $P_{ET}O_2$ and $P_{ET}CO_2$ at desired input levels. Independently controlled solenoid valves delivered the desired volumes of O_2, CO_2, and N_2 as determined by an error reduction algorithm incorporating $P_{ET}O_2$, $P_{ET}CO_2$, and inspiratory and expiratory tidal volume from the last breath. Levels of desired $P_{ET}O_2$ for both mild-HH and severe-HH were individualized to the average PaO_2 measured during the last minute and a half of the maximal breath-hold. Levels of $P_{ET}CO_2$ during the mild-HH trial were individualized to the $PaCO_2$ achieved at ~50% of the maximal apnea. Levels of $P_{ET}CO_2$ during the severe-HH trial were targeted for +10 mmHg from the mild-HH. Each condition (mild-HH and severe-HH) lasted 5 minutes, and the severe-HH trial was performed immediately following mild-HH, while continually maintaining hypoxia. Arterial and jugular venous blood draws were taken following 4.5 min in each stage.

Measurements

Blood gases, oximetry and metabolites: Measurements of arterial and jugular venous PO_2, PCO_2, O_2 saturation (SO_2%), glucose (Glu) and lactate (La) were analyzed immediately following each draw using a commercially available cassette based analyzer (ABL90 Flex, Radiometer, Copenhagen, Denmark). Each measure required <2 mL of blood. Arterial (radial) and jugular venous draws were taken at the same time.

Catecholamines: Blood samples (7 mL) were collected into tubes containing ethylenediaminetetraacetic acid (K-EDTA) and centrifuged at 600*g* for 10 min at 4°C. Plasma (2 mL sample volume) was transferred into cryovial tubes and immediately snap-frozen under liquid nitrogen (N_2, Cryopak CP100, Taylor-Wharton, Theodore, AL, USA) and then stored at -80°C prior to analysis. Analysis was performed within 1 month of receipt of the sample. All chemicals including reagents and standards were of the highest available purity from Sigma-Aldrich® (UK). Plasma concentrations of adrenaline, noradrenaline and dopamine were measured by reverse-phase ion pair high performance liquid chromatography [Gilson ASTED.XL (Anachem)] with electrochemical detection [ESA Coulochem II (ESA Analytical)] (Bouloux *et al.*, 1985). The CV was 5% for noradrenaline at 4.8 nmol/L and 8% for adrenaline at 1.0 nmol/L.

Cardiovascular: Heart rate (HR) was obtained from the R-R intervals measured from a three-lead ECG. Beat-to-beat arterial blood pressure was measured by finger photoplethysmography (Finometer PRO, Finapress Medical Systems, Amsterdam,

Netherlands) normalized to manual cuff measurements of the brachial artery. Direct intra-radial arterial pressure and jugular venous pressure were also recorded, however, due to the frequency of blood sampling, measures were available for ~5 to 10 second bins around each blood draw. Online calculations of stroke volume were obtained using the three-element nonlinear arterial model of the arterial blood pressure waveform (Finometer). Cardiac output was then derived from the product of stroke volume and HR.

Cerebrovascular: Cerebral blood velocity of the right middle cerebral artery (MCAv) and left posterior cerebral artery (PCAv) were measured using a 2-MHz pulsed transcranial Doppler ultrasound system (Spencer Technologies, Seattle, WA). A specialized headband fixation device (model M600 bilateral head frame, Spencer Technologies) was used to secure the probes in position. Signal quality was optimized using standardized search techniques that produce test-retest reliability of ~3% and 2% for MCAv and PCAv, respectfully. MCAv was insonated through the left temporal window, at a depth 1 cm distal to the MCA-anterior cerebral artery bifurcation. PCAv was insonated at the P1 segment through the right temporal window.

Volumetric blood flow of the right internal carotid artery (QICA) and left vertebral artery (QVA) was concomitantly measured using duplex vascular ultrasound (Terason 3000, Teratech, Burlington, MA). The right ICA was insonated ~ 2cm from the carotid bifurcation, while the left VA was insonated at the C5-C6 or C5-C4 space depending on individual anatomy. Care was taken to standardize the insonation location between measures within subjects. The steering angle was fixed to 60-degrees among all trials, and the sample volume was placed in the center of the vessel adjusted to cover the

entire vascular lumen. All files were collected as an AVI file for offline analysis at 30 Hz using custom designed software (Woodman *et al.*, 2001). Simultaneous measures of luminal diameter and velocity over a minimum of 10 cardiac cycles were used to calculate flow.

Calculations

Under the assumption of symmetrical blood flow of contralateral ICA and VA arteries, global CBF (gCBF) was calculated from:

$$\mathrm{gCBf}(\mathrm{ml.min^{-1}}) = (\mathrm{QICA} \cdot 2) + (\mathrm{QVA} \cdot 2)$$

Due to the onset of involuntary breathing movements (IBMs) during the latter ~60% of the breath-hold (struggle phase), which encompasses large movements of the chest wall and movement of the sternocleidomastoid muscles, we were unable to attain reliable QICA and QVA values in all subjects for the entire breath-hold. We were, however, able to reliably track ICA and VA diameter, up to the breath-hold termination. As such, where simultaneous measures of ICA or VA velocity with diameter were missing, gCBF was derived from incorporating changes in MCAv (indicative of ICA velocity) and PCAv (indicative of VA velocity) with correction for diameter. That is, the % change in MCAv or PCAv was estimated to equate with ICA or VA velocity changes (r^2=0.7 where simultaneous measures available). The inclusion of changes in diameter measurements assured that gCBF was not underestimated using the metrics of ΔMCAv and ΔPCAv only (Coverdale *et al.*, 2014).

Arterial content of oxygen (CaO_2) and venous content of oxygen (CvO_2) were calculated using the equations:

$$\mathrm{CaO_2(ml.dl^{-1})} = [\mathrm{Hb}] \cdot 1.36 \cdot \frac{\mathrm{SaO_2(\%)}}{100} + 0.003 \cdot \mathrm{PaO_2}$$

$$\mathrm{CvO_2(ml.dl^{-1})} = [\mathrm{Hb}] \cdot 1.36 \cdot \frac{\mathrm{SvO_2(\%)}}{100} + 0.003 \cdot \mathrm{PvO_2}$$

Where 1.36 is the affinity for oxygen to hemoglobin for a given arterial saturation, and 0.003 is the percentage of oxygen dissolved in the blood. Values are expressed as ml of O_2 per 100 ml of blood ($ml.dl^{-1}$).

Cerebral delivery of oxygen (CDO_2) was calculated from:

$$\mathrm{CDO_2(ml.min^{-1})} = \mathrm{CaO_2} \cdot \frac{\mathrm{gCBF}}{100}$$

The cerebral metabolic rate of oxygen ($CMRO_2$) was calculated from:

$$\mathrm{CMRO_2(ml.min^{-1})} = (\mathrm{CaO_2} - \mathrm{CvO_2}) \cdot \frac{\mathrm{gCBF}}{100}$$

Cerebral oxygen, glucose and lactate extraction fraction was calculated from the arterial-venous oxygen content difference, divided by the arterial oxygen, glucose or lactate content, respectively, and then multiplied by 100.

The oxidative glucose index (OCI), which provides an estimation of oxidative versus non-oxidative metabolism (see (Ainslie *et al.*, 2014)), was calculated from below. In short, a reduction of 6 reflects the presence of non-oxidative metabolism. This was then converted to a percent OCI by dividing by 6 and then multiplying by 100.

$$\mathrm{OCI}\ (\%) = \frac{\dfrac{CaO_2 - CVO_2}{(Glu_a - Glu_v) + 0.5(Lac_a - Lac_v)}}{6} X\ 100$$

Statistical analysis

Mean values ± standard deviations (SD) are presented. Baseline measures were acquired during quite rest prior to the preparatory apneas (~15 minutes before the maximal apnea), and one minute before the end-tidal forcing trials. Baseline measures were averaged over one minute around the baseline blood draw. Mean values for MAP (Finometer corrected with intra-arterial) HR, and gCBF during the apnea were averaged over 20 seconds around the blood draws.

Statistical analysis for both apnea and the end-tidal forcing was performed using one-way repeated measures ANOVA. When a main effect was observed, pre-planned paired comparisons were performed to baseline only using two-tailed Student t-tests. A Bonferroni adjustment was applied to correct for multiple comparisons (six stages of the breath hold, and three stages of end-tidal forcing). When significant, Cohen's d (d) was calculated for effect size of the primary outcome variable ($CMRO_2$).

RESULTS

Apnea

Maximal apnea times ranged from 3:36 to 7:26 min with an average of 5:14 min.

*Blood gases, oximetry and metabolites***:** Arterial and jugular venous blood gas, oximetry, pH, and metabolite data are presented in Table 1. To illustrate the near normalization of arterial with venous blood at the end of the apnea**,** arterial and jugular venous PO_2 from baseline to percent time 100 of the apnea are presented in Figure 1. As expected there was a significant main effect for all blood gas variables (p all <0.05). Significant post-hoc comparisons to baseline are denoted in Table 1.

*Hemodynamics***:** Absolute heart rate (HR), mean arterial pressure (MAP), stroke volume (SV), cardiac output (CO), and global cerebral blood flow (gCBF) are presented in Table 2. There was a significant main effect of for all variables (p all <0.05). Despite a significant reduction in CO from baseline, the MAP was significantly elevated throughout the entire apnea (p all <0.05), indicating an elevated total peripheral resistance. The significant reductions in indices of SV from percent time 20 to 80 of the apnea (p all <0.05) are attributable to the glossopharyngeal insufflation prior to the start of the apnea. As expected, gCBF was significantly reduced at the onset of the apnea, and then was progressively elevated by ~70% at percent time 100 of the apnea. All significant post-hoc comparisons to baseline are denoted in Table 2.

Cerebral metabolic dynamics and oxygen delivery: Cerebral delivery of oxygen (CDO_2), oxygen extraction (O_2 Ext), lactate extraction (Lac Ext), glucose extraction (Glu Ext), OCI and, $CMRO_2$ are presented in Table 3. Individual $CMRO_2$ at baseline and percent time 100 of the apnea are presented in Figure 2. There was a significant main effect of CDO_2 ($p<0.01$), O_2 Ext ($p<0.01$), Glu Ext ($p<0.01$), OCI ($p=0.02$), and $CMRO_2$ ($p<0.01$). Compared to baseline, the $CMRO_2$ was significantly reduced by ~29% at percent time 100 of the apnea ($P<0.01$, $d=1.18$), but similar at all other time points (p all >0.05). The O_2 Ext was increased at percent time 20 of the apnea, and significantly decreased at the latter half of the apnea (p all <0.05). The CDO_2 fell below baseline values at percent time 20 of the apnea ($p<0.01$), above baseline at percent time 60 and 80 of the apnea (p both <0.01) and was similar to baseline at percent time 100 of the apnea. All significant post-hoc comparisons to baseline are denoted in Table 3. Although there was a main effect of OCI, no post-hoc comparisons were significantly different. There was no correlation between the absolute or change in $PaCO_2$ or PaO_2 with the change in $CMRO_2$ from baseline to percent time 100 of the apnea.

Catecholamines: The radial arterial plasma adrenaline and noradrenaline increased by ~380% (670±380 to 2012±1020 pmol/L) and ~483% (1893±673 to 8260±4375 pmol/L) from baseline to percent time 100 of the apnea, respectively (p both <0.01). Likewise, the jugular venous plasma adrenaline and noradrenaline increased by ~258% (584±258 to 1762±801 pmol/L) and ~385% (1860±577 to 8559±3707 pmol/L) (p both <0.01). The

arterial-venous adrenaline and noradrenaline differences were not significantly different from baseline to percent time 100 of the apnea (p both >0.05).

Subset study: Mild and severe hypercapnic hypoxic breathing

Three participants were unable to complete the end-tidal forcing trial due to ventilatory volumes during the severe-hypercapnic hypoxia (severe-HH) that exceeded the capacity of the end-tidal forcing system (i.e. >100 L/min). The sample size for the hypoxic breathing trial was in turn reduced from 14 to 11 subjects.

Blood gases, oximetry and metabolites: Arterial and jugular venous blood gas, oximetry, pH, and metabolite data are presented in Table 4. By design, there was a significant increase in $PaCO_2$, and reduction in PaO_2, SaO_2 and pH in both mild-HH and severe-HH from baseline. There was no main effect of glucose or lactate (p both >0.05).

*Hemodynamics***:** Absolute HR, MAP, SV, CO, and gCBF are presented in Table 5. Except for SV, all measures in both conditions were significantly elevated from baseline (p all <0.05).

Cerebral metabolism and oxygen delivery: Absolute CDO_2, O_2 Ext, Glu Ext, Lac Ext, $CMRO_2$, and OCI are presented in Table 6. Individual $CMRO_2$ are presented in Figure 3. There was a significant main effect of $CMRO_2$ ($p<0.01$). Compared to baseline, post-hoc

comparisons revealed no change in $CMRO_2$ during mild-HH ($P>0.05$); but a significant ~17% reduction in the severe-HH condition ($P=0.04$, $d=0.87$). The OCI was unchanged from baseline in both conditions. As expected in both mild-HH and severe-HH the O_2 Ext was significantly reduced from baseline, and the CDO_2 was significantly elevated (p all <0.05).

DISCUSSION

This study reports the first measures of cerebral metabolism in humans during a prolonged dry apnea lasting on average greater than five minutes. A marked reduction in $CMRO_2$ was observed immediately prior to the termination of the apnea (~29%, d=1.18), compared to baseline measures. In contrast, there was no change at any time point in the indirect estimation of non-oxidative metabolism, despite a ~380% increase in arterial adrenaline concentrations from baseline to the end of apnea. With manipulation of end-tidal gases, mild hypercapnic hypoxia had little influence on $CMRO_2$; however, hypoxia combined with severe hypercapnia significantly reduced the $CMRO_2$ by ~17% (d=0.87). We interpret these findings to indicate that the increased levels of hypercapnia and reduced pH, in part, explain the reduction in $CMRO_2$ near the termination of apnea.

The notion of a reduced $CMRO_2$ resulting from hypercapnia is not novel, but has remained debatable (Yablonskiy, 2011). With obvious implications in general medicine and anesthesia (e.g. (Waaben *et al.*, 1989)), the impact of $PaCO_2$ on $CMRO_2$ deserves attention. Unfortunately, results in human studies with similar MRI-based experimental designs and in the absence of anesthesia have remained inconsistent. For example, Jain et al., (Jain *et al.*, 2011) report no change in $CMRO_2$ with a ~8 mmHg increase in end-tidal CO_2; Chen et al., (Chen and Pike, 2010) report a ~7% reduction in $CMRO_2$ with a ~9 mmHg increase in end-tidal CO_2; and Xu et al., (Xu *et al.*, 2012) report a ~13% reduction in $CMRO_2$ with a ~7 mmHg increase in end-tidal CO_2. Using arterial and cerebral venous sampling, the present study provides evidence for reductions in $CMRO_2$ with

hypercapnia, at least in the presence of hypoxia. The pH-dependent activity of phosphofructokinase (the enzyme responsible for the phosphorylation of fructose 6-phosphate in glycolysis) provides mechanistic support for reductions in $CMRO_2$ with hypercapnia. Indeed, an accumulation of glucose 6-phosphate and fructose 6-phosphate is shown in rats exposed to acute hypercapnia, which cannot be explained by glycogen breakdown (Folbergrova *et al.*, 1975). Additionally, hypercapnia has consistently been shown to depress cortical activity (Dulla *et al.*, 2005; Zappe *et al.*, 2008; Thesen *et al.*, 2012) and some speculate that a reduced brain temperature from the increased blood flow may account for some of the reduction in $CMRO_2$ (i.e. due to the Q10 effect (Bain *et al.*, 2015b)). Nonetheless, the bulk of literature now collectively supports a $PaCO_2$ modulation of $CMRO_2$. The end-tidal forcing data in the present study may indicate this modulation is dose dependent, whereby a threshold change in $PaCO_2$ is required.

During apnea, chemosensory stimuli and baroreflex inhibition from increased intrapulmonary and transpulmonary pressures (exacerbated with glossopharyngeal insufflation) significantly increase muscle sympathetic nerve activity (burst frequency, magnitude and recruitment) (Heusser *et al.*, 2010; Steinback *et al.*, 2010b), and therefore catecholamine release. Ascribed to the known effect of catecholamines on muscle metabolism (Tank and Lee Wong, 2015), an increase in whole-body anaerobic metabolism is often held integral to the mammalian dive reflex (Blix and Folkow, 2011). Whether the cerebral tissue significantly partakes in the non-oxidative metabolism is unclear, but it is generally accepted that like in the muscle, adrenaline (but not noradrenaline) increases the cerebral non-oxidative metabolism (Seifert *et al.*, 2009). The

~380% increase in arterial adrenaline from baseline to the termination of apnea therefore offers potential for an increased cerebral non-oxidative metabolism (Seifert *et al.*, 2009). However, there was no reduction in the indirect estimation of non-oxidative carbohydrate use (OCI) at any time point during the apnea compared to baseline. In fact non-oxidative metabolism trended above 100% from percentage time 20 to 60 of the apnea. An oxidative carbohydrate index above 100% may indicate the presence of carbon oxidation from sources other than arterial glucose or lactate. Based on animal studies, these data may in turn indicate an allosteric glycogen release from the astrocytes (Saez *et al.*, 2014).

Under the same metabolic paradigm whereby adrenaline increases non-oxidative metabolism, adrenaline should also increase the oxidative metabolism (Tank and Lee Wong, 2015). For example, intra-venous adrenaline infusion (~8ug/ml/min), with concurrent increases in MAP to assure amine passage across the blood-brain barrier, significantly increases the $CMRO_2$ in humans (King *et al.*, 1952). However, when adrenaline concentrations are such that the glycogenolytic and glycolytic flux is increased to impact the $CMRO_2$, circulating blood glucose would be elevated in adjunct (Seifert *et al.*, 2009; Tank and Lee Wong, 2015). Therefore, the slightly reduced arterial glucose, reduced $CMRO_2$, and unchanged oxidative carbohydrate index collectively suggests that the ~380% increase in adrenaline was insufficient to elicit noticeable metabolic changes – perhaps not surprising given that when non-oxidative metabolism does increase, plasma adrenaline may be increased >10-fold, e.g., during exhaustive exercise (Pott *et al.*, 1996; Tank and Lee Wong, 2015). Of note, anaerobic-tolerant animals adapt to hypoxia by slowing the oxidative metabolism, rather than increasing the non-oxidative metabolism

(Hochachka *et al.*, 1996). Our findings reveal that a similar metabolic outcome is initiated in humans during prolonged apnea.

Although we place hierarchy on the impact of $PaCO_2$ on $CMRO_2$, oxygen-conserving reflexes attending the dive-reflex cannot be dismissed. Indeed it is conceivable that the $CMRO_2$ may be reduced to a greater extent had the apnea been performed in water, with activation of the trigeminal nerve and therefore a stronger oxygen-conserving / dive reflex (Schaller *et al.*, 2009; Lemaitre *et al.*, 2015). However, it appears that the activation of the trigeminal nerve is more responsible for blood redistribution (from the periphery to the brain), and bradycardia, without directly influencing the $CMRO_2$ (Reis *et al.*, 1997; Schaller *et al.*, 2009; Lemaitre *et al.*, 2015). Reductions in $CMRO_2$ associated with oxygen-conserving reflexes may relate more to mechanisms observed in rapid ischemic preconditioning (Gidday, 2006; Schaller *et al.*, 2009). Here, neural inhibitory factors, for example adenosine and ATP sensitive potassium channels, are likely responsible, as evidenced in the anoxic tolerant turtle (Perez-Pinzon *et al.*, 1993). It is difficult to speculate on these potential oxygen-conserving mechanisms in the present study, however, given that the average level of acidosis was greater in the severe-hypercapnic hypoxia trial than it was at the termination of apnea (arterial pH: 7.30 vs. 7.34, respectively), oxygen-conserving reflexes independent of acidosis are likely to have also contributed to the reduction in $CMRO_2$ during apnea. It should also be emphasized that the metabolic changes associated with $PaCO_2$ described herein are in the background of hypoxia. Nevertheless, although acidosis decreased the oxygen saturation for the same PaO_2 during severe-hypercapnic hypoxia compared to mild-hypercapnic hypoxia (via the

Bohr effect, Table 5), it is unlikely that this would have influenced the $CMRO_2$. Indeed, we have previously demonstrated using the same measurement technique that similar eucapnic oxygen saturations (~70%) of 15-minute duration do not alter the $CMRO_2$ (Ainslie *et al.*, 2014). Surprisingly, at the termination of apnea, neither the PaO_2 nor the $PaCO_2$ was independently correlated with the reduction in $CMRO_2$. This may point to the individual variability in the $CMRO_2$ response to extreme blood gas changes, an interacting facet of hypoxia and hypercapnia, or the impact of other poorly defined brain oxygen-conserving reflexes in humans (Schaller *et al.*, 2009).

In summary this study presents a clear reduction in $CMRO_2$ immediately prior to the termination of a prolonged apnea, attributable in part to hypercapnia. In contrast, there appears to be no change in non-oxidative metabolism, despite a 3 to 4-fold increase in arterial adrenaline concentrations. To our knowledge, this is the first study to examine the cerebral metabolism in healthy humans during prolonged apnea yielding some of the most extreme levels of hypoxia and hypercapnia reported to date. It is suggested that hypercapnia promotes brain oxygen-conservation, and therefore provides a protective mechanism against severe hypoxia relating to apnea.

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Competing Financial Interests statement

All authors declare no financial or competing interests

Figure legends

Figure 1. Mean ± standard deviation of absolute arterial (PaO_2) and internal jugular venous (PvO_2) partial pressure of oxygen throughout the duration of the maximal apnea.

Figure 2. Individual data of the absolute cerebral metabolic rate of oxygen ($CMRO_2$) before the apnea (Baseline) and at apnea termination (100% Apnea). Dashed line with open circles denotes mean data. Compared to baseline, the $CMRO_2$ was significantly reduced by ~29% at percent time 100 of the apnea ($P<0.01$, $d=1.18$).

Figure 3. Individual data of the absolute cerebral metabolic rate of oxygen ($CMRO_2$) at baseline, during mild-hypercapnic hypoxia (mild-HH), and severe-hypercapnic hypoxia (severe-HH). Dashed line with open circles denotes mean data. There was a significant ~17% reduction in the severe-HH condition ($P=0.04$, $d=0.87$) compared to baseline.

Table 1. Arterial (radial) and cerebral venous (internal jugular bulb) measures of the partial pressure for oxygen (PO_2) and carbon dioxide (PCO_2), oxygen saturation (SO_2), Hematocrit (Hct), Hemoglobin (tHb), Glucose (Glu), Lactate (Lac), and pH at baseline and during 20% increments of the maximal apnea. * Denotes significant difference from baseline

	PO_2 (mmHg)	*PCO_2 (mmHg)*	*SO_2 (%)*	*Hct (%)*	*tHb (mmol/L)*	*Glu (mmol/L)*	*Lac (mmol/L)*	*pH*
Baseline								
Arterial	92.4□9.1	40.9□3.3	97.8□0.9	44.4±3.1	14.5±1.0	5.6□0.8	0.8□0.3	7.41□0.02
Venous	31.1□2.6	51.8□3.6	62.1□5.1	44.1□□□□	□□□□□0.9	4.9□0.8	0.8□0.3	7.36□0.02
20%								
Arterial	108.8□11.5*	32.4□5.1*	99.0□0.4*	44.3±3.2	14.4□1.0	5.3□0.6	0.9□0.2	7.48□0.04*
Venous	24.7□3.6*	47.6□3.5*	50.5□8.1*	45.3□□□□	14.8□1.1	4.6□0.6	0.9□0.2	7.40□0.02*
40%								
Arterial	84.2□16.0	39.6□4.3	96.9□1.9	44.8±4.2	14.6□1.4	5.3□0.6	0.9□0.2	7.42□0.02
Venous	30.7□3.9	50.0□3.1	61.2□6.5	45.5□□□□	14.9□1.2	4.8□0.6	0.9□0.3	7.38□0.02
60%								
Arterial	56.9□11.0*	46.1□5.0*	89.2□5.1*	45.4±4.4	14.8□1.5	5.3□0.7	1.0□0.3	7.38□0.03*
Venous	32.4□4.0	52.5□3.4	63.7□6.0	45.7±3.8	14.9□1.2	4.9□0.6	1.0□0.3	7.36□0.02
80%								
Arterial	45.8□9.7*	49.7□4.7*	80.6□7.8*	45.5±3.7*	14.8□1.2*	5.5□0.6	1.0□0.3	7.36□0.02*
Venous	30.6□3.5	54.9□3.7	59.5□6.0	45.7□□□□*	14.9□1.0*	5.1□0.6	1.0□0.3	7.34□0.02*
100%								
Arterial	32.6□8.8*	53.4□4.8*	60.9□14.0*	45.8±3.4*	14.9□1.1*	5.4□0.7	1.1□0.3*	7.34□0.02*
Venous	25.2□5.9	57.8□4.7*	45.6□13.0*	46.0□□□□□	15.0□1.2*	5.1□0.6	1.2□0.4*	7.32□0.02*

Table 2. Heart rate (HR), stroke volume (SV), cardiac output (CO), mean arterial pressure (MAP), and the global cerebral blood flow (gCBF) at baseline and during 20% increments of the maximal apnea. * Denotes significant difference from baseline

	HR (b/min)	*SV (ml)*	*CO (L/min)*	*MAP (mmHg)*	*gCBF (ml/min)*
Baseline	64±11	108±21	6.78±1.65	88±8	610±153
20%	80±17*	58±16*	4.52±1.47*	92±10	469±140*
40%	77±16*	62±13*	4.54±1.03*	99±11*	613±196
60%	66±16	81±15*	5.11±1.46*	115±21*	828±267*
80%	59±14	95±18	5.41±1.52*	131±21*	991±325*
100%	56±15	96±33	5.17±1.91*	139±23*	1041±318*

Table 3. Cerebral oxygen delivery (CDO_2), oxygen extraction fraction (O_2 Ext), lactate extraction (Lac Ext), glucose extraction (Glu Ext), oxidative carbohydrate index (OCI), and metabolic rate of oxygen ($CMRO_2$) at baseline and during 20% increments of the maximal apnea. * Denotes significant difference from baseline

	CDO_2 (ml/min)	*O_2 Ext (%)*	*Lac Ext (%)*	*Glu Ext (%)*	*OCI (%)*	*$CMRO_2$ (ml/min)*
Baseline	122±30	37±6	-2±11	12±3	83±13	45±11
20%	92±24*	47±10*	-3±17	12±6	127±75	43±10
40%	120±34	36±7	-3±20	9±6	125±61	41±10
60%	152±48*	28±7*	0±15	7±4*	139±94	41±12
80%	163±50*	26±4*	-3±17	7±2*	92±37	41±11
100%	128±36	25±9*	-2±13	5±3*	92±57	32±12*

Table 4. Arterial (radial) and cerebral venous (internal jugular bulb) measures of the partial pressure for oxygen (PO_2) and carbon dioxide (PCO_2), oxygen saturation (SO_2), Hematocrit (Hct), Hemoglobin (tHb), Glucose (Glu), Lactate (Lac), pH and the oxidative carbohydrate index (OCI) at baseline and hypoxic breathing with mild and severe levels of hypercapnia. * Denotes significant difference from baseline

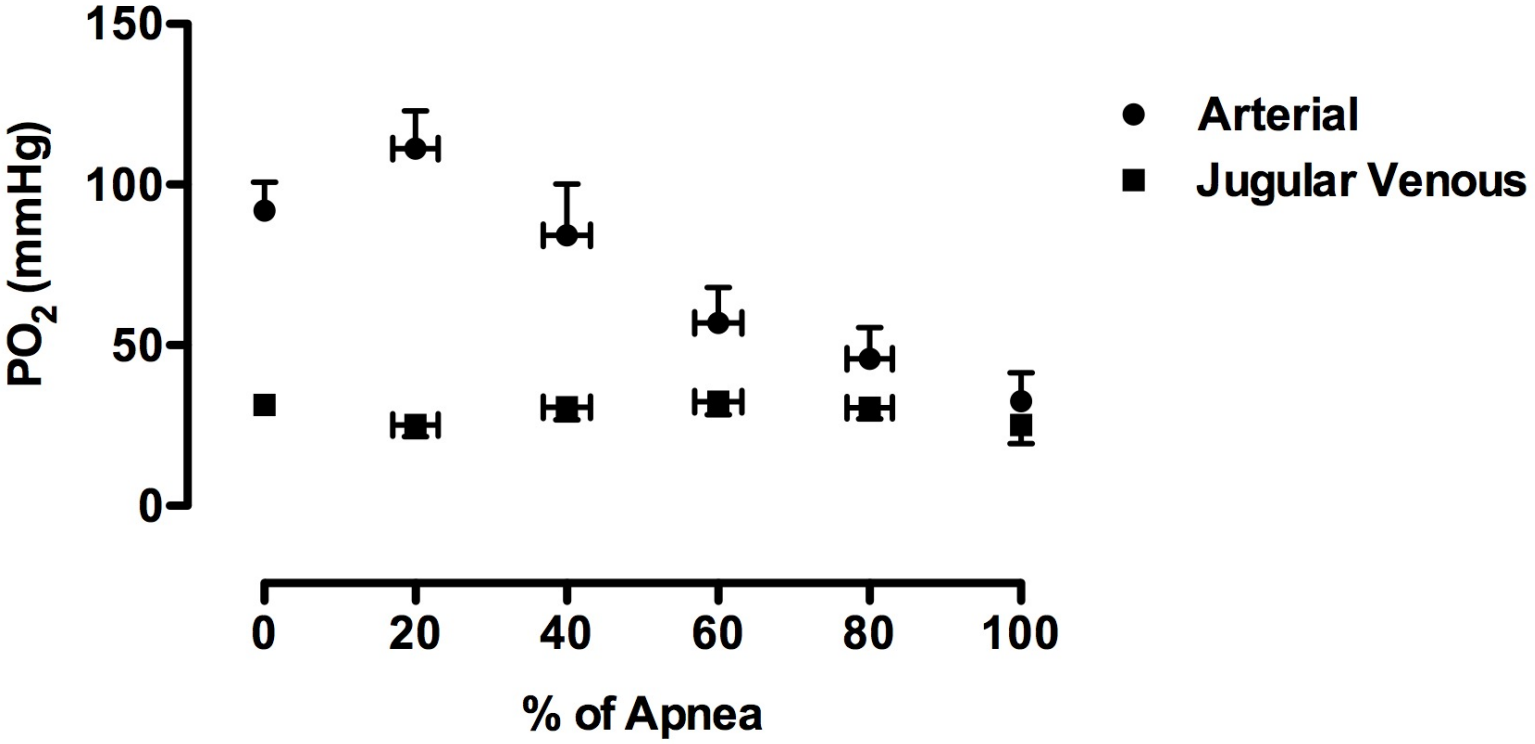
	PO_2 (mmHg)	*PCO_2 (mmHg)*	*SO_2 (%)*	*Hct (%)*	*tHb (mmol/L)*	*Glu (mmol/L)*	*Lac (mmol/L)*	*pH*
Baseline								
Arterial	91.6±13.7	38.6±5.1	98±1	43.9±3.7	14.3±1.2	5.4±0.5	0.9±0.3	7.43±0.05
Venous	29.5±4.1	50.1±4.2	60±5	44.6±4.1	14.6±1.3	4.9±0.6	1.0±0.3	7.37±0.04
Mild-HH								
Arterial	38.9±7.8*	46.3±3.2*	75±8*	44.7±3.8*	14.6±1.2*	5.3±0.5	0.9±0.3	7.38±0.03*
Venous	28.0±3.4	52.6±2.6*	54±7*	44.6±3.4	14.6±1.1	5.0±0.5	0.9±0.3	7.35±0.02*
Severe-HH								
Arterial	38.0±8.9*	58.7±4.3*	68±12*	45.3±3.4*	14.8±1.1*	5.3±0.4	0.8±0.3	7.30±0.02*
Venous	30.8±5.5	62.3±4.1*	55±11*	45.2±3.6	14.8±1.2	5.2±0.4	0.9±0.3	7.29±0.02*

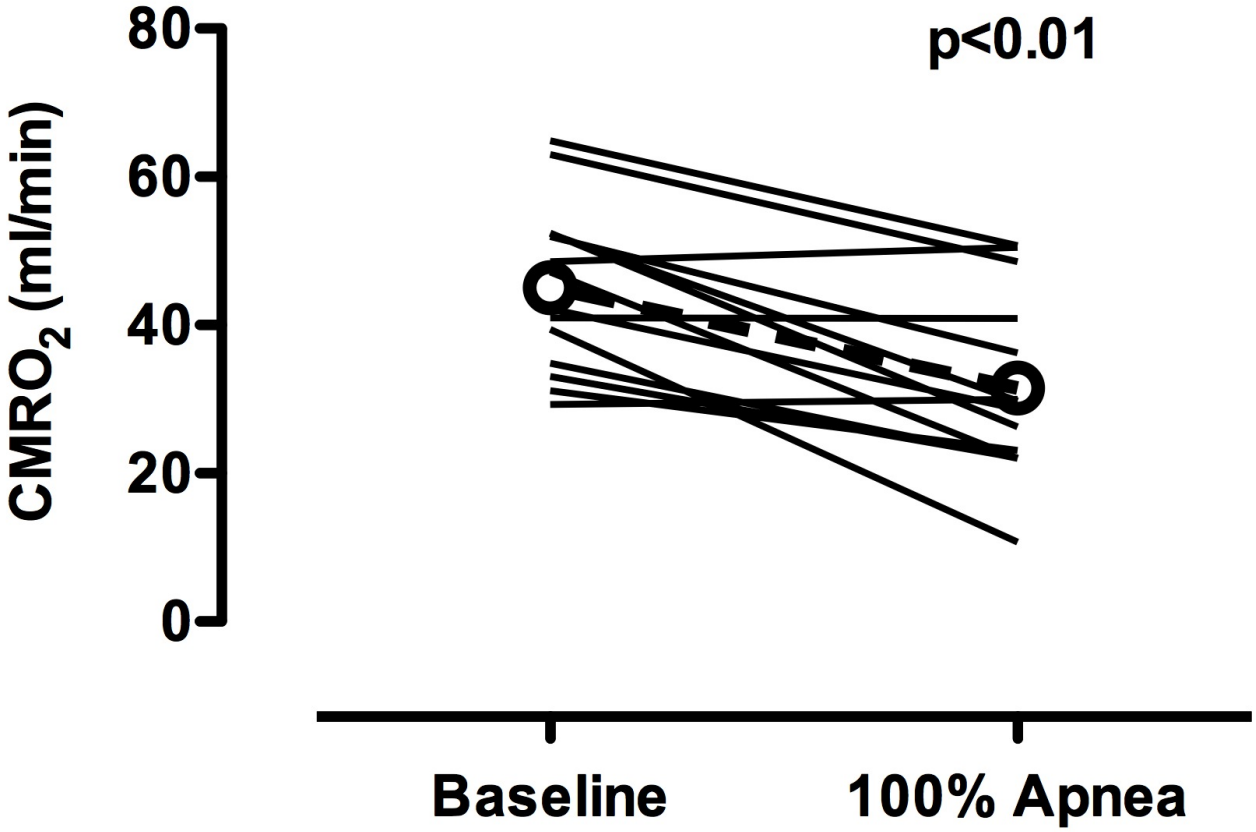
Table 5. Heart rate (HR), stroke volume (SV), cardiac output (CO), mean arterial pressure (MAP), and the global cerebral blood flow (gCBF) during baseline and hypoxic breathing with mild and severe levels of hypercapnia. * Denotes significant difference from baseline

	HR (mmHg)	*SV (ml)*	*CO (L/min)*	*MAP (mmHg)*	*gCBF (ml/min)*
Baseline	69±18	102±18	7±1	89±6	593±151
Mild-HH	88±14*	104±17	9±2*	102±11*	983±204*
Severe-HH	94±16*	107±15	10±3*	113±18*	1340±343*

Table 6. Blood flow in the right internal carotid artery (QICA) and left vertebral artery (QVA), flow velocity in the middle (MCA) and posterior (PCA) cerebral artery, total cerebral delivery of oxygen (CDO_2) and total cerebral metabolic rate of oxygen ($CMRO_2$) during baseline and hypoxic breathing with mild and severe levels of hypercapnia. * Denotes significant difference from baseline

	CDO_2 (ml/min)	*O_2 Ext (%)*	*Lac Ext (%)*	*Glu Ext (%)*	*OCI (%)*	*$CMRO_2$ (ml/min)*
Baseline	117±32	38±7	-5±6	10±5	88±16	44±13
Mild-HH	149±32*	28±5*	2±8	7±3	80±18	41±11
Severe-HH	186±53*	19±4*	-5±15	3±3*	80±26	34±11*


150
100
50
0
PO2 (mmHg)
0
20
40
60
80
100
% of Apnea
Arterial
Jugular Venous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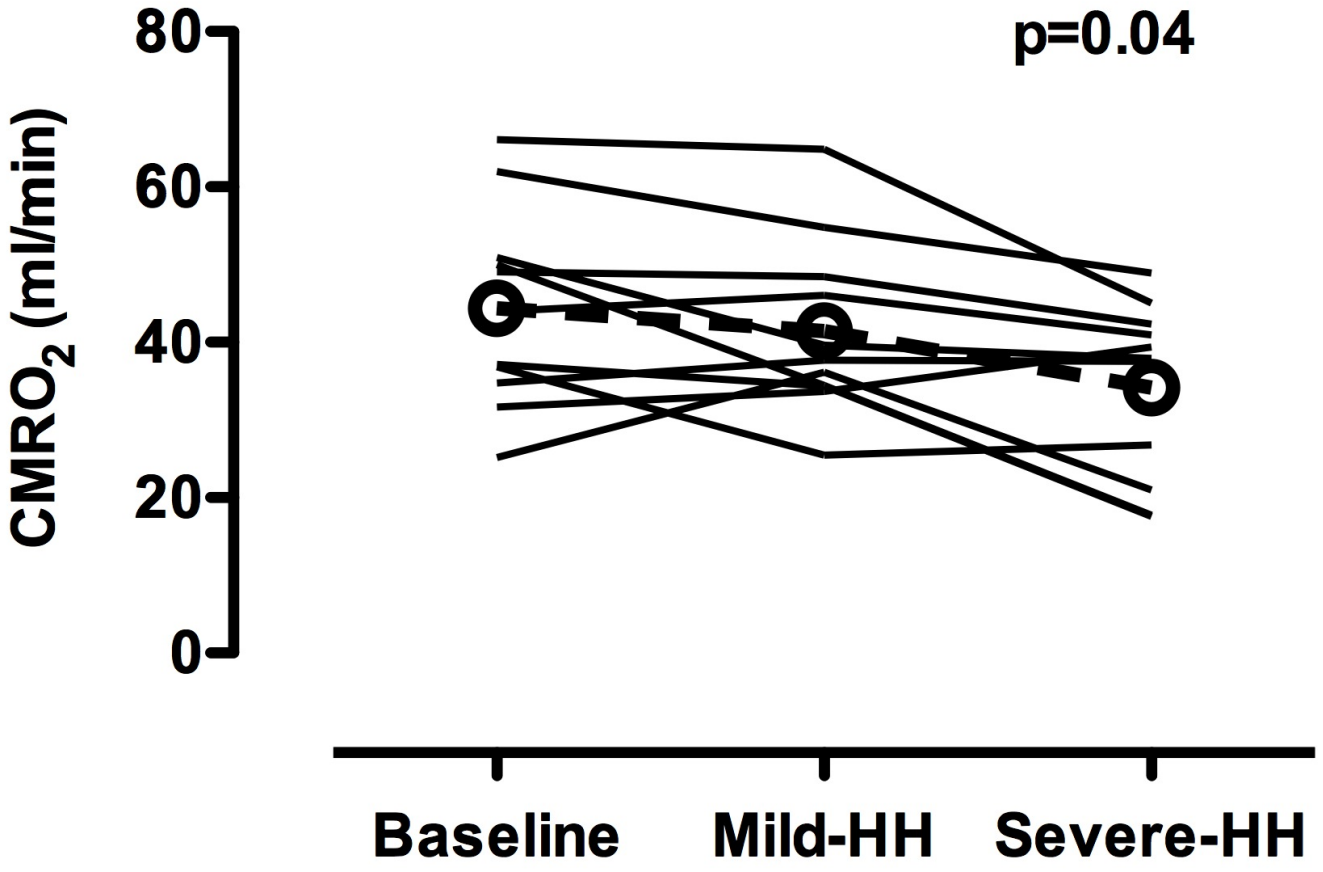
CMRO2 (ml/min)
Baseline
100% Apnea


p=0.04
80
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CMRO2 (ml/min)
Baseline
Mild-HH
Severe-HH