HYPOXIA IN AQUATIC SPORTS

Joshua T. Royal

Doctoral Dissertation
Jožef Stefan International Postgraduate School
Ljubljana, Slovenia

Supervisor:	Prof. Igor B. Mekjavić Department of Automatics, Biocybernetics and Robotics Jožef Stefan Institute, Ljubljana, Slovenia
Co-Supervisors:	Dr. Adam C. McDonnell Department of Automatics, Biocybernetics and Robotics Jožef Stefan Institute, Ljubljana, Slovenia
	Dr. Jernej Kapus, Faculty of Sport, University of Ljubljana Ljubljana, Slovenia

Evaluation Board:

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MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL

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HIPOKSIJA V VODNIH ŠPORTIH

Doktorska disertacija

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Dr. Jernej Kapus

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Abstract

The present thesis considers the premise that breath-holding in aquatic sports, primarily swimming, in order to enhance performance, can potentially cause adaptation to tissue hypoxia. To investigate this theory, a series of studies needed to be performed:

I. Analysis of variability in individuals' responses to hypoxia, to evaluate the range of degrees acclimation to the stimulus. Data from three bed rest projects (LunHab project: 10-d bedrest; FemHab project: 10-d bed rest; PlanHab project: 21-d bed rest) were combined for a total of 31 participants that completed two bed rest experimental conditions in each project: i) normoxic bed rest (NBR), and ii) hypoxic bed rest (HBR).
II. Introduction and establishment of a carbon monoxide rebreathing technique in our laboratory to assess the total haemoglobin mass (Hbmass). 22 participants were tested for Hbmass from both the antecubital vein and fingertip for measures of validity. 13 participants returned for a second identical visit where reliability was assessed.
III. Assessment of markers of hypoxic adaptation in a group of swimmers against full controls, terrestrial exercise controls (cross-country skiers), and breath-holding non-exercise controls (apnoea divers). Participants were evaluated for differences in blood oxygen-carrying capacity (aerobic capacity and Hbmass) and chemosensitivity (hypoxic and hypercapnic ventilatory response).
IV. Longitudinal analysis of swimmers and control participants over the course of a swimming season to assess seasonal variation. Of Study III's participants, 5 control participants and 4 swimmers returned for two further bouts of testing during the 2021/22 swimming season.

The results of the above studies were as follows:

I. Sex had an impact on the erythropoietin (EPO) response in NBR and HBR, whereas bed rest duration had no effect. Relative EPO responses are not sufficient indicators of the resultant increased production of reticulocytes and red blood cells.
II. Fingertip capillary blood sampling was an acceptable alternative to venous blood for the calculation of Hbmass and blood volumes in terms of validity, but not reliability.
III. Despite differences in aerobic capacity between all athletic groups and the control group, no differences were found in the haematological values or chemosensitivity to hypoxia and hypercapnia
IV. The seasonal variation seen in controls and swimmers was similar, suggesting that training load changes over the course of a season have no effect on the values measured and are more likely due to typical seasonal lifestyle changes, measurement and/or biological error, and biological variation.

From these results, it was concluded that the swimmers in the present study were not exposed to a hypoxic stimulus of sufficient intensity to invoke hypoxic adaptation. This is observed also when comparing their values to both full and terrestrial exercise controls. The present thesis has also elucidated the importance of reporting individual values as well as means and standard deviations in physiological research.

Povzetek

Pričujoča doktorska disertacija obravnava predpostavko, da zadrževanje diha pri vodnih športih, predvsem plavanju, z namenom izboljšanja zmogljivosti vodi v prilagoditev na mišično hipoksijo. Delo smo izvedli v seriji štirih raziskav:

Raziskava I: Analiza variabilnosti odzivov posameznikov na hipoksijo. Razpon prilagoditve na hipoksijo je bil ovrednoten na podlagi podatkov, pridobljenih iz treh študij večdnevnega ležanja v postelji oz. »bed rest« (2 x 10 dni, 1 x 21 dni). V analizo smo vključili rezultate 31 preiskovancev, ki so opravili dva eksperimentalna pogoja (normoksični bed rest = NBR in hipoksični bed rest = HBR).

Raziskava II: Veljavnost in zanesljivost metode izračuna skupne mase hemoglobina (Hbmass) na podlagi predihavanja ogljikovega monoksida. Da bi ocenili veljavnost metode, smo preiskovancem (N = 22) Hbmass izmerili na podlagi vzorcev krvi, odvzetih iz dveh različnih mest: antekubitalne vene in konice prsta. Zanesljivost metode je bila preverjena s ponovitvijo meritve pri 13 preiskovancih.

Raziskava III. Ovrednotenje kazalcev hipoksične prilagoditve pri plavalcih, smučarskih tekačih, potapljačih na vdih in osebah, ki se z vodnimi športi ne ukvarjajo (kotrolna skupina). Športniki različnih športov in kontrolna skupina so bili primerjani glede zmogljivosti prenosa kisika v krvi (aerobna zmogljivost in Hbmass) in kemosenzitivnosti (hipoksični in hiperkapnični ventilacijski odziv).

Raziskava IV. Longitudinalna analiza plavalcev in oseb, ki se z vodnimi športni ne ukvarjajo. Da bi izmerili sezonsko variacijo tekom celotne plavalne sezone, se je 9 preiskovancev (4 plavalci in 5 oseb, ki se z vodnimi športi ne ukvarjajo) iz raziskave III udeležilo dveh dodatnih testiranj v plavalni sezoni 2021/22.

Z raziskavami smo ugotovili, da spol vpliva na odziv eritropoetina (EPO) med NBR in HBR, medtem ko trajanje »bed resta« nima učinka. Izkazalo se je, da relativni EPO odzivi niso zanesljivi kazalci povečane proizvodnje retikulocitov in rdečih krvničk (raziskava I). Metoda izračuna Hbmass in volumna krvi z uporabo kapilarne krvi iz konice prsta in ne venske krvi je veljavna, ne pa tudi zanesljiva (raziskava II). Športniki različnih športov (plavalci, potapljači na vdih in smučarski tekači) so imeli znatno višjo aerobno zmogljivost kot preiskovanci v kontrolni skupini, čeprav ni bilo razlik v njihovih hematoloških kazalcih ter kemosenzibilnosti na hipoksijo in hiperkapnijo (raziskava III). Sezonska variacija med plavalci in kontrolno skupino je bila primerljiva, kar nakazuje, da obremenitvene spremembe v vadbi med sezono ne vplivajo znatno na izmerjene spremenljivke, ampak so le-te verjetneje posledica tipičnih sezonskih sprememb življenjskega sloga, napake v meritvah in/ali biološke variacije (raziskava IV).

Na podlagi rezultatov zaključujemo, da plavalci, ki so sodelovali v naših raziskavah, niso bili izpostavljeni dovolj intenzivnim hipoksičnim dražljajem, da bi povzročili hipoksično prilagoditev. To je opazno tudi pri primerjavi njihovih rezultatov z drugimi športniki (potapljači na vdih in smučarski tekači) in kontrolno skupino. Pričujoča doktorska disertacija je razjasnila pomembnost poročanja individualnih vrednosti poleg povprečij in standardnih odstopanj v fizioloških raziskavah.

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Abbreviations

BV	...	Blood volume
CAP	...	Capillary blood
CI	...	Confidence interval
CO	...	Carbon monoxide
COHb%	...	Carboxyhaemoglobin saturation levels
CoV	...	Coefficient of variation
EPO	...	Erythropoietin
[Hb]	...	Haemoglobin concentration
Hbmass	...	Total haemoglobin mass
HBR	...	Hypoxic bed rest
Hct	...	Haematocrit
ICC	...	Intraclass correlation coefficient
IPS	...	International Postgraduate School
IQR	...	Interquartile range
JSI	...	Jožef Stefan Institute
NBR	...	Normoxic bed rest
$nCO_{absorbed}$	...	Number of CO molecules absorbed
nHb_{tagged}	...	Number of tagged haemoglobin molecules
nHb_{total}	...	Number of total haemoglobin molecules
O_2	...	Oxygen
P_{atm}	...	Ambient pressure
P_IO_2	...	Partial pressure of inspired oxygen
POST	...	Post-intervention
PRE	...	Pre-intervention
PV	...	Plasma volume
R	...	Ideal gas constant ($8.314\ J \cdot K^{-1} \cdot mol^{-1}$)
RBC	...	Red blood cells
RBCV	...	Red blood cell volume
Rct	...	Reticulocytes
SARS-CoV-2	...	Severe acute respiratory syndrome coronavirus 2
SD	...	Standard deviation
SaO_2	...	Arterial blood O_2 saturation
SpO_2	...	O_2 saturation from pulse oximetry
T	...	Temperature
TE%	...	Typical measurement error
$VCO_{absorbed}$	...	Volume of absorbed CO gas
VEN	...	Venous blood

Chapter 1

Introduction

1.1 Thesis Structure

This thesis outlines a series of studies conducted to assess physiological adaptation to hypoxia in aquatic athletes.

The Introduction (Chapter 1) presents an overview of the existing research on hypoxic stimuli and adaptation during aquatic sports. The importance of establishing the degree of hypoxic stimulus and adaptation in aquatic sports is also discussed. This chapter also introduces the issues that arise when trying to measure pulse oximetry in water.

Study I (Chapter 2) addresses individual variability in acclimation to hypoxia in both males and females. While research in the area of sports and exercise physiology tends to report values as that of the mean and standard deviation of the cohort, this chapter highlights the importance of reporting individual values and the magnitude of variability between them.

Study II (Chapter 3) examines the carbon monoxide rebreathing method; a protocol that is used to determine haemoglobin mass and intravascular volumes. The measurement of haemoglobin mass allows researchers to track changes in oxygen-carrying capacity without the influence of hydration status. Commonly, in the absence of a trained phlebotomist, researchers may draw capillary blood instead of performing a venepuncture. This chapter therefore examines the validity and reliability of using capillary blood for the calculation of haemoglobin mass when compared to venous blood.

Study III (Chapter 4) compares ventilatory and haematological measures in athletes from different sporting disciplines with the aim of deciphering differences between acclimatisation to hypoxia and exercise.

Study IV (Chapter 5) explores longitudinal analysis in a group of competitive swimmers and control participants that were assessed throughout a swimming season.

Chapter 6 acknowledges the previous work of Woorons and colleagues, and critically analyses their findings.

Chapter 7 provides an overall conclusion from the work conducted within the framework of the present thesis.

1.2 Scientific Background

1.2.1 Hypoxia During Sport and Exercise

Since the aerobic performance reductions attributed to altitude that were anticipated and observed at the 1968 Olympic Games in Mexico City (Jokl et al., 1969; Pugh, 1967), hypoxic training has gained popularity as a technique of physical preparation for

competition. Although the fraction of oxygen (F_IO_2) in the air at altitude is the same as at sea level, the reduced barometric pressure (P_B) causes a reduction in the partial pressure of oxygen ($PO_2 = P_B \times F_IO_2$) in the air (Kenney et al., 2015). Reduced ambient PO_2 decreases the PO_2 gradient between the lungs and capillary blood; this, in turn, reduces the blood oxyhaemoglobin saturation and causes tissue hypoxia.

Long-term exposure to hypoxic conditions (either through ambient air altitude or artificially), can cause a physiological adaptation as the body tries to match the stimuli it is facing. Previous research shows that chronic exposure to hypoxia results in numerous mechanisms of adaptation, namely erythropoiesis (Scholz et al., 1990), increased capillary density (Hoppeler & Vogt, 2001), metabolic change (Vogel et al., 2015) and chemosensitivity changes (Richalet et al., 2012). The degree of adaptation is influenced by genetic variances between individuals and populations, with those who have lived in high-altitude environments for generations often exhibiting enhanced adaptation (Weil, 2003; Weil et al., 1971).

In conventional training programs, hypoxia is often used as a stimulus to drive haematological and respiratory adaptations (Brugniaux et al., 2006). When used in conjunction with exercise, these adaptations assist in maximising oxygen delivery to the working muscles. Multiple forms of hypoxia/normoxia time ratios have been tested for their effectiveness: i) Live High: Train Low (Wehrlin et al., 2006), ii) Live Low: Train High (Debevec et al., 2010), iii) Live High: Train High (Heinicke et al., 2005; Kounalakis et al., 2013) and iv) Intermittent Hypoxic Exposure (Humberstone-Gough et al., 2013).

Most commonly, hypoxia is induced via a decrease in the quantity of inspired oxygen either through environmental (altitude; PiO_2) or artificial means (i.e., hypoxic chamber; hypobaric chamber) (Vogt et al., 2001). The majority of hypoxic research has been conducted in this area due to its aforementioned potential detrimental work/exercise performance effects. Hypoxia from hypoxaemia is often found in medical conditions where severe Chronic Obstructive Pulmonary Disorder (COPD) and cystic fibrosis patients often suffer from persistently low oxygen levels (Lopez et al., 2006). However, hypoxaemia is also found in athletes during exercise (exercise-induced hypoxaemia; EIH), which in terrestrial sports is often attributed to 3 mechanisms: relative hypoventilation, ventilation to perfusion mismatching and diffusion limitation.

1.2.2 Hypoxic Stimuli in Aquatic Sports

The development of hypoxia during aquatic exercise in humans was first reported by Craig (1961), who studied the dangers of prolonged breath-holds during simulated swimming exercise. This research was furthered by Davies and colleagues (1995), who monitored PO_2 and PCO_2 during synchronised swimming competition breath-holds. Their research confirmed that breath-holding when combined with increased CO_2 production (hypercapnia), augments the magnitude of hypoxia. Miyasaka et al. (2002) who monitored arterial oxygen saturation during freestyle sprint swimming, found reductions as large as 14% in blood oxygen saturation measured through pulse oximetry (SpO_2) after just 100 metres. Voluntary prolonged breath-holding may be utilised in order to maximise streamlining and maintain velocity (Pedersen & Kjendlie, 2006). This prolonged breath-holding, in conjunction with high exertion exercise, has been shown to reduce SpO_2, therefore invoking tissue hypoxia. Over an intense training period where SpO_2 is intermittently reduced, repeated tissue hypoxia may invoke physiological adaptations commonly also seen at altitude.

1.2.3 Changes in Chemosensitivity in Aquatic Athletes

As reduced breathing frequency plays an essential role in the degree of tissue hypoxia experienced in aquatic sports, the hypoxic and hypercapnic ventilatory drives of participants are potentially crucial components (Ferretti et al., 1991). Ventilatory adaptations to hypercapnia and hypoxia have been reported for activities requiring breath-holding, such as apnoea diving, synchronised swimming, and competitive swimming (Costalat et al., 2014; Davis et al., 1987; Delapille et al., 2001; Florio et al., 1979; Grassi et al., 1994; Masuda et al., 1981; Ohkuwa et al., 1980). Repeated and prolonged exposure to hypercapnia and hypoxia reduce the gain of the chemoreceptors responsible for conveying information regarding hydrogen ions (H+; reflecting P_aCO_2) and partial pressure of oxygen in the arterial blood (Grassi et al., 1994), thus eliciting the ventilatory adaptation to hypercapnia and hypoxia (Dempsey & Smith, 2014).

A similar ventilatory adaptation to hypoxia, but without the adaptation to hypercapnia is observed in lowlanders venturing to high altitudes. Adaptation to altitude results in a decreased ventilatory response to hypoxia, with no effect on the ventilatory sensitivity to hypercapnia (Sato et al., 1994). Adaptation would allow athletes to experience a more intense or more extended duration period of tissue hypoxia whilst training. Table 1.1 shows the results from various Hypoxic Ventilatory Response (HVR) and Hypercapnic Ventilatory Response (HCVR) studies in the aquatic sports of interest. Few studies monitoring HVR and HCVR values in these sports exist, especially in synchronised swimming and competitive swimming. The results which do exist come from studies utilising different methods and small sample populations. Common to these studies is the inconclusiveness of, and contradiction between values, making comparisons between studies difficult and thus establishing concrete inferences.

One important consideration to make when deciding on chemosensitivity tests to perform is the concentration of "control" gases to ensure the ventilatory response is based on the change in the desired gas. In the present thesis, the HVR tests consisted of one normoxic (low intensity) exercise phase, followed by three hypoxic (low intensity) exercise phases. During the normoxic exercise phase, the $P_{ET}CO_2$ was measured throughout, the value that each participant plateaued at was then considered their "baseline" which would be met during their hypoxic bout through supplemental CO_2.

Table 1.1: Articles based on the HCVR and HVR of different aquatic sportspeople.

Study	**No. and Gender**	**Mean ± SD or range of age (years)**	**Results**
Grassi et al. (1994)	Elite Divers (1M, 2F), Control (6M, 3F)	23 - 65	ED blunted HCVR, unchanged HVR
Bjurstrom and Schoene (1987)	Synchro swimmers (10F), Control (10F)	16 - 24	SS increased Lung Volume, unchanged HCVR, blunted HVR
Masuda et al. (1981)	Breath Hold Divers (5F), Non-Divers (5F)	50.4 ± 9.7, 47.6 ± 5.3	Lower HVR in BHD, unchanged HCVR
Delapille et al. (2001)	Breath Hold Divers (8F), Non-Divers (8F)	30.5 ± 8.4, 27.7 ± 8.2	Lower HCVR in BHD
Ohkuwa et al. (1980)	Untrained (10M), Sprint Swimmers (17M), Long-Distance Swimmers (11M)	19.9 ± 1.2, 18.9 ± 1.9, 20.2 ± 2.1	No significances in HCVR between groups
Rebuck and Read (1971)	Sprint swimmers (2M), Middle- & long-distance swimmers (3M)	18 ± 3	Higher HCVR in Sprinters
Costalat et al. (2014)	Breath Hold Divers (7M), Controls (7M)	37.3 ± 12.8, 31.9 ± 5.6	Higher HVR in BHD, indifferent HCVR between groups

Reduced oxygen (O_2) availability in the tissues, as a consequence of lowered partial pressure of O_2 (PO_2) in the ambient air, stimulates the renal release of the hormone erythropoietin (EPO) (Scholz et al., 1990). This EPO release promotes erythropoiesis in the bone marrow. After red blood corpuscles have matured into Reticulocytes (Rcts), they are released into the circulating blood. The reference value for Rcts is typically 0.5% – 2.5% of the total erythrocytes circulating in the blood (Banfi et al., 2006; Koepke & Koepke, 1986). The addition of these now matured RBCs increases the O_2 carrying capacity of the blood due to the increase in total RBC volume, haematocrit (Hct) and haemoglobin concentration ([Hb]).

1.2.4 Impact of the COVID-19 Pandemic

The COVID-19 pandemic, which engulfed the world starting in late 2019, profoundly affected various aspects of this thesis. Specifically, the restrictions imposed during the pandemic directly or indirectly influenced Sections 1.3.2, and Chapters 3, 4, and 5 of this thesis. The imposition of COVID-19 restrictions led to limited access to laboratory and swimming pool facilities during the initial testing phases. Furthermore, these restrictions resulted in a significant reduction in participant numbers, adversely affected the historical quality of athlete training, and, in certain instances, led to participants contracting COVID-19 between testing phases. The long-term cardiorespiratory implications of such infections remain uncertain.

1.3 Problem Identification

1.3.1 Haematological Measures

Haemoglobin is typically measured and reported as haemoglobin concentration ($g.L^{-1}$). The issue with this measure is that it is a concentration relative to plasma volume, which itself is relative to an individual's size, body composition, hydration status and state of training. In training intervention studies, this has led to the appearance of "pseudo-anaemia" in some athletes (Bartsch, Mairbaurl, & Friedmann, 1998), such is the increase in their plasma volume. Using the optimised carbon monoxide rebreathing method (Schmidt & Prommer, 2005), researchers have been able to measure Hbmass in absolute terms. Implementation of this method should eliminate the effects of plasma volume changes on haemoglobin values. Although many studies exist comparing haematological values of competitive swimmers pre- and post-hypoxic training intervention, to the authors' knowledge the only study that exists to date comparing swimmers Hbmass to other athletes and controls is that by Heinicke et al., (2001) and no such information exists for synchronised swimmers. In apnoea divers, Hbmass was shown to be identical between trained apnoea divers and untrained SCUBA divers (Prommer et al., 2007), although the authors suggested that long-term effects of apnoea training on Hbmass still need to be demonstrated in longitudinal studies.

1.3.2 Pulse Oximetry

For the assessment of tissue hypoxia during terrestrial exercise, a measurement of arterial blood oxygen saturation is used (SaO_2). This is acquired noninvasively using a pulse oximeter, to predict arterial blood oxygen saturation based on measurements obtained from infrared sensors applied to the skin (SpO_2). Pulse oximetry is based on two physical principles. The first, is the presence of a pulsatile signal generated by arterial blood, which is relatively independent of non-pulsatile arterial blood, venous and capillary blood, and other tissues. The second is that oxyhaemoglobin (HbO_2) and reduced haemoglobin (Hb) have different absorption spectra (Tobin, 1998). In short, a light transmitter shines red and infrared light through the skin onto the peripheral capillaries. Due to the difference between the amount of light absorbed by haemoglobin carrying different amounts of O_2, the O_2 saturation of the capillary blood is calculated by the varying light signals collected by the photodiode.

Very few studies to date have investigated reductions in SaO_2 during aquatic sports. This is primarily due to technological limitations faced when trying to collect SpO_2 data in water, resulting in swimming studies that only measure it intermittently during rest

periods or during distances that are 400 metres or less (Miyasaka et al., 2002; Spanoudaki et al., 2004; Woorons et al., 2014). In apnoea divers to avoid this issue, some studies have tested divers during cycling ergometry exercise with participants' faces submerged in a container of water (Andersson & Evaggelidis, 2009; Andersson et al., 2002; Lindholm et al., 2007). More recently, due to technological advancements, the monitoring of SpO_2 during continuous swimming has become possible (Trincat et al., 2017; Woorons et al., 2014), albeit with the limitation of the inability to perform flip turns. However, at the time of writing, development is underway for wireless waterproof pulse oximetry which allows SpO_2 measurements to be taken over a prolonged period during aquatic sports without inhibition.

Due to the previous limitations for monitoring SpO_2, researchers have tried to use alternative measures to assess whether a potential hypoxic stimulus exists in these breath-holding sports. Kapus et al. (2010; 2009; 2008) investigated the effects of reduced breathing frequency (RBF) in terrestrial exercise compared to that of spontaneous breathing (SB). This was achieved by timing the respiratory rate during exercise with a metronome. At higher intensities, RBF induced a significant reduction in the end-tidal pO_2 and an increase in end-tidal pCO_2, resulting in a significant reduction in oxyhaemoglobin saturation. In addition, these studies provide valuable insight into the contribution of reduced breathing frequency concomitant with exercise to inducing hypoxic and hypercapnic stimuli.

1.3.2.1 In-ear pulse oximetry – Pilot testing

1.3.2.1.1 Initial Testing of the Cosinuss° Two Device

A pilot study was conducted to evaluate the only device for measuring SpO_2 that would be of practical use during swimming. SpO_2 data during swimming exercise was collected using an in-ear pulse oximeter model Cosinuss° Two (Cosinuss GMBH, Munich, Germany). This device has three sensors which collected heart rate, oxygen saturation, and temperature data from the ear canal proximal to the tragus (Figure 1.1). As the device had no local storage capacity, all data was sent to its receiver unit (named LabGateway) within a 10-metre radius via a Bluetooth connection. Data was sent then by the LabGateway over either a Wi-Fi or mobile data connection.

Figure 1.1: Cosinuss° Two in-ear pulse saturation monitor (right) and LabGateway receiver unit (left).

The Cosinuss device was initially tested in a terrestrial pilot study in conditions similar to those of Kapus et al. (2010) prior to it being used with participants in a swimming pool. Two participants performed two 10-minute bouts of a constant-load cycling exercise, one whilst breathing spontaneously, and the other with a reduced breathing protocol. The reduced breathing protocol involved a two-second single exhale and inhale period followed

by four seconds of breath-holding. The results were similar to that shown in previous research (Kapus et al., 2008; Kapus et al., 2013), with SpO_2 reduced during the reduced breathing exercise period compared to spontaneous breathing (Figure 1.2).

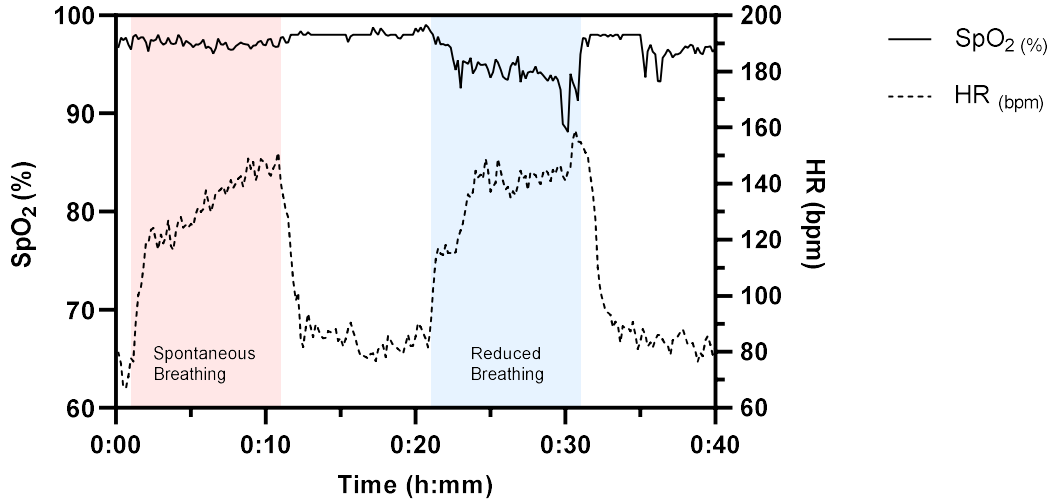

Figure 1.2 Heart rate (HR) and oxyhaemoglobin saturation (SpO_2) responses measured with the Cosinuss° Two device during two 10-s exercise bouts, separated by a 10-s rest period (white areas). The responses in the red highlighted area are for exercise conducted with spontaneous breathing and the blue highlighted area for exercise conducted with reduced breathing.

1.3.2.1.2 Challenges with Bluetooth Connection and Signal Range

The Bluetooth signal from the Cosinuss° Two device may travel a maximum of 10 metres if unimpeded. Submerging the device in water will significantly reduce this distance. Further, if one LabGateway system was to be stationed to the side of a 25 or 50-metre swimming pool then the connection between the LabGateway and the Cosinuss would be lost and therefore data collection would become patchy. Therefore, following the initial testing phase, the next key step was to assess how quickly the device would reconnect to the receiver after losing the signal. To evaluate this required a research assistant to walk 25 metres back and forth at a constant intensity into and out of the range of the LabGateway, at the same pace as a swimmer would complete the distance typically during aerobic swimming (1:30 – 1:45 per 100m). The data from this pilot test (Figure 1.3) were deemed unusable. On many occasions, the connection would not be reinstated within the ~20-second period the participant was walking within the connection area during each 50-metre lap. When the device successfully reconnected, the data recorded did not appear to be realistic and therefore untrustworthy. Changes in HR were noted in the range of up to 30 bpm in a matter of seconds despite the participant exercising at a consistent intensity. During the latter stages of the test (10^{th} to 20^{th} minute), the Cosinuss° Two device could not connect to the LabGateway for minutes at a time. This intermittent connection led to a complete disconnection and ultimately the devices timed out and shut themselves down.

The results of this second pilot test indicated that positioning a single receiver unit (LabGateway) at the mid-length point on one side of the pool was not a viable option. Therefore, for testing in the pool, it was determined that the receiver unit would need to

remain within the range of the device at all times to avoid the data irregularities that occur with these dropouts.

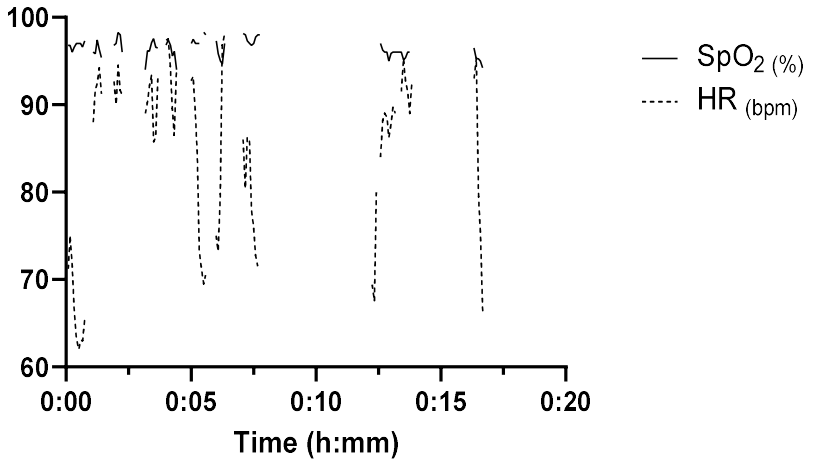

Figure 1.3: HR and SpO_2 data from intermittent Bluetooth connection test.

1.3.2.1.3 Waterproofing and Pool Testing

To assess the device in the pool required it to be waterproofed, this was achieved simply by the application of both a swimming hat and a latex or neoprene ear band over the device. In a final pilot study, the device was evaluated in a swimming pool on 2 occasions during which a swimmer performed various distances and intensities of front crawl swimming. During this period, two researchers oversaw the receiver unit's distance from the swimmer at any given time and recorded the distance and time of the efforts the swimmer was completing. Recording the distance and time as well as the heart rate and SpO_2 was to confirm associations of higher swimming intensity with an exaggerated reduction in SpO_2. Unfortunately, during this pilot testing period, no data was acquired, and the device would disconnect and fail to reconnect as soon as the swimmer's head was submerged. The unsuccessful data collection is likely due to one of several factors, most notably either the unavoidable distortion of the sensor when wearing swimming caps to waterproof the device, or the inability to transmit over a Bluetooth signal in water. Typically, in terrestrial conditions, Bluetooth signal travels as far as 10 metres, however in water due to the density of water molecules, this distance is reduced to a few centimetres.

1.3.2.1.4 Collaboration and Future Directions

Finally, as a result of the intermittent reliability of the Cosinuss monitor, a collaboration was initiated with another research institution in Erfurt, Germany (CiS Forschungsinstitut für Mikrosensorik GmbH). This research group were also developing in-ear pulse oximetry technology. To date, this research team have also struggled to achieve a reliable data transfer from their device without creating personalised ear moulds for each device. Local data storage and noise reduction, however, are two major concerns that need to be addressed prior to any device being released for this purpose.

Finally, a prototype system (SeaBear Diving Technology) was also tested. Due to the prototype nature of this system, it was not streamlined and thus impeded swimming.

Common to all systems tested was the need for substantial post-processing of data. Thus, none of the systems would be of any practical benefit to an athlete during training.

1.3.2.2 Fingertip pulse oximetry – Pilot testing

The methods of collecting SpO_2 and HR during swimming described by Miyasaka et al. (2002), which were one of the initial motivations for this research, have been replicated in a pilot study by our research group. HR and SpO_2 were collected in a single participant for an hour-long swimming session using a finger pulse oximeter (3100 WristOx, Nonin Medicals, Minnesota, USA), and waterproofed by using a latex dry glove (SI TECH AB, Brastad, Sweden). After a period of 15 minutes, the device failed to collect further data and therefore the testing period was ended.

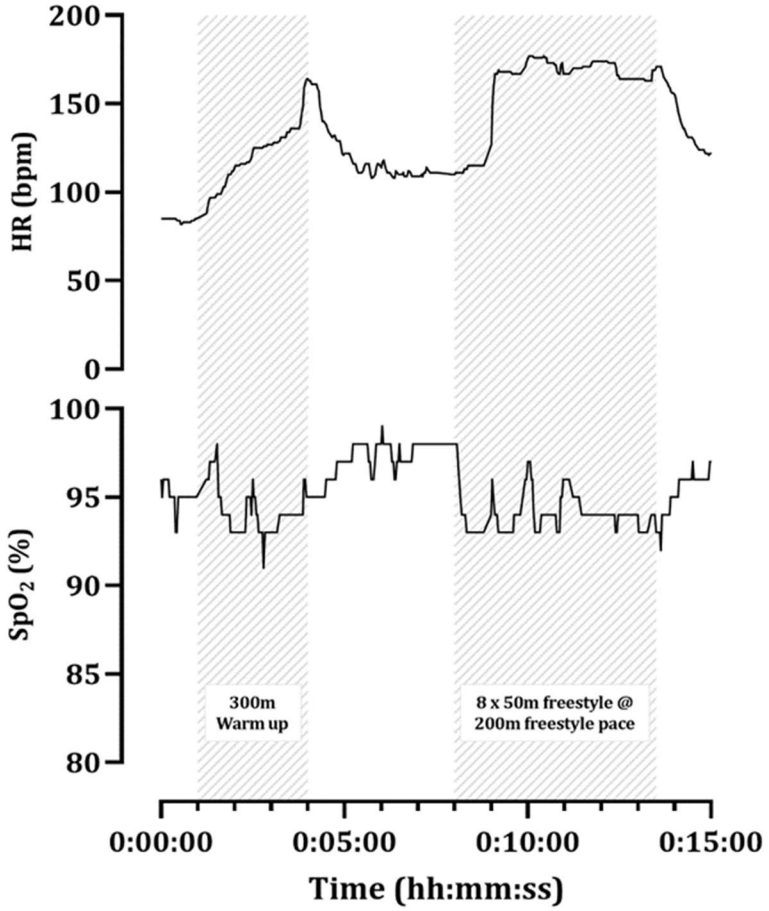

Figure 1.4: Pilot study heart rate and pulse saturation data from during swimming. Hatched area signifies the period when the participant was swimming.

Pilot data (Figure 1.4) showed a reduction in SpO_2 with a concomitant rise in HR during periods of swimming, however, did not show the same degree of hypoxic stimuli as seen by Miyasaka et al. (2002). The reason for a smaller drop in SpO_2 could be due to a multitude of factors such as exercise intensity, athlete training status, or testing protocol. These results do highlight the need for an improvement in swimming pulse oximetry technology. The use of fingertip pulse oximetry for the measurement of SpO_2 during swimming is less than ideal due to the high degree of movement artefacts (noise) and the large amount of motion required by the arms, altering the data collected (Poets & Stebbens, 1997). Therefore, monitoring S_pO_2 from the forehead or torso may be considered a preferential location due to the reduced degree of motion noted in these locations compared to that in the finger-tip during each swimming stroke (Olstad & Zinner, 2020).

1.3.3 Physiological Adaptations to Aquatic Sports

Both adherence to an exercise training protocol and hypoxic acclimatisation can lead to increases in the oxygen-carrying capacity of the blood. However, to the author's knowledge, there is limited literature on quantifying the hypoxic load of swimming, synchronised swimming, and apnoea diving training, and the subsequent physiological changes that may occur. Furthermore, these potential changes have not been extensively compared to the changes that result from typical training and fitness adaptations. It is possible that aquatic sports with a breath-holding element may induce a significant hypoxic stimulus, and that continuous exposure to this stimulus during exercise may have several consequences: 1) it may limit performance and training capacity, as has been observed in live-low train-high studies (McLean et al., 2014). 2) it may induce hypoxic acclimatisation, which may benefit performance due to the haematological adaptations, 3) it may identify individual variability of athletes in their adaptation to the hypoxic exercise. These issues could have implications for how training in aquatic sports, especially competitive swimming, is approached.

If a hypoxic stimulus is present during swimming exercise, then the effectiveness of hypoxic training camps, where athletes go to improve the efficiency of oxygen transport and utilisation, could be called into question. Robach et al. (2006) compared 13 days of identical supervised training in highly trained swimmers in a live-high train-low (LHTL) intervention to a control group. The LHTL group spent a total of 13 nights at altitude, the first 5 at a simulated altitude of 2500m, and the following 8 nights at a simulated altitude of 3000m. The altitude that the control subjects slept at, and all participants trained at was 1200m. No significant differences were found between the two groups for Hct, [Hb] and EPO values. Hbmass and Rct values after 5 nights at 2500m altitude and 8 nights at 3000m altitude were significantly larger in the LHTL group compared to the controls. However, these gains in oxygen-carrying capacity were only short-term and not reflected in improvements in maximal aerobic capacity ($VO_{2\,max}$). These findings are in line with other studies, where hypoxic acclimatisation protocols result in improved oxygen-carrying capacity of the blood, without concomitant increases in maximal aerobic capacity or sporting performance (Bonne et al., 2014; Gough et al., 2012). This lack of improvement in performance despite increases in oxygen-carrying capacity raises questions about the effectiveness of hypoxic training and suggests that other factors, such as the ability to use oxygen effectively at a cellular level, may be more important determinants of performance. This highlights the need for further research to better understand the physiological adaptations to hypoxic training and their impact on swimming performance, which is the focus of the series of studies presented in this thesis.

1.3.4 Individual Variation

It is commonplace in the fields of exercise and environmental physiology that data are reported as Means ± Standard Deviations. Sometimes reporting in this format can mislead the reader about the full spread of data. Figure 1.5 depicts reanalysed data discussed in further detail in Chapter 2, if data were to be reported as just mean ± standard deviation, the reader would potentially mistakenly believe none of the participants experienced a reduction in erythropoietin (EPO) below baseline after the intervention. The inclusion of individual data or measures of individual variability (Hopkins, 2015; Williamson et al., 2017) helps further the understanding of the responses to an intervention. Individual variability is derived from three sources: error within the measurement, biological error, and biological variation.

Typical error of measurement (TE%) is a culmination of all statistical "noise" (Atkinson & Batterham, 2015) that exists from the equipment used in the measure, the methodological protocol, and the research team experimenting. Whereas biological error is derived from the noise from environmental factors such as the diurnal cycle, quality of sleep and diet, and psychological stress (Chrzanowski-Smith et al., 2020).

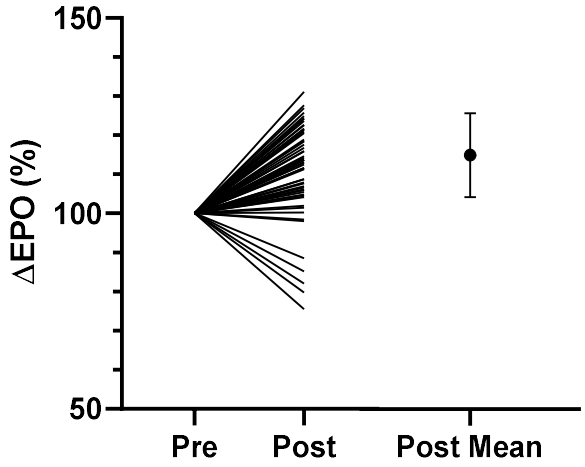

Figure 1.5: Percentage change (Pre-Post) of erythropoietin (EPO) in inactive individuals exposed to a simulated altitude of 4000m for 10- or 21-days. The filled circle represents the Mean ± SD Post data. These data are discussed in more detail in Chapter 2.

To gain greater insight into how individuals' physiological adaptations to hypoxia may differ, it was decided that reanalysis of previous hypoxic acclimatisation was required, from the perspective of evaluating the responses of individuals rather than groups only. This was to be completed prior to the testing of any of the selected athletic groups. For this purpose, data from three hypoxic confinement studies were reanalysed for the following reasons. Firstly, the data from these studies showed male and female responses during hypoxic acclimatisation of an identical length (10 days). Secondly, these studies allowed for comparison of hypoxic exposure length (10 days vs 21 days). Finally, all studies contained both hypoxic and normoxic confinements. This allowed for analysis of the effects of hypoxia, with the exclusion of unknown external environmental factors (exercise level, dietary intake, sleep regulation, etc.).

1.4 Aims and Hypotheses

The overall aim of the present thesis was to examine whether adaptation to hypoxia is present in athletes participating in aquatic sports, with special reference to the haematological and ventilatory changes that potentially occur in competitive swimmers.

This thesis consists of four studies, each testing a set of null hypotheses. If the null hypothesis is rejected, the corresponding alternate hypothesis must be accepted. The sets of hypotheses for each study are listed below:

Study I (Chapter 2): Assess the individual variability in the cascade of haematological responses to inactivity and hypoxia.

- Null hypothesis 1 ($H_0$1): There is no individual variability in the haematological responses to hypoxic confinement.
- Alternate hypothesis 1 (H_A1): There is significant individual variability in haematological responses to hypoxic confinement.

- $H_0$2: Individual variability in the haematological responses to hypoxic confinement is not affected by the duration of the hypoxic exposure.
- H_A2: Individual variability in haematological responses to hypoxic confinement is affected by the duration of the hypoxic exposure.

- $H_0$3: Individual variability in the haematological responses to hypoxic confinement is not affected by gender.
- H_A3: Individual variability in haematological responses to hypoxic confinement is affected by gender.

Study II (Chapter 3): Examine whether blood sampling from a fingertip capillary or an antecubital vein during a carbon monoxide rebreathing

- $H_0$4: Sampling from the fingertip capillary or an antecubital vein does not affect the validity of resultant determinations of haemoglobin mass or intravascular volumes.
- H_A4: The validity of determinations of haemoglobin mass or intravascular volumes are influenced by the location of blood sampling.

- $H_0$5: Sampling from the fingertip capillary or an antecubital vein does not affect the reliability of resultant determinations of haemoglobin mass or intravascular volumes.
- H_A5: The reliability of determinations of haemoglobin mass or intravascular volumes is influenced by the location of blood sampling.

- $H_0$6: Participant posture does not affect the measurements of haematological variables collected from venous blood.
- H_A6: Participant posture influences the measurements of haematological variables collected from venous blood.

Study III (Chapter 4): Assess differences in the hypoxia-induced physiological adaptations across various aquatic sporting modalities.

- $H_0$7: There is no difference in markers of hypoxic acclimatisation in athletes participating in various aquatic sporting modalities.
- H_A7: There are significant differences in markers of hypoxic acclimatisation in athletes participating in various aquatic sporting modalities.

Study IV (Chapter 5): Assess the effect of a training season on ventilatory and haematological measures.

- $H_0$8: Longitudinal data shows markers of hypoxic acclimatisation are not significantly different between controls and swimmers.
- H_A8: Longitudinal data shows markers of hypoxic acclimatisation are significantly different between controls and swimmers.

- $H_0$9: Training phase does not influence swimmers' markers of hypoxic acclimatisation.

H_A9: Training phase influences swimmers' markers of hypoxic acclimatisation.

Chapter 2

Heterogeneity of Haematological Response to Hypoxia and Short-Term or Medium-Term Bed Rest

Foreword

Prior to evaluating aquatic athletes, an assessment of an individuals' acute physiological responses to hypoxia and resultant physiological adaptation over time should be conducted. Therefore, the present chapter performed retrospective analyses on an existing data set displaying hypoxic acclimatisation. The dataset chosen was a combination of the data from three hypoxic confinement studies (Debevec et al., 2016; Keramidas et al., 2017; Keramidas et al., 2016). Each study comprised a prolonged period of inactivity (bed rest) in either normobaric normoxic or normobaric hypoxic (simulated altitude of 4000m) conditions. This allowed the reanalysis to be centred around the differences in individuals' responses to a single stressor (hypoxia).

The bedrest experimental model is used to simulate the effects of weightlessness encountered by astronauts in space. Specifically, the effects of inactivity and unloading of the weight-bearing limbs. Since it is anticipated that the ambient conditions within future space habitats will be hypoxic, a series of studies were conducted to compare the adaptation of physiological systems to inactivity in normoxic and hypoxic conditions. The results of these studies were used to assess the heterogeneity of the haematological responses to hypoxic inactivity.

The findings to this chapter were published in the Frontiers in Physiology under the title "Heterogeneity of Hematological Response to Hypoxia and Short-Term or Medium-Term Bed Rest" authored by Joshua T. Royal, Ola Eiken, Michail E. Keramidas, Adam C. McDonnell, and Igor B. Mekjavic.

2.1 Introduction

The haematological changes that occur with severe inactivity were first reported by Taylor et al. (1945), who observed a 9.3% loss of blood volume, concomitant with a 15.5% contraction of plasma volume (PV) in healthy young males as a consequence of three-weeks of bed rest. Their results also revealed significant individual variability in the haematological responses to bed rest, but these were not explored. The observed normoxic bed rest (NBR)-induced hypovolemia was attributed to the prolonged cephalad fluid shift (CFS) that stimulates central volume carotid, aortic and cardiac receptors, releasing atrial natriuretic peptide (ANP) in turn causing diuresis and natriuresis and a resultant decrease in PV (Fortney et al., 2010). Renal release of the hormone erythropoietin (EPO) is inhibited by the resultant increase in central venous pressure during CFS bed rest (De Santo et al., 2005; Kirsch et al., 1984). Gunga et al. (1996) reported a rapid decline of EPO in the initial 24 hours ($p < 0.01$) due to the initial increase in central venous pressure. Thereafter it returns gradually to pre-bed rest levels. Additionally, despite the EPO suppression found in the first 24 hours of NBR, some individuals experience concomitant increases in the concentrations of reticulocytes (Rct) and red blood cells (RBC) (Ryan et al., 2016).

Reduced oxygen (O_2) availability in the tissues resulting from a lower partial pressure of O_2 in the ambient air stimulates the renal release of EPO (Scholz et al., 1990), which in turn promotes erythropoiesis in the bone marrow. When a red blood corpuscle has matured to a Rct, it is released into the circulating blood. The fraction of Rcts is typically 0.5% – 2.5% of the total RBCs circulating in the blood (Banfi et al., 2006; Koepke & Koepke, 1986). Once matured to RBC, the blood's O_2 carrying capacity is increased due to the rise in total RBC volume. PV decreases during hypoxic acclimation; however, studies that report this variable tend to report a large degree of interstudy variability (Heinicke et al., 2003; Sawka et al., 1996). Theoretically, as PV reduction is seen in both prolonged hypoxia and bed rest via different mechanisms, hypoxic bed rest (HBR) should produce compounded PV loss compared to hypoxia or bed rest alone (Keramidas et al., 2016; Loeppky et al., 1993).

The concept of individuals being either responders or non-responders in response to an intervention is commonplace in physiology. The categorisation of individuals into these groups is based on observing a response that exceeds the typical error of the measurement (Montero & Lundby, 2017). Ge et al. (2002), reported substantial individual variability in the EPO responses after several 24-hour hypoxic exposures at a range of simulated altitudes. The authors also commented that individuals who had the largest responses to lower simulated elevations also had the largest responses to higher altitudes. Similar mean increases and a significant correlation between individuals' haemoglobin mass responses after normobaric and hypobaric hypoxic live-high train-low interventions have been reported (Hauser et al., 2017). A moderate altitude (~2100m) training camp attended by 12 Australian-Football players for 2 consecutive years found that individuals' EPO responses to the same stimuli were not consistent from one year to the next (McLean et al., 2013). Of note, this finding was also present in the variability in the haemoglobin mass response of Finnish endurance athletes (Nummela et al., 2020), and German elite swimmers (Wachsmuth et al., 2013), both attributing this intraindividual variability to the lack of consistency and monitoring of athletes prior to each altitude exposure. Therefore, the categorisation of individuals into "responders" or "non-responders" after a single intervention is unreasonable and should not be considered an unchanging and distinguishable trait. In each of these studies, the authors stress the importance of individual evaluation of haematological variables in response to hypoxic exposures.

Levels of oestrogen and progesterone change over the course of the menstrual cycle. Oestrogen typically peaks during the late follicular phase, close to the start of ovulation, driving plasma volume expansion (Øian et al., 1987). However, in the late luteal phase, levels of progesterone increase, causing natriuresis and resulting in body fluid loss (Maffei et al., 1999). Reports investigating haematological changes during the menstrual cycle have described reductions (Javaid et al., 2007; Ofojekwu et al., 2013; Vellar, 1974), or no change (Kim et al., 1993; Lebrun et al., 1995) in Hb concentration during the early follicular compared to the luteal phase. However, it should be noted that Hb concentration's stability is dependent upon the intra-extracellular fluid movements. Fortney et al. (1994), in a review of their previous series of studies, reported large fluctuations of PV and red cell mass within the menstrual cycle. The absolute PV and red cell mass were measured using a technetium radioisotope technique during the follicular and luteal phases of each woman's menstrual cycle. A peak increase in PV was observed within two days of the estimated ovulation day, preceded by a decreased PV lasting 1 to 3 days. Fortney and colleagues (1994) also reported that in both sexes, PV was significantly reduced post-bed rest compared to pre; however, a greater degree of blood volume and PV loss was noted in males than females. While the menstrual cycle has a varying effect on PV, previous studies have reported no effect on red blood cell volume or haemoglobin mass (Aguree et al., 2020; Chapman et al., 1997; Malipatil & Patil, 2013; Reeves et al., 2001). Keller and colleagues (Keller et al., 2020) identified that although there was no significant change in haemoglobin mass across the menstrual cycle, the coefficient of variation (CoV) for haemoglobin mass over the duration of a single menstrual cycle was 4.1%, which is above the typical CoV commonly reported when using the carbon monoxide rebreathing technique (2.2%) and thus may be under-reported.

Common to the aforementioned studies is that individual variability is noted by presenting the standard deviation in the haematological changes observed in response to bed rest, however, it is not discussed or expounded. Due to the potential for large individual variation in the responses to hypoxia and/or inactivity, the prescription of training protocols should consider individuals' physiological systems' responses to hypoxic adaptation. The present study aimed to assess the individual variability in the cascade of haematological responses to normoxic bed rest and hypoxic bed rest. We hypothesized that the process of acclimation as reflected in the haematological changes observed in participants exposed to bed rest alone or in combination with hypoxia would not be the same for all participants.

2.2 Methods

2.2.1 Ethical Approval

Subjects' written informed consent was obtained prior to each project, and they were informed that they were free to withdraw their consent at any time. The procedures were approved by the Committee for Medical Ethics at the Ministry of Health (Republic of Slovenia; approval numbers: 205/2/11 and 88/04/12) and conformed to the standards set by the Declaration of Helsinki (PlanHab: NCT02293772), except for the registration of the LunHab and FemHab projects in a database.

2.2.2 Study Design

Data used for these analyses were derived from 3-bed rest projects: two 10-day bed rest projects (LunHab: male participants; FemHab: female participants) and one 21-day bed

rest project (PlanHab: male participants). Each project comprised two experimental interventions: normobaric normoxic bed rest (NBR) and normobaric hypoxic bed rest (HBR, simulated altitude of 4000m, partial pressure of inspired oxygen (P_IO_2) ≈ 91 mmHg). In each intervention, participants were confined to one floor of the Olympic Sports Centre Planica (Rateče, Slovenia) situated at an altitude of 940m (P_IO_2 = 133 mmHg). A horizontal position was maintained throughout all interventions, and participants could only use one pillow for head support. Additionally, all activities were conducted in the horizontal position (i.e., hygiene, toilet, etc.), with the exception that during meals, they were allowed to rest on an elbow. Inclusion and exclusion criteria for PlanHab, LunHab, and FemHab have previously been described in detail (McDonnell et al., 2019; McDonnell et al., 2020; Mekjavic et al., 2020) and followed the guidelines recommended by the European Space Agency (Heer et al., 2009). Concerning prior altitude exposure, participants were excluded if they had been to altitudes above 2,000 m within two months prior to the start of an intervention. The participants' physical characteristics are presented in Table 2.1. The detailed methodologies for each of these projects have been reported previously (Ciuha et al., 2015; Keramidas et al., 2016; McDonnell et al., 2019; Mekjavic et al., 2020; Salvadego et al., 2016).

Table 2.1: Physical characteristics of participants that completed both NBR and HBR interventions in the 10-d LunHab, FemHab projects, and the 21-d PlanHab project (Note: n = number; M = males; F = females).

STUDY	**n**	**Sex**	**Age (yrs)**	**Height (m)**	**Weight (kg)**
LunHab	8	M	23.4 ± 1.7	1.78 ± 0.07	74.1 ± 14.1
FemHab	12	F	26.1 ± 3.7	1.69 ± 0.06	59.5 ± 8.8
PlanHab	11	M	25.4 ± 3.6	1.80 ± 0.04	79.9 ± 13.6

The fraction of O_2 in the Planica facility was maintained using a Vacuum-Pressure Swing Absorption system (VSPA, B-Cat, Tiel, The Netherlands). Samples of air from within each of the hypoxic rooms and common areas were analysed at 15-minute intervals for O_2 and carbon dioxide content throughout the interventions. Should the O_2 levels be above the target level, the introduction of a hypoxic gas mixture was initiated. In contrast, should the O_2 levels decrease below the pre-set value, the system would terminate further delivery of hypoxic gas to that room. If the O_2 did not return to the required level, delivery of external ambient (normoxic) air would be initiated, concomitant with the triggering of an audible alarm. In addition, each participant wore a personal portable (clip-on type) O_2 analyser (PGM-1100; Rae Systems, San Jose, California), providing immediate feedback of the F_IO_2 of the surrounding air and with an alarm alerting the user to the lower than anticipated O_2 fraction. As a result of the VPSA monitoring system, the F_IO_2 was tightly controlled throughout all hypoxic interventions (LunHab: 0.144 ± 0.001, PlanHab: 0.141 ± 0 .004, FemHab: 0.142 ± 0.001). As a result, the partial pressure of O_2 in each project was the following: LunHab: 91.6 ± 0.14 mmHg; PlanHab: 89.6 ± 0.4; FemHab: 90.4 ± 0.4 mmHg).

2.2.3 Measurements

Oxygen saturation measured using pulse oximetry (SpO_2) was measured daily in the morning after waking (07:00) in all interventions using a finger pulse oximeter (3100 WristOx, Nonin Medicals, Minnesota, USA).

Venous blood was drawn from an antecubital vein at specific time points during each bed rest intervention; details of the exact blood sample timings may be found below in the Data Processing section. Blood samples were collected just after waking and prior to ambulation (relevant to the Pre and Post bed rest data collection) following an overnight fast. Approximately 200 ml of blood was collected per participant in LunHab and FemHab, with 516.5 ml of blood drawn per participant in PlanHab.

Blood samples for erythropoietin (EPO) analysis when collected (EDTA vacutainers) were allowed to coagulate for 20 mins, then centrifuged, and subsequently, aliquoted serum was frozen at - 80°C for future analyses. EPO concentration was determined by sandwich enzyme-linked immunoassay (Quantikine IVD EPO ELISA; R&D Systems, Minneapolis, MN) using 100 µL of serum. Optical density was quantified on a SPECTRAmaxTM PLUS384 microplate spectrophotometer (Molecular Devices Corporation, 1311 Orleans Drive, Sunnyvale, California) set at 450 nm and corrected at 600 nm. The estimated CoV of the analysis was 2.2%.

Hb, Hct, RBCs, and Rct counts were analysed with an automated laser-based haematology analyser (Advia 120; Siemens, Munich, Germany) within 8 hours of blood sampling using clinical laboratory standards. All haematological variables were determined in duplicate by researchers blinded to the nature of the interventions.

Changes in PV were estimated from the Dill and Costill equation using Hct and Hb values (Dill & Costill, 1974). This approach was deemed appropriate for qualitative uses for this manuscript due to the concomitant changes in plasma renin concentration highlighted during a previous analysis of the PlanHab data (Keramidas et al., 2016). Thus, any differences in PV between HBR and NBR in the three projects indicate qualitative variations in the response and do not permit us to draw firm conclusions regarding the PV changes' exact magnitude.

2.2.4 Data Processing

The current study is an amalgamation of the results from three research projects designed to assess the separate and combined effects of hypoxia and bed rest on multiple physiological systems and the participants' psychological status. Therefore, the data analysis was not included in the original design of the studies, namely, to assess individual variation and the chronological changes in the haematological variables. The three projects were similar in design and protocol. The experimental schedules have been reported previously for the LunHab (McDonnell et al., 2019) and FemHab (McDonnell et al., 2020) projects. Due to minor changes in the experimental schedules, the haematological sampling frequency is not consistent across the three projects.

Each of the three projects consisted of three interventions where participants would experience one of the three conditions (NBR, HBR, and Hypoxic Ambulatory). Due either to participant dropouts, methodological error or human error, the Hypoxic Ambulatory data were too incomplete to compare with the NBR and HBR interventions. A minimum washout period of one month and three months was instituted between interventions for the 10-d (LunHab and FemHab) and 21-d (PlanHab) projects, respectively. Due to the sample sizes involved and the possibility of carryover at some physiological level between interventions despite the washout periods, the current data set was considered inappropriate for quantifying true individual response (SD_{IR}) (Williamson et al., 2017). As a result, the data presented in this study does not allow us to make conclusions as to the source of the variability; however, the current study highlights the importance of providing measures of individual variability when presenting results. As a result, the primary purpose of the study was to investigate the variability in the presented data and highlight the

importance of providing measures of individual variability in the hemopoietic cascade of adaptation to bed rest.

The blood sampling draws for each project were:

- LunHab: Pre (Day -1) and Post (Day R1)
- FemHab: Pre (Day -2), During (Days 2 and 6), and Post (Days R1 and R2)
- PlanHab: Pre (Day -2), During (Days 2, 5, 14, and 21), and Post (Days R2 and R4)

On Day 1 of each intervention, participants woke at 07:00, and continued with their assigned daily routine, following which they entered into the intervention, either HBR or NBR at 09:00. The participants then conducted 10 (240 hours: LunHab & FemHab) or 21 (504 hours: PlanHab) days in that intervention. Thus, upon waking on the morning of R1, prior to re-ambulation at 09:00, the participants were still in their designated intervention when a blood sample was collected at 07:00. Therefore, as there was no R1 data collection point for PlanHab, in order to ensure all Post blood draws were collected before re-ambulation, the Post values were collected on R1 for both LunHab and FemHab (R1) and Day 21 for PlanHab.

2.2.5 Statistical Analyses

Data are expressed as individual responses, mean ± SD, or as ranges and interquartile values. Statistical analyses were undertaken using SPSS (Version. 25, IBM, New York, USA) with significance set as $p \leq 0.05$. To assess whether a significant statistical change had occurred in the pre- to post-intervention haematological values, a paired samples *t*-test was applied to the means. In the current analyses, participants who completed both NBR and HBR interventions were included and paired-sample *t*-tests were used to distinguish differences between Δ(Pre-Post) values.

A One-way ANOVA was used to assess for significance between the NBR and HBR Δ(Pre-Post) values between projects (e.g., LunHab NBR vs. FemHab NBR vs. PlanHab NBR). The between-variable relationship strength was calculated using Pearson's or Spearman's correlation analysis. Correlation analysis was used to assess potential relationships between SpO_2 and EPO throughout the intervention. In all studies, potential relationships between pre-intervention haematological values and both absolute and relative degrees of change to post-intervention were investigated. Correlation coefficients were applied as recommended (Cohen, 2013) (strong ≥ 0.60; moderate ≥ 0.40 - < 0.59; weak ≥ 0.20 - < 0.39).

A two-way repeated measures ANOVA was employed to assess the effect of time (Pre– vs Post-bed rest) and condition (normoxia and hypoxia) within each bed rest project (LunHab, FemHab, and PlanHab). A two-way mixed-model ANOVA was employed to determine whether differences in the haematological markers existed due to the duration of comparable interventions (FemHab and PlanHab). In addition, post-hoc analyses using a Bonferroni corrected independent (between studies) and paired t-tests (within studies) were performed and reported where appropriate.

2.3 Results

The Δ(Pre-Post) values (mean, SD, overall range, and interquartile range) of EPO, Rct, RBC, and PV from each intervention for LunHab, PlanHab, and FemHab are presented in Table 2.2. The duration of bed rest did not have a statistical effect on any of the haematological variables, nor did participant sex ($p > .05$). No correlations were found

between the Δ(Pre-Post) bed rest haematological responses to NBR and HBR in EPO, Rct, or RBC (Fig. 2.1).

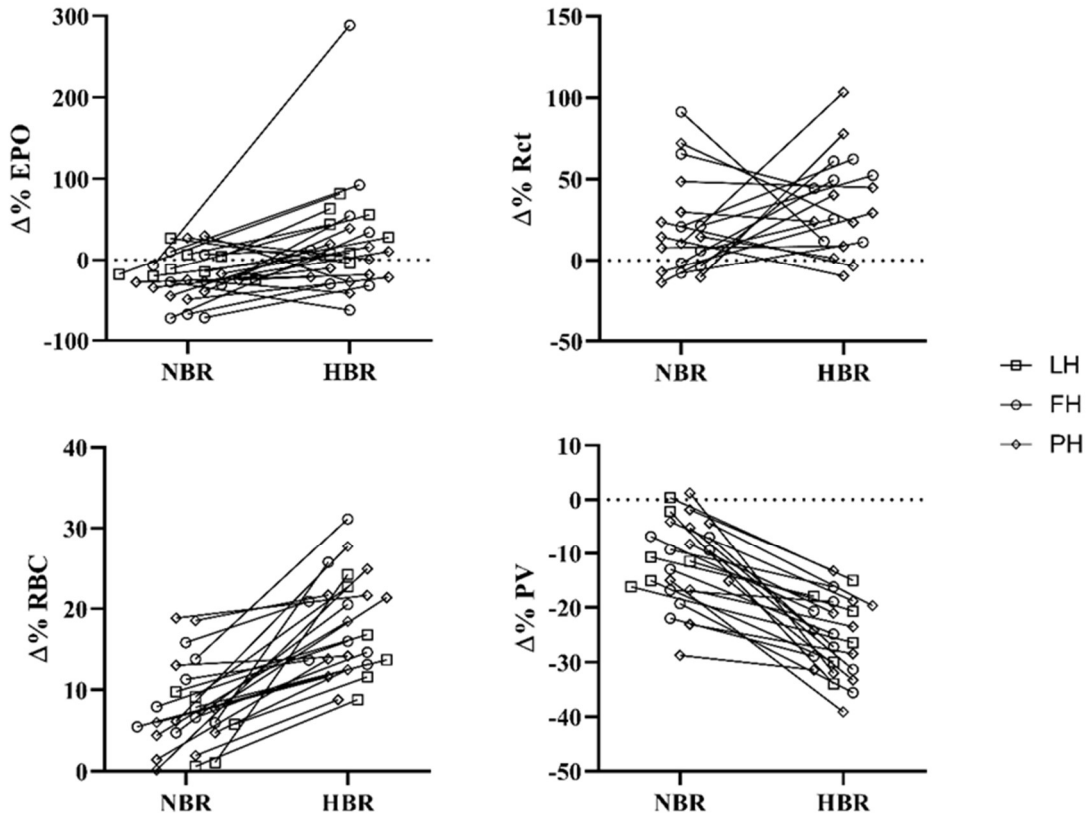

Figure 2.1: Individual relative (Δ(Pre-Post)) responses of erythropoietin (Δ%EPO), reticulocytes (Δ%Rct), red blood cells (Δ%RBC), and plasma volume (Δ%PV) for NBR and HBR trials. Squares: LunHab; Circles: FemHab and Diamonds: PlanHab.

2.3.1 SpO_2 and EPO Response Relationship

Correlation between SpO_2 and EPO values throughout both the NBR and HBR interventions in FemHab and PlanHab were assessed (Fig. 2.2). SpO_2 presented in Figure 2.2 was collected on the same day as the corresponding blood draw. In PlanHab HBR, a significant moderate negative correlation was identified (r = -0.561, $p < 0.001$). No significant relationship was discovered in either FemHab intervention (HBR: r = -0.252, p = 0.117; NBR: r = 0.200, p = 0.271) or in PlanHab NBR (r = 0.143, p = 0.253).

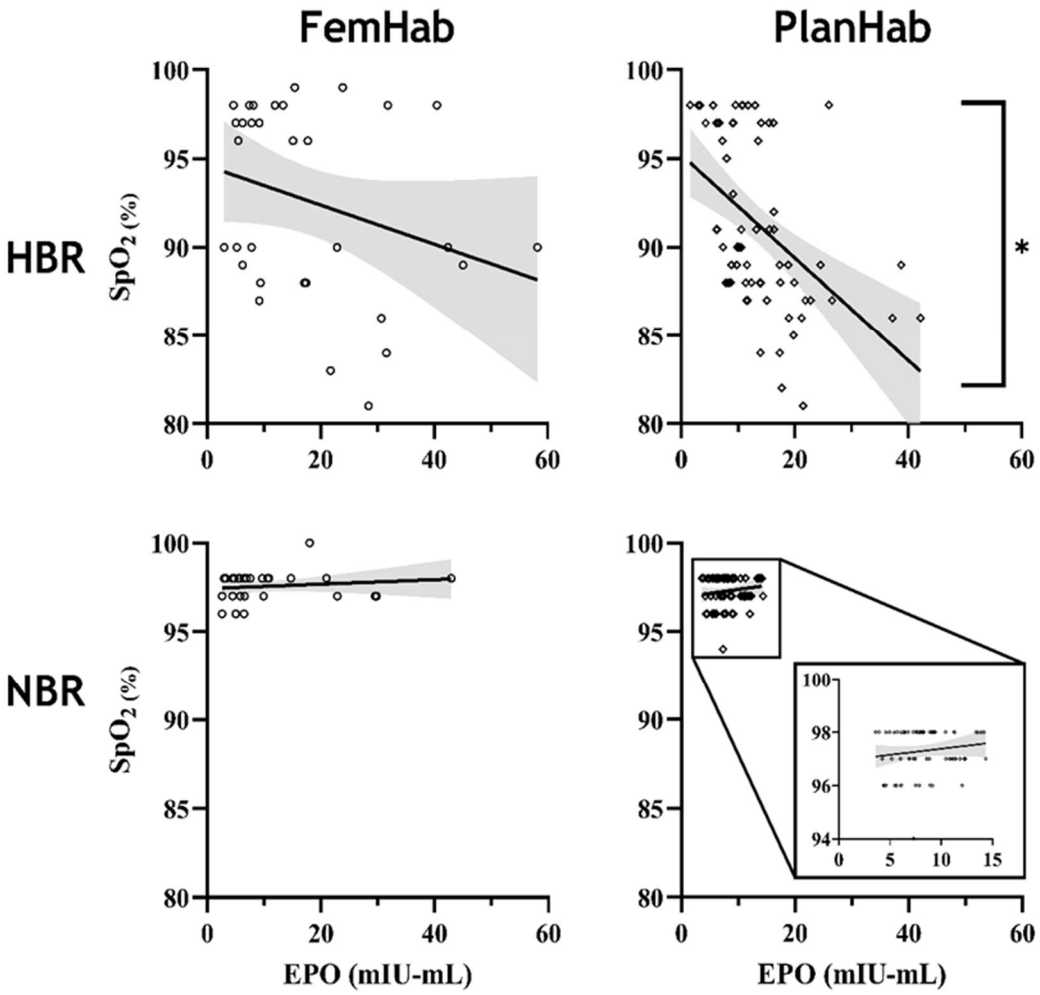

Figure 2.2: Correlation analysis between EPO and SpO_2 values throughout the FemHab and PlanHab studies. SpO_2 values were measured on the same days as EPO values were taken in each respective study. *denotes significant correlation.

Table 2.2: Bed rest Δ(Pre-Post) values in EPO concentration (ΔEPO), number of reticulocytes (ΔRct), number of red blood cells (ΔRBC), and plasma volume (ΔPV) during NBR and HBR interventions in all three projects (**Note:** LunHab - 6 males (8 for EPO), FemHab - 8 females, PlanHab - 11 males; IQR – Interquartile Range; * denotes significance between Δ(Pre-Post) values for condition ($p \leq 0.05$); † denotes significance in Δ(Pre-Post) values within study between normoxic and hypoxic gases ($p \leq 0.05$), ‡ denotes significance in the Δ(Pre-Post) EPO response in NBR between LunHab and FemHab.

		LunHab			**FemHab**			**PlanHab**		
		NBR	**HBR**	***p* †**	**NBR**	**HBR**	***p* †**	**NBR**	**HBR**	***p* †**
ΔEPO ($mIU \cdot mL^{-1}$)	Mean	-0.59 †‡	2.18*†	.036	-6.06*‡	1.70	.179	-2.21*†	-1.28†	.504
	SD	1.51	2.22		6.79	13.83		2.45	3.11	
	Range	4.60	7.10		17.39	49.00		7.23	9.72	
	IQR	2.03	2.78		12.97	10.91		1.37	4.91	
	*p**	.306	.028		.040	.739		.014	.204	
ΔRct ($\times 10^9 \cdot L^{-1}$)	Mean	**N/A**			16.40	27.38*	.364	8.94*	22.92*	.138
	SD				25.53	13.62		12.37	23.30	
	Range				77.70	36.00		36.40	74.10	
	IQR				29.75	26.75		22.50	40.10	
	*p**				.112	.001		.038	.009	
ΔRBC ($\times 10^{12} \cdot L^{-1}$)	Mean	0.28*†	0.81*†	.016	0.40*†	0.88*†	.002	0.38*†	0.92*†	.001
	SD	0.20	0.33		0.16	0.28		0.32	0.33	
	Range	0.48	0.83		0.44	0.67		1.03	1.02	
	IQR	0.41	0.64		0.29	0.56		0.51	0.56	
	*p**	.018	.018		<.001	<.001		.003	.009	
ΔPV (%)	Mean	-5.08	-13.30		-8.01	-15.86		-6.18	-14.51	
	SD	3.73	4.22		3.85	4.42		5.27	4.34	
	Range	8.86	11.18		10.34	12.50		15.72	14.86	
	IQR	7.65	7.80		7.25	7.30		7.49	6.81	

2.3.2 Changes in Erythropoietin (ΔEPO)

While mean EPO peaked in FemHab and PlanHab on Day 2 of HBR, in FemHab (Fig. 2.3A), only six of the twelve participants peaked on Day 2. Four of the FemHab participants' peak EPO values did not increase above baseline for the HBR. All but one of FemHab's participants' EPO values reduced to lower than baseline on Day R2.

The results of the PlanHab project (Fig. 2.3D) indicate that eleven of the twelve participants' EPO peaked on Day 2. All participants' EPO was reduced to lower than pre-HBR levels on Day R2. At the group level, significance was detected between Δ(Pre-Post) EPO values in LunHab HBR ($p = 0.028$), FemHab NBR ($p = 0.040$) and PlanHab NBR ($p = 0.014$). The within-project ranges for EPO Δ(Pre-Post) during HBR were -0.5 to 6.6 $mIU \cdot mL^{-1}$ (LunHab), -25.34 to 23.66 $mIU \cdot mL^{-1}$ (FemHab) and -7.15 to 2.57 $mIU \cdot mL^{-1}$ (PlanHab). The within-project ranges for EPO Δ(Pre-Post) during NBR were -2.2 to 2.4 $mIU \cdot mL^{-1}$ (LunHab), -16.41 to 0.98 $mIU \cdot mL^{-1}$ (FemHab) and -4.47 to 1.77 $mIU \cdot mL^{-1}$ (PlanHab). Pearson's correlation analyses showed that there were no correlations between

NBR and HBR Δ(Pre-Post) EPO (LunHab: r = -0.162, p = 0.702; FemHab: r = 0.469, p = 0.241; PlanHab: r = -0.229, p = 0.050).

2.3.3 Changes in Reticulocytes (ΔRct)

Group mean Rct peaked in FemHab on Day 6 and on Day 5 in PlanHab (Fig. 2.3B and E) due to the sampling timeline. In FemHab, 2 of the participants' Rct values peaked on Day R2. However, in PlanHab, 4 participants did not reach their maximum Rct concentration on the same day as the group mean peak value. After the Rct peak, these values gradually reduced to baseline levels in both projects.

At the intervention level, significance was discovered between pre- and post-bed rest Rct values in FemHab HBR (p = 0.001), PlanHab NBR (p = 0.038) and PlanHab HBR (p = 0.009). From HBR Δ(Pre-Post), the range for each data set was 6.70 to 42.90 $\times 10^9 \cdot L^{-1}$ (FemHab) and -8.2 to 65.9 $\times 10^9 \cdot L^{-1}$ (PlanHab). No significance was found in the FemHab NBR intervention (p = 0.112).

The inter-participant range for the changes in Rct NBR Δ(Pre-Post) -6.50 to 71.20 $\times 10^9 \cdot L^{-1}$ (FemHab) and -8.70 to 27.70 $\times 10^9 \cdot L^{-1}$ (PlanHab). Pearson's correlation analyses showed there were no correlations between the Δ(Pre-Post) values in Rct for NBR and HBR (FemHab: r = -0.280, p = 0.501; PlanHab: r = -0.218, p = 0.520).

2.3.4 Changes in Red Blood Cell volume (ΔRBC)

RBC peaked in FemHab HBR on Day R1 despite half of the participants' peak scores occurring on Day 6 (Fig. 2.3C). In PlanHab HBR, the peak in RBC was on Day 14 of the intervention, although 5 of the 11 participants' peaks were on a day other than Day 14 (Fig. 2.3F). In both studies, after the group peak in RBC, values dropped to around that observed at baseline. At the group level, significance was discovered between Pre- vs. Post-bedrest RBC values in all data sets (LunHab, FemHab, and PlanHab) and conditions (NBR and HBR) ($p < 0.05$). NBR 0.03 to 0.51 $\times 10^{12} \cdot L^{-1}$ (LunHab), 0.24 to 68 $\times 10^{12} \cdot L^{-1}$ (FemHab) and 0.01 to 1.04 $\times 10^{12} \cdot L^{-1}$ (PlanHab). In HBR the Δ(Pre-Post) ranges for each data set were -0.11 to 1.27 $\times 10^{12} \cdot L^{-1}$ (LunHab), 0.62 to 1.29 $\times 10^{12} \cdot L^{-1}$ (FemHab) and 0.48 to 1.33 $\times 10^{12} \cdot L^{-1}$ (PlanHab). Pearson's correlation analyses showed there were no correlations between the Δ(Pre-Post) changes in RBC for NBR and HBR (LunHab: r = 0.161, p = 0.761; FemHab: r = 0.355, p = 0.388; PlanHab: r = 0.247, p = 0.464).

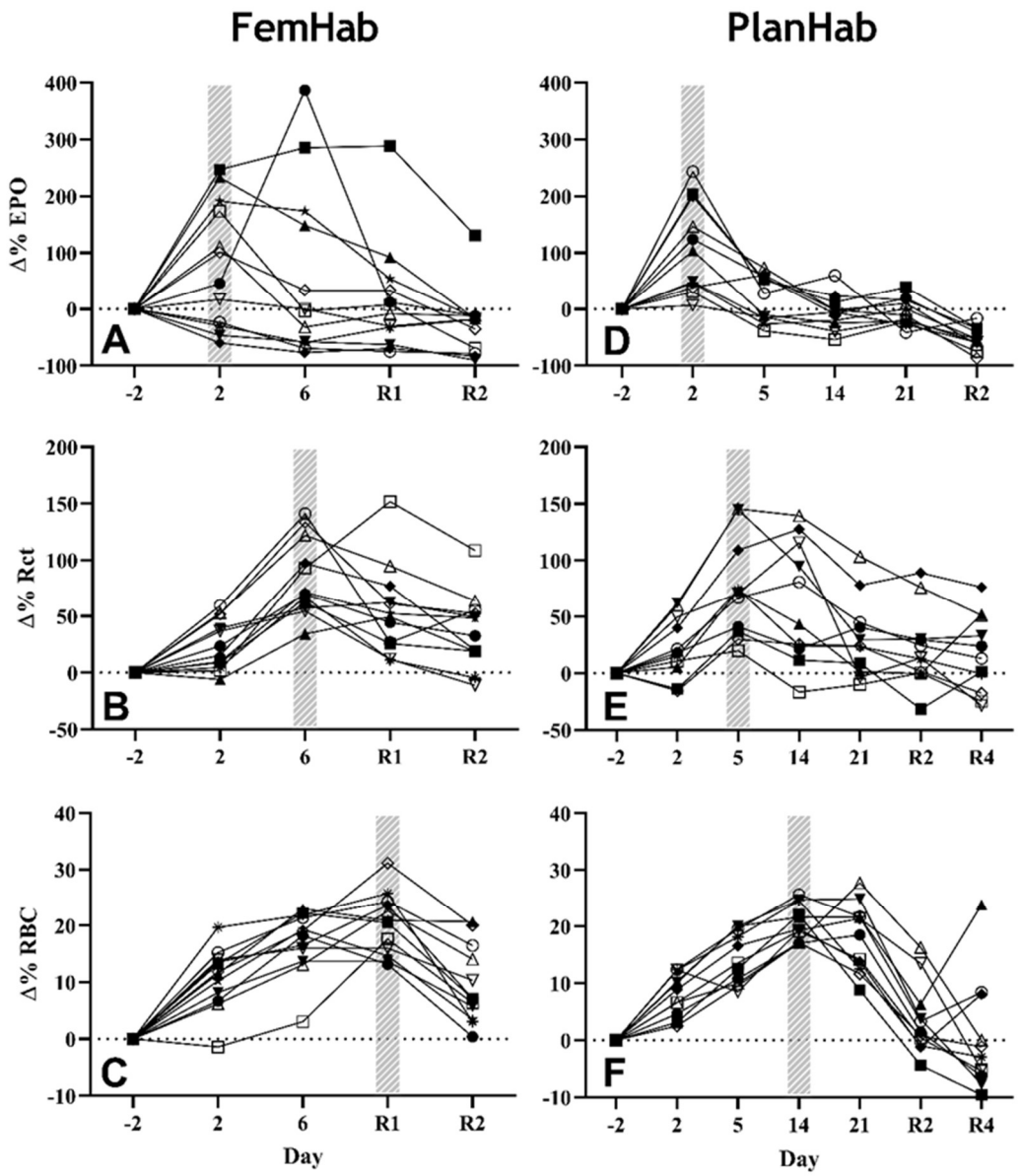

Figure 2.3: Baseline corrected relative individual changes (Δ%) of erythropoietin (ΔEPO; A and D), number of reticulocytes (ΔRct; B and E), and number of red blood cells (ΔRBC; C and F) during the hypoxic bed rest (HBR) trials in the FemHab and PlanHab projects, respectively. The hatched columns indicate the highest mean group value for each variable. Each different symbol represents an individual participant that completed the intervention.

2.3.5 Baseline Corrected Responses

The only correlation between mean peaks in the measured haematological variables during Pearson's correlation analysis was a significant positive moderate correlation between FemHab HBR Rct and RBC ($r = 0.597$, $p = 0.040$). No other correlation existed between the mean peaks in the measured haematological variables as either absolute or relative changes from baseline ($p > 0.05$; Fig. 2.4).

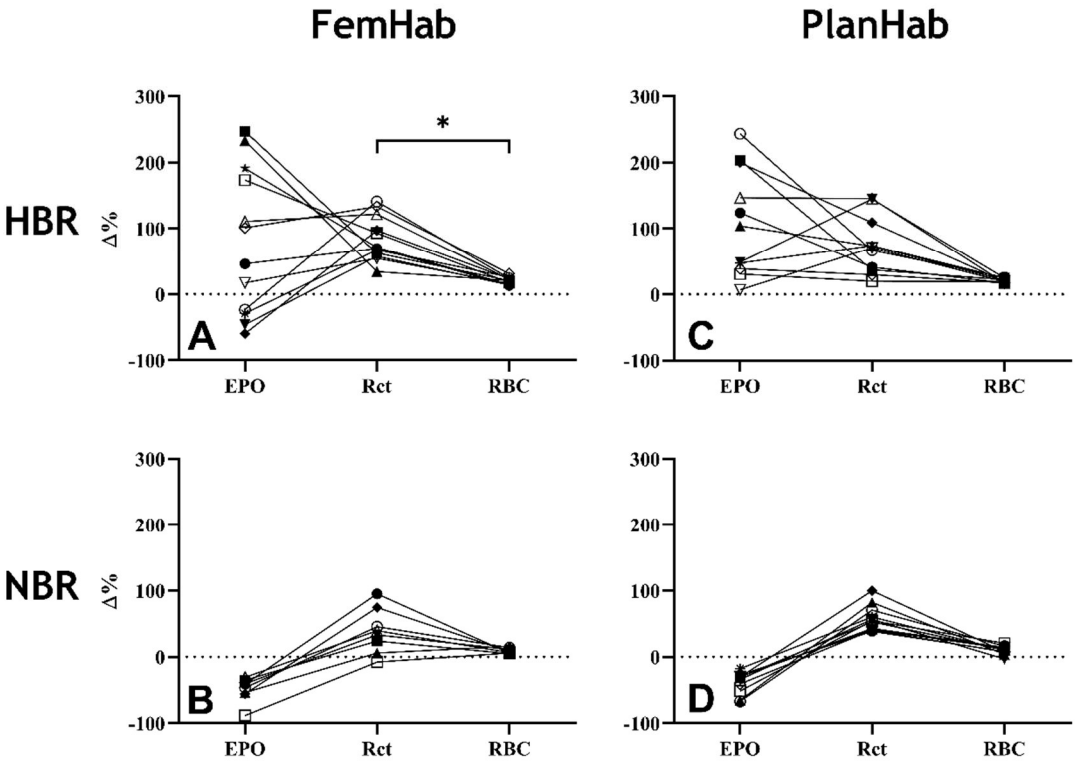

Figure 2.4: Relationships between individuals' ΔEPO, ΔRct, and ΔRBC in HBR (A and C) and NBR (B and D) in FemHab and PlanHab on the days of the highest mean group change (Δ%) for each variable during the HBR confinement. * denotes significant correlation between relative responses in FemHab HBR Δ(Pre-Post) Rct and RBC ($p \leq 0.05$). Each different symbol represents an individual participant that completed the intervention.

EPO, Rct and RBC significantly changed over the duration of the HBR in both FemHab and PlanHab (FemHab EPO: $F_{(2.106,\ 23.166)} = 3.037$, $p = 0.027$, $\eta^2 = 0.216$; FemHab Rct: $F_{(2.007,\ 22.074)} = 18.823$, $p < 0.001$, $\eta^2 = 0.631$; FemHab RBC: $F_{(4,\ 44)} = 37.919$, $p < 0.001$, $\eta^2 = 0.775$; PlanHab EPO: $F_{(2.007,\ 20.068)} = 30.176$, $p < 0.001$, $\eta^2 = 0.751$; PlanHab Rct: $F_{(6,\ 60)} = 13.802$, $p < 0.001$, $\eta^2 = 0.580$; PlanHab RBC: $F_{(2.485,\ 24.850)} = 30.243$, $p < 0.001$, $\eta^2 = 0.752$) (Fig. 2.3).

2.3.6 Changes in Plasma Volume (ΔPV)

Any differences found in ΔPV between HBR and NBR in the three projects (LunHab, PlanHab, FemHab: Table 2.2, Fig. 2.5) are purely speculative due to the calculation methods employed and do not permit us to draw firm conclusions regarding the exact magnitude of PV changes. Pearson's correlation analyses revealed no correlations in the ΔPV between NBR and HBR (LunHab: $r = 0.209$, $p = 0.691$; FemHab: $r = 0.385$, $p = 0.346$; PlanHab: $r = 0.489$, $p = 0.127$).

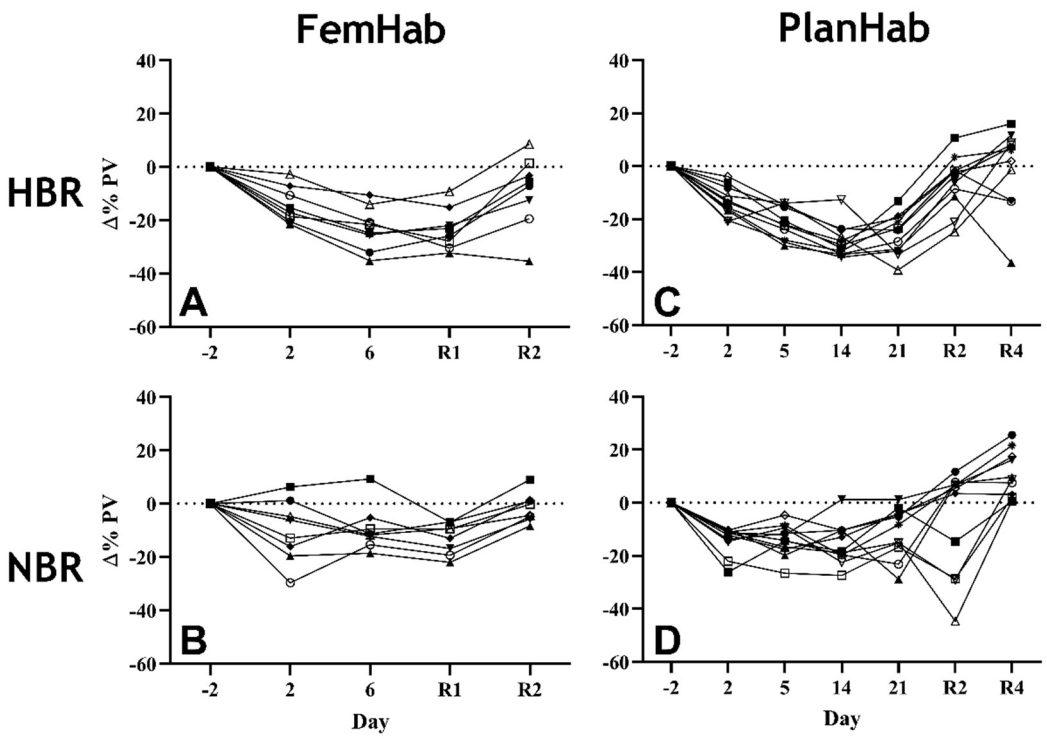

Figure 2.5: Individual changes in PV relative to pre-intervention baseline values during NBR and HBR interventions in FemHab and PlanHab studies. Each different symbol represents an individual participant that completed the intervention.

2.4 Discussion

It is well-documented that both genotypic and phenotypic factors influence the responses of individuals exposed to hypoxia (Beall, 2014; Moore, 2001). The principal finding of the present study is that for the same hypoxic stimulus, these factors affect the cascade of haematological responses among inactive participants differentially. Namely, for a given hypoxic exposure (approximately P_IO_2 = 90 mmHg) of 10- and 21-day duration, we observed the resultant responses of arterial O_2 saturation, which placed in motion the cascade of events from the release of EPO to the resultant increased production of Rcts and finally RBCs. The latter being essential for the ability of blood to bind O_2. Hypoxic acclimatization and training protocols strive to achieve an optimal outcome of the last event in this cascade, namely an increase in red blood cells. This is considered essential for maintaining the performance of lowlanders at high altitudes (i.e., alpinists), or for improving their sea-level performance (i.e., athletes). Surprisingly, the substantial individual variation observed in the first steps (SpO_2, EPO) of the cascade in HBR, gradually diminishes towards the last step of the cascade (RBC), as evident in Fig. 2.4.

2.4.1 SpO_2 and EPO Response Relationship

A significant negative correlation between SpO_2 and EPO only existed in the PlanHab HBR intervention (Fig.2.2). FemHab NBR had a larger variation in EPO response than values at the same SpO_2 in PlanHab, with some females eliciting a response equal or greater to that of males and females in HBR. In females, the hypoxic ventilatory response has been

stated to be significantly more extensive during the luteal versus the follicular phase (Takano, 1984). As the hypoxic ventilatory response is an indicator of the chemosensitivity to hypoxia, this change in chemosensitivity may affect the EPO response at a given SpO_2. Since no control was in place, and monitoring of females' menstrual phase in the FemHab study was limited, it cannot be verified whether the females with exaggerated EPO responses during NBR, despite no change in SpO_2, were also in the luteal phase at that time. The higher degree of variation in females' EPO response may also be a contributing factor as to why no statistical correlation was observed in HBR during FemHab.

2.4.2 Magnitude of the Haematological Responses

The EPO response to NBR and HBR in females appears to be considerably larger than in males (Table 2.2). Additionally, bed rest duration appears to have no impact on the heterogeneity of haematological responses (Table 2.2). Our analyses also show that the resultant haematological changes (Rct and RBC) that occur during NBR and HBR are not proportional to the EPO level when individual responses are considered. Considering hypoxia as the stimulus for the haematological changes, we demonstrate the heterogeneity of the cascade of responses to this stimulus, from arterial O_2 saturation (Fig. 2.2) to increased EPO release and production of Rct and RBC (Fig. 2.3). The increase in EPO concentration (Fig. 2.3A, D) within the first days of exposure is followed by an increase in Rct concentration (Fig. 2.3B, E) by Days 5 (FemHab) and 6 (PlanHab), finally resulting in an increase in RBC (Fig. 2.3C, F) by Days R1 (FemHab) and 14 (PlanHab). Qualitative analysis of the individual responses indicates a large degree of individual variation in these responses' magnitude and kinetics. Finally, these responses need to be considered from the perspective of the plasma volume changes, which are largely affected by individual variation itself. Intriguingly, despite the large range of EPO responses observed in both studies, the range of RBC concentration, the last step in this cascade, is substantially lower. These issues are discussed in further detail below.

The magnitude of an individual's relative EPO or Rct response is not necessarily indicative of the size of their relative Rct or RBC response, respectively (Fig. 2.4). The reason the relative increases across the haematological variables are not consistent, could be due to scale relativity; however, the increases in these haematological variables are most likely primarily attributable to increases in haemoconcentration from hypovolemia. Rct fraction of total RBC volume is typically 0.5% – 2.5% (Banfi et al., 2006; Koepke & Koepke, 1986); therefore, an increase of 100% in Rct concentration would hypothetically only account for a consequential RBC increase of 1% - 5%. Reductions in RBC volume seen in Days R2 (FemHab), 21, and R2 (PlanHab) initially seem implausible due to the typical lifespan of circulating RBC and are most likely due to the sudden rise in PV seen in the latter half of the interventions (Fig. 2.5).

The magnitude of EPO production is largely dependent on the level of an individual's hypoxic stress (Chapman et al., 2014); however, the increase in EPO level during a fixed hypoxic stimulus between participants varies considerably (Ploszczyca et al., 2018), as demonstrated in Fig. 2.2 and 2.4. The differences in the spread of individual responses to NBR and HBR shown in Fig. 2.4 indicate that the majority of variability seen in the haematological variables is due to the mechanisms responding to hypoxic acclimation rather than bed rest. Inter-individual variation in EPO response has previously been observed by Klausen and colleagues (1996), with some participants having a serum EPO response almost 10x greater than others after 42 hours at an altitude of 4350 m. Chapman et al., (2010) also found a large variation in the EPO response when taking 26 elite distance runners to 2500 m elevation for 20 hours (ΔEPO – 2.9 ng.ml-1 to 20.5 ng.ml-1; -19.9 to 415.4%). Variation amongst participants in the magnitude of the EPO response to a fixed

hypoxic stimulus could potentially be influenced by a multitude of factors. Disparities in participants' carotid body chemosensitivity, hypoxic ventilatory drive, haemoconcentration, or renal blood flow at the moment of renal EPO release, and factors that are potentially hereditary traits, may explain why inter-individual variation is often more common than intra-individual variation (Collins et al., 1978; Scoggin et al., 1978).

2.4.3 Haematological Kinetic Response

The timing of the mean group peaks in EPO, Rct, and RBC in Fig. 2.3 concur with previous reports (Banfi et al., 2006; Scholz et al., 1990). A reduction in EPO level after the initial peak is apparent in both FemHab and PlanHab studies (Fig. 2.3A, D), a finding that is in line with other altitude studies (Ploszczyca et al., 2018). However, the underlying mechanisms for this reduction are still not entirely clear. Lundby et al. (2009) noted that hypoxic inducible factor-1 (HIF-1) peaks within the first hours of hypoxic exposure and then reduces gradually to pre-hypoxic exposure levels. The authors speculated that the response to the initial hypoxic stimulus might diminish the degree of cellular hypoxia. Ploszczyca et al. (2018) have also suggested that the gradual reduction in EPO over a prolonged hypoxic exposure is likely associated with the reduction in HIF-1. However, the rate of decrease in EPO is not equivalent to the lesser reduction in circulating Rcts (Rusko et al., 2004). Therefore, the authors suggested that the reduction in serum EPO after the initial peak is due to the establishment of an equilibrium between EPO production and consumption for Rct creation.

2.4.4 Sex

Absolute and relative EPO responses had considerably greater inter-individual variability (Table 2.2, Fig. 2.3 and 2.4) in the female bed rest study than the two male bed rest studies and no statistical mean differences in the relative responses. Distribution values (SD, range and IQR; Table 2.2) in the FemHab study are all larger in both NBR and HBR interventions compared to their male counterparts. The relative EPO responses of females to hypoxia in comparison to LunHab and PlanHab studies appear to have large variability, which diminishes when compared to previous studies with prolonged hypoxic stimuli (Ploszczyca et al., 2018). Females tend to have lower [Hb] than males, meaning their concentration of arterial O_2 at any given O_2 saturation is usually lower (Murphy et al., 2010). Chapman et al. (2010) speculated that this hypothetically means females have a larger EPO response to hypoxia than males. Although in the present study, no differences were found in EPO between males and females at the group level, the variability in EPO response found in FemHab could be due to individuals' being at different phases of their menstrual cycle as no control was implemented on menstrual cycle phase during the bed rest interventions. Stachenfeld (2008) suggested that oestrogens play an important role in stabilizing body fluid volume. During a normal menstrual cycle, oestrogens potentially function to counter the fluid loss effects of progestins, maintaining PV. In response to long-duration stress, such as prolonged bed rest (greater than 5-7 days) or weightlessness, oestrogens are speculated to stabilize the vascular compartment (Fortney et al., 1988). A manner to potentially reduce EPO variability in females would be through the control of oestradiol and progesterone. Contraception has been shown to control levels of oestrogen but not progesterone so is not ideal. The use of a gonadotropin-releasing hormone agonist or antagonist for reproductive function suppression with a controlled administration of oestradiol and progesterone would negate hormonal fluctuations (Stachenfeld, 2008), and in turn, potentially reduce variability. Tracking the menstrual cycle and hormonal changes throughout the intervention would not change the level of variability however it would

allow researchers to identify if larger EPO responses were concurrent with changes in either oestradiol or progesterone.

Until the age of puberty, males and females have similar increases in Hbmass during their development. After puberty has begun, Hbmass increases exponentially in males, whilst remaining at a rate similar to pre-puberty in females (Prommer et al., 2018). It is believed the source of this change in rate is due to the role of androgens in males' puberty (Hero et al., 2005). The introduction of testosterone causes a rightward shift in the EPO-Hb relationship curve as well as a new physiological "set point" (Bachman et al., 2014). Mancera-Soto et al. (2021), claim that during the most sensitive phase of puberty, an increase in testosterone plasma of 1 ng/ml is correlated to an increase in haemoglobin mass of ~ 65g. The effects of testosterone on individual variability in men compared to women; however, is currently unknown and would require further examination. Furthermore, Goodrich et al. (2020) demonstrated that variations in haemoglobin mass across groups of varying athleticism and sex were more closely related to lean body mass than whole body mass. Once normalized for lean mass, as seen previously variations were greatly diminished. In addition, they also pointed to deficiency as a strong determinator of lower haemoglobin mass. Further, iron deficiency anaemia was identified to be far more prevalent in women of childbearing age due to blood and iron loss during the menstrual cycle (Fernandez-Jimenez et al., 2020). Both of these mechanisms may explain to a certain extent the potential sex difference noted in the current manuscript.

2.4.5 Plasma Volume Changes

Prolonged bed rest and hypoxia independently both cause reductions in PV (Table 2.2; Fig. 2.5). Bed rest duration or participant sex do not appear to contribute to the inter-individual variability of the PV response. Bed rest's horizontal positioning stimulates receptors in the upper body after the CFS, which, in turn, results in the release of ANP, causing diuresis and natriuresis. PV changes during hypoxic acclimation have considerable variability and appear to be mediated primarily by changes in oncotic pressure (Siebenmann et al., 2017). The majority of the variability in the PV response to hypoxic acclimation is attributed to the variability in the concomitant changes in total circulating protein (TCP) during hypoxic acclimation. Young et al. (2019) speculated that this decrease in TCP could be attributed to both reduced plasma protein synthesis and the leaking of plasma proteins into the extravascular space. The combination of both of these mechanisms results in approximately twice the reduction of PV in comparison to NBR alone at the intervention level (Table 2.2). The changes in PV seen in NBR have been speculated to modulate the production and release of EPO (Gunga et al., 1996). Keramidas et al. (Keramidas et al., 2016) concluded that tissue O_2 saturation is a primary mediator for the degree of renal EPO synthesis and that PV is a secondary factor. Bed rest duration appears to have little to no impact on the amount of individual variation in PV seen in the Δ(Pre-Post) and day-to-day values (Table 2.2).

2.4.6 Limitations

Using haemoglobin mass (Hbmass) as opposed to haemoglobin concentration ([Hb]) and RBC would eliminate the effects of PV retraction on measuring the variability in changes to blood O_2 carrying capacity. Haemoglobin mass typically has a measurement error of around 2% in well-trained research teams (Gough et al., 2011; Siebenmann et al., 2017; Steiner & Wehrlin, 2011). The Dill and Costill (1974) equation, utilized in this study, is deemed appropriate for the calculation of plasma and serum biomarkers; however, not for whole blood biomarkers (Matomäki et al., 2018). Future research should consider using a

direct tracer-dilution method to study PV changes and draw firm conclusions as to the exact extent of PV changes.

The debate between hypo- and normobaric hypoxic continues and is sometimes overlooked when discussing hypoxic exposure in general. There are many discrepancies in the responses of physiological systems between normobaric hypoxia and hypobaric hypoxia which have been attributed to the differences in barometric pressure (Millet & Debevec, 2020) and may indeed be worth further study. Despite this, Hauser et al. (2016) found similar Hbmass responses to live-high train-low interventions in normobaric and hypobaric hypoxia. Wide variability existed in individual responses to both intervention types after the same hypoxic dose and after 18 days post-intervention. The heterogeneity of haematological responses to inactivity and hypobaric hypoxia may differ either in source or in magnitude from that of inactivity and normobaric hypoxia and require further investigation.

Chapter 3

Validity and Reliability of Capillary Blood vs Venous Blood for the Assessment of Haemoglobin Mass and Intravascular Volumes

Foreword

The results of Chapter 2 highlight two issues, which are fundamental to this thesis. Firstly, the responses of individuals to a hypoxic stressor can differ significantly, and therefore the data of individuals should be displayed in addition to the mean and standard deviation, especially in situations with low sample sizes. Secondly, a method of measuring blood oxygen-carrying capacity is required to assess haematological adaptation to hypoxia without the influence of changes in hydration status. Hydration status and more notably plasma volume can change due to changes in training intensity and volume (Green et al., 1991; Kargotich et al., 1998). This chapter describes the implementation of a novel method, the carbon monoxide rebreathing protocol, to measure the total mass of haemoglobin of an individual, uninfluenced by changes in hydration. Before using this measurement in the planned analyses of aquatic athletes, the reliability of this measurement should be established. Previous iterations of the carbon monoxide rebreathing method use capillary blood draws and a seated posture. Therefore, this chapter will also assess the effect of posture on collected blood variables as well as the validity and reliability of the use of capillary blood draws as an alternative to venous blood draws when using the carbon monoxide rebreathing protocol.

The findings to this chapter were published in the Frontiers in Physiology under the title “Validity and reliability of capillary vs. Venous blood for the assessment of haemoglobin mass and intravascular volumes” authored by Joshua T. Royal, Jason T. Fisher, Tinkara Mlinar, Igor B. Mekjavic, and Adam C. McDonnell.

3.1 Introduction

Measurements of total haemoglobin mass (Hbmass; g or $g \cdot kg^{-1}$), red blood cell volume (RBCV), total blood volume (BV) and plasma volume (PV) may be used to monitor an individual's health status (Otto et al., 2017). Hbmass calculation offers an insight into absolute blood oxygen-carrying capacity and is often used to examine the effects of exercise training interventions (Heinicke et al., 2001; Rønnestad et al., 2021), hypoxic exposures (Hahn et al., 2001; Pugh, 1964) and altitude training (Gore et al., 1998; Gough et al., 2012; Schmidt & Prommer, 2008). Hbmass is more accurate and thus more informative than other measures like haematocrit (Hct) or haemoglobin concentration ([Hb]; $g \cdot mL^{-1}$), which may potentially misrepresent actual content as a consequence of posture and hydration status changes (Dill & Costill, 1974; Durussel et al., 2013).

Venepunctures require a phlebotomist in order to obtain a successful blood draw. While all blood drawing techniques carry an inherent risk, the likelihood of a vasovagal reaction is approximately 1% and lower with capillary draws (WHO, 2010). This risk is further reduced with repeat visits. Capillary blood draws are often regarded as a cost-effective, quick and simple blood draw method and often less painful than a venepuncture. While easier in theory for the layperson to conduct, the fingerstick lancing technique draws blood from the capillaries, venules and arterioles, as well as interstitial fluid and may lead to a haemolysed sample. However, such contamination of the blood sampled may be reduced with good blood flow and by eliminating the first blood drop of the draw (Krleza et al., 2015). Comparisons between venous and capillary blood for a multitude of variables have been made over the last 100 years (Andresen & Mugrage, 1938; Duke & Stofer, 1922; Fliervoet et al., 2022; Kellenberger et al., 2022; Mohammed et al., 2010). Haemoglobin concentration and Hct values in adults and children are typically higher when the sample is drawn from the capillary rather than the vein (Hütler et al., 2000; Moe, 1970; Neufeld et al., 2002), which highlights a potential risk of using capillary and venous blood interchangeably. Various iterations of the carbon monoxide (CO) rebreathing method are frequently implemented (Table 1). Each iteration of the protocol has details which differ between research groups. Commonly, these are the rest period prior to rebreathing, the rebreathing time itself, the dosage of CO, the timing of blood sampling, the sampling technique (capillary or venous), the number of replicate analyses and posture.

The influence of posture on blood values is widely reported (Astolfi et al., 2020; Lippi et al., 2015; Maughan et al., 2001). Despite this, one major discrepancy between protocols is the posture assumed prior to and during CO rebreathing, for example, a seated position is utilised in the Schmidt and Prommer (2005) and Hütler et al. (2000) methods, whereas in the Siebenmann et al. (2017) and Burge and Skinner (1995) methods, a supine position, with or without legs raised was assumed, respectively. To investigate this, Durussel et al. (2013) conducted a postural analysis prior to performing the "Schmidt & Prommer" CO rebreathing method. Participants assumed a supine position for 10 minutes before moving to a seated position for a further 10 minutes, with venous blood draws after each stage. No significance was found in [Hb], Hct or resultant intravascular volume values as a result of the participant's posture. However, critically, there are other studies (Diaz et al., 1979; Hagan et al., 1978) that indicate plasma volume expansion occurs in the supine position as a result of a cephalad fluid shift when moving from upright to supine, which should be considered. In order to further clarify this matter, the present study aims to replicate the idea behind the work of Durussel et al. (2013) whilst altering the protocol. Firstly, by measuring a baseline value immediately prior to the first posture and secondly, by increasing the participants' time spent in each posture. Namely, 20 minutes compared to 10 minutes was chosen as this is the length of time recommended to maintain the supine

position in the protocol chosen for the present manuscript (Siebenmann et al., 2017) and critically, it is longer than the recommended duration (> 15 minutes) to allow vascular volumes to stabilise (Smith et al., 1994; Tan et al., 1973).

Finally, Durussel et al. (2013) performed the CO rebreathing protocol described by (Prommer & Schmidt, 2007) (Table 1) and found that the carboxyhaemoglobin saturation levels (COHb%) at baseline (BL) and at 8 minutes after the onset of the 2-minute CO rebreathing (6 minutes post-CO rebreathing), from capillary blood to be consistently higher than those estimated from venous blood. However, importantly, no significant difference in the ΔCOHb% (Post COHb% - Pre COHb%) obtained by either method was noted. Good repeatability was found in Hbmass determination from both capillary and venous blood samples, which supports other research groups' findings using this protocol (Gore et al., 2006; Prommer & Schmidt, 2007). Hütler and colleagues, (2000) reported that despite finding significantly higher [Hb] and Hct values in capillary blood samples, these variables were highly correlated across sampling techniques ([Hb]: r = 0.90, Hct: r = 0.93). Additionally, they noted that ΔCOHb% (10^{th} min - 0 min; CAP = 6.24 ± 0.59%, VEN = 6.26 ± 0.60%) and Hbmass values (959 ± 106 g vs 962 ± 110 g; r = 0.987) were statistically indifferent across sampling techniques (Hütler et al., 2000).

Finally, Siebenmann et al., (2017) utilised the micromethod to assess Hct prior to rebreathing and obtain the other values pertinent to calculating Hbmass from venous blood. Therefore, research comparing Hbmass and intravascular volume calculated from only capillary or venous blood samples, while conducting the supine CO rebreathing method described by Siebenmann et al., (2017) is scarce. Kellenberger et al. (2022) performed a comparison between capillary and venous blood, however, a test-retest reliability analysis, which would reflect the typical measurement error, is crucially missing from the Kellenberger et al., (2022) analysis.

Therefore, the aims of the present study are as follows:

- Compare the validity and reliability of values obtained from capillary blood draws compared to venous blood draws.
- Assess the effect of posture (seated vs. supine) upon haematological values relevant to Hbmass assessment prior to the start of a CO rebreathing protocol.
- Provide practical implications of the present study for clarity for researchers considering the assessment of Hbmass and intravascular volumes.

Table 3.1: Previous iterations of the CO rebreathing protocol utilised for the assessment of Hbmass and intravascular volumes. (Note: VEN = venous blood, CA*P* = capillary blood, BW = Body weight, CO = Carbon monoxide, COHb% = Carboxyhaemoglobin, O_2 = Oxygen).

#	Citation	Rebreathing Protocol					Blood Sampling				
		Rest period (min)	**Position**	**Pre-breathing 100%O_2 (min)**	**CO dose (mL)**	**Rebreathing time (min)**	**Sampling location**	**Timing (min)**	**Replicate analysis**	**Blood Analyser**	**Coefficient of Variation (%)**
1	(Thomsen et al., 1991)	15	Supine	2	50	10	Antecubital veins from each side of the body (2 x 2 mL)	Pre, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15	2 x sample	Radiometer OSM3	2.3-2.6% (Hb)
2	(Burge & Skinner, 1995)	20	Seated	4	50-90	10	Antecubital vein (2 mL)	Pre (2 samples), 10 min. (single post sample)	4 – 6 x per sample	Radiometer OSM3	0.8%
3	(Hütler et al., 2000)	15	Seated	None	1mL·kg^{-1} BW + 10mL (100 mL max)	Continued until plateau or decrease in COHb% observed in CAP and VEN	VEN: Antecubital vein (1mL) CAP: earlobe (Volume not indicated)	VEN: Pre, 2, 4, 6, 8, 10, 12 min. CAP: Pre, 1, 3, 5, 7, 9, 11 min.	3 x per sample	Radiometer OSM3	VEN: 3.0±1.3% (Hbmass); 3.6±1.5% (RBCV); PV: 5.2±2.6% (PV) CAP: 3.3±1.6% (Hbmass); 3.6±1.8% (RBCV); PV: 5.1±9.8% (PV)
4	(Prommer & Schmidt, 2007;	15	Seated	None	1mL·kg^{-1} BW (99.5% CO)	CO bolus followed by rebreathing of		Pre (-1 min), 1, 2,	No replicate info, 1 x	Radiometer ABL 520	1.7-2%

	Schmidt & Prommer, 2005)					3.5 L O_2 for 2 minutes.	VEN: Antecubital vein (~2 mL) CAP: earlobe (85µL)	4, 6, 8, 10, 12.5, 15	per sample Study II, duplicate [Hb] only		
5	(Siebenmann et al., 2017)	20 (500mL water)	Supine with legs elevated	4	1.0 mL·kg^{-1} BW (f) 1.5 mL·kg^{-1} BW (m) (99.997% CO)	10	Antecubital vein (2 mL heparinised syringe)	Pre rebreathing, 10 minutes	4 x per sample	Radiometer ABL 800 Hct micromethod	1.49%
6	Oberholzer et al., 2020)	20 (500mL water)	Supine with legs elevated	4	0.5 – 1.5 mL·kg^{-1} BW 99.997% CO	10	2 mL	Pre (0 min), 6, 8, 10 minutes	4 x per sample	Radiometer ABL 800 Hct micromethod	1.5-6.5%
7	Kellenberger et al., 2022	15	Seated	None	1.0 mL·kg^{-1} BW (f) 1.2 mL·kg^{-1} BW (m) (100 mL Max)	2	CAP: earlobe 3 at baseline, Single sample after that (cf. Fig 1.)	Pre (0min), 2, 4, 6, 8, 10 12 minutes	1 x each of 3 capillary samples at baseline 1 x per other sample	Radiometer ABL 800	N/A
		20	Supine	1	1.0 mL·kg^{-1} BW (f) 1.2 mL·kg^{-1} BW (m)	10	VEN: Antecubital vein (2 mL heparinised syringe) CAP: earlobe 3 at baseline, Single sample after that (cf. Fig 1.)	Pre (0min), 6, 8, 10 minutes 2 x per venous sample	2 x per venous sample 1 x each of 3 capillary samples at baseline 1 x other samples	Radiometer ABL 800	N/A

3.2 Methods

The participants' written informed consent was obtained prior to the study, at which point they were informed that they were free to withdraw from the study and/or terminate a trial at any time. The procedures were approved by the National Committee for Medical Ethics at the Ministry of Health (Republic of Slovenia; approval number: 0120-401/2020/8) and conformed to the standards set by the Declaration of Helsinki, except for registration in a database.

3.2.1 Experiment 1: Validity and Reliability

Paired venous and capillary blood draws were analysed from 22 adults (18 males, 4 females) aged 29.1 ± 6.4 years. Of the 22 participants, 13 performed the experiment twice to assess the reliability of both blood drawing techniques. These repeat measurements were conducted at the same time of the day, separated by a minimum of 48 hours and a maximum of one week. Participant inclusion criteria were: 18 years of age or older, non-smoker and without any diagnosis of a medical condition. Twenty-six participants were initially included, however, 4 were excluded due to the loss of blood samples as a result of clotting, reduced or no blood flow and as such could not be included in the analysis.

3.2.1.1 Carbon Monoxide Rebreathing (CO rebreathing)

The assessment of Hbmass and PV was conducted according to the same CO rebreathing protocol and equations described in detail by Siebenmann et al. (2017). Briefly, participants entered the laboratory in a hydrated state, >2 hours post-prandial, where their height (179.4 ± 7.0 cm) and weight (79.9 ± 10.0 kg) were immediately recorded. Participants consumed 500 mL of water before lying supine with their lower legs raised to facilitate complete blood mixing. Immediately thereafter, simultaneous baseline (BL) blood samples were drawn via venepuncture from an antecubital vein (4.5 mL lithium heparin vacutainer) and via the fingerstick procedure from the middle finger (100 µL). Prior to the collection of each capillary blood sample, the participant's hand was pre-warmed for 5 minutes using a bespoke heating box. The participants remained in the same supine position, breathing ambient air for 20 minutes. During this period, a personalised dosage of 99.9% chemically pure CO (SIAD S.p.A., Bergamo, Italy) was prepared based on the participant's weight (males: 1.5 $mL \cdot kg^{-1}$; females: 1.2 $mL \cdot kg^{-1}$) using a 140 mL syringe. Participants were then connected to an open-loop circuit (Fig. 1; blue), where they breathed 100% oxygen (O_2) for 4 minutes to initiate denitrogenation of the lungs and other tissues, with expired air leaving the circuit into the room. Before transitioning to the closed-loop (Fig. 1; orange), a second set of blood draws were taken and marked as pre-rebreathing (PRE). Following the blood draws, the participants fully exhaled before being switched to the closed-loop circuit by a sliding valve (Hans Rudolph Inc., Kansas, USA) to avoid overfilling the 6 L rebreathing bag (P3 Medical, Bristol, UK). Participants breathed 100 % O_2 within the closed-loop circuit to allow them to settle and secure a good seal on the mouthpiece. At the end of this 30-second period, their personalised dose of CO was introduced. The syringe was flushed 3 times while connected to the closed circuit to ensure complete delivery of the CO dose.

Participants rebreathed this gas mixture for 10 minutes. During the 10-minute rebreathing period, exhaled carbon dioxide (CO_2) was absorbed by soda lime within the circuit and one-way air valves ensured even mixing of the gas mixture and its proper movement through the system. Additional O_2 was added to the closed-circuit *ad libitum*

depending on the participants' rate of O_2 consumption. At the end of the 10-minute rebreathing period, the participants again fully exhaled before being disconnected from the closed loop and connected to the open-loop circuit. Thus, all of the unabsorbed CO in the participant's lungs was expired - except that in the residual volume - into the rebreathing bag for later analysis. Immediately after moving the participant to the open circuit, a final set of blood draws was completed and labelled post-rebreathing (POST).

The remaining gas in the closed-loop circuit was assessed for volume using a calibrated syringe (remaining gas + 1.28 L circuit dead space) and a flow meter (S43OA Spirometric Module, KL Engineering Co., California, USA) and for CO concentration in parts per million (ppm) with a flue gas analyser (model DC710, TPI Europe Ltd., West Sussex, UK). The remaining unabsorbed CO in the closed-loop circuit was then calculated as a percentage of the original dosage.

All blood samples were immediately analysed using an ABL80 FLEX CO-OX Blood gas analyser (Radiometer Medical, Brønshøj, Denmark). The parameters of interest were haematocrit (Hct), haemoglobin concentration ([Hb]) and carboxyhaemoglobin concentration (COHb%). A single capillary tube containing 100 µL of blood could only be analysed once as 85 µL is required by the ABL 80. Venous blood samples were gently and thoroughly mixed for 30 seconds before being analysed in duplicate with the average used for the follow-up analysis. The following equations were used to calculate Hbmass, PV, BV and RBCV.

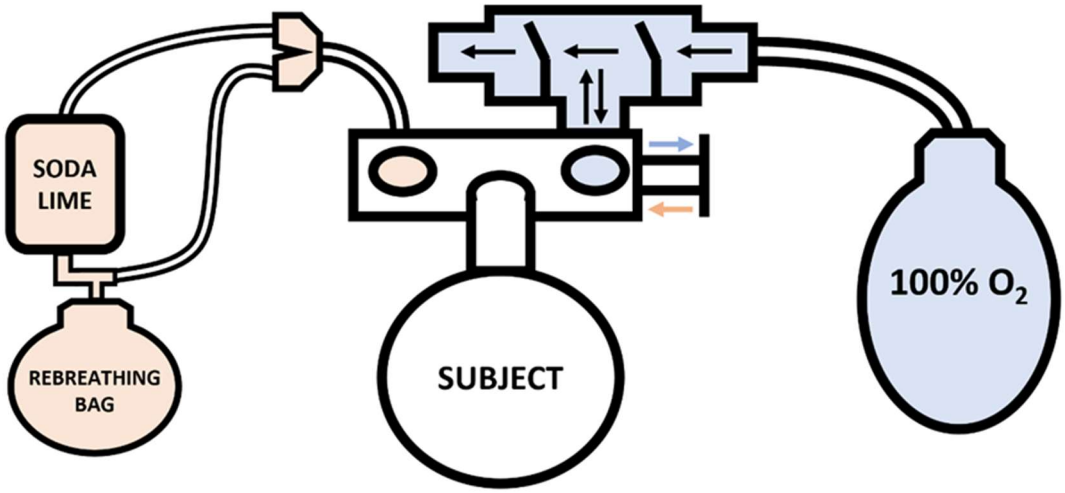

Figure 3.1: Schematic of the Hbmass CO rebreathing setup. (Note: Closed-loop circuit - orange, Open-loop circuit – blue).

Haemoglobin mass was derived using the following set of equations in the following order:

$$nCO_{absorbed} = P_{atm} \times VCO_{absorbed} \div (R \times T) \tag{3.1}$$

$$nHb_{tagged} = \frac{nCO_{absorbed}}{4} \tag{3.2}$$

$$nHb_{total} = \left(\frac{nHb_{tagged}}{\Delta COHb\%}\right) \times 100\% \tag{3.3}$$

$$Hbmass = nHb_{total} \times 6.44 \times 10^4\ g/mol \tag{3.4}$$

where,
$nCO_{absorbed}$ = number of CO molecules absorbed (mole)
P_{atm} = ambient pressure (standard atmospheres)
$VCO_{absorbed}$ = volume of absorbed CO gas (L)
R = ideal gas constant (0.0821 *L*atm/(mol*K)*.
T = temperature (K)
nHb_{tagged} = number of tagged haemoglobin molecules (mole)
nHb_{total} = number of total haemoglobin molecules (mole)
ΔCOHb% = change in carboxyhaemoglobin concentration (%)
6.44×10^4 g/mol = molar mass of haemoglobin (g/mole)

Intravascular volumes (L) were determined through the following equations:

$$RBCV = Hb_{mass} \times \frac{Hct}{Hbc} \tag{3.5}$$

$$BV = \frac{RBCV}{Hct} \tag{3.6}$$

$$PV = BV - RBCV \tag{3.7}$$

where,
RBCV = Red blood cell volume (L)
Hbmass = haemoglobin mass (g)
BV = Total blood volume (L)
PV = Plasma Volume (L)
Hct = Haematocrit (decimal value not %)
[Hb] = Haemoglobin concentration (g/L)

3.2.2 Experiment 2: Postural comparison

Fourteen adults (3 females, 11 males; height: 180.6 ± 6.7 cm, weight: 75.4 ± 8.3 kg, aged 21.0 ± 6.0 years) were included in a separate experiment designed to assess the effect posture (seated vs supine with raised legs) may have on the following variables of interest [Hb], Hct and COHb%, which are used in the calculation of Hbmass. The same inclusion criteria were followed for both experiment 1 and 2. Additionally, the participants followed identical instructions to experiment 1 regarding food and liquid consumption prior to entering the laboratory. The participants were fully informed regarding the experimental procedures and then they provided written informed consent prior to having their height and weight recorded.

The participants were instructed to sit quietly and immediately upon sitting, a venous blood sample was drawn from an antecubital vein (marked as timepoint vein 0: V0) via venepuncture (4.5 mL lithium heparin vacutainer). They then remained seated for 20 min, at which point a second blood draw (time point vein 1: V1) was taken. The participants were subsequently moved to a supine position with their legs raised (identical to the resting supine position in experiment 1). The participants remained in this position for 20 min, after which a final blood sample was collected (time point vein 2: V2). The participants did not perform a CO rebreathing protocol after this postural comparison. A schematic detailing the experimental protocol is displayed in Figure 3.2.

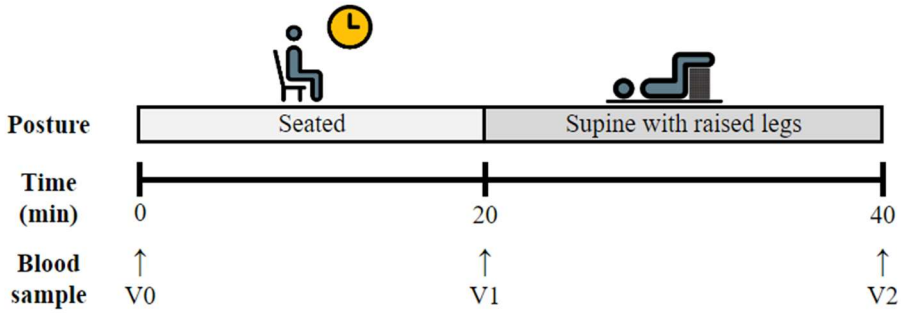

Figure 3.2: Schematic of the experimental protocol for Experiment 2: Postural Analysis. Venous blood sampling time points are listed as V0 (Pre-Seated), V1 (Post-Seated/Pre-Supine) and V2 (Post-Supine).

3.2.3 Statistical Analysis

Data are presented as Mean ± SD unless otherwise indicated. All statistical tests were performed unblinded to experimental conditions with SPSS (v25, IBM, New York, USA). GraphPad Prism (v8.4.2, GraphPad Software, San Diego, USA) was used for data visualisation. The significance level on all tests was set as $p < .05$, *a priori*. The paired *t*-test was used to identify differences between capillary and venous blood and the difference between BL and PRE time-points in certain analytes (Hct, [Hb], and COHb%). The between-variable (capillary vs venous) relationship strength was calculated using Pearson's correlation analysis and correlation coefficients were applied as recommended (Cohen; strong ≥0.60; moderate ≥0.40 − <0.59; weak ≥0.20 − <0.39). The intraclass correlation coefficient was utilised to assess test-retest correlation. The CO rebreathing typical measurement error (TE%) for each sampling site (venous or capillary) was calculated as a percentage of the mean value for each variable (Hopkins, 2000). Bland Altman plots (Bland & Altman, 1986) were used to compare venous and capillary blood samples and to compare Hbmass and PV from the two repeated tests as part of the repeatability analysis.

3.3 Results

As expected, given the low dose of CO inhaled, all participants completed the protocol with no signs of CO toxicity (highest COHb% = 10.65%). The analyte values determined from each blood sampling site (capillary and venous) and each time-point (BL, PRE and POST) are presented in Table 2. The Bland-Altman analysis (Fig. 3) contain the individual data of analytes between capillary and venous blood. The mean unabsorbed CO percentage remaining in the circuit from the CO rebreathing protocol was 1.74 ± 0.42 % (cf. Siebenmann et al. (2015); unabsorbed CO ranges from 0.8-5.1%).

3.3.1 Validity

The COHb% obtained from capillary blood was significantly lower than that in venous blood at all time-points ($p < .001$). However, there were no significant differences present in the ΔCOHb% ($p = .101$) in either CAP or VEN. All analytes obtained from capillary and venous blood were significantly correlated ($r > .678$, $p < .001$) except for COHb% at BL ($r = .238$, $p = .143$). No statistical differences were detected between any of the resultant Hbmass (absolute and relative) or intravascular volumes (RBCV, BV, and PV)

calculated from venous or capillary blood (Table 2). The Bland-Altman analysis (Fig. 3) further described the bias in the resultant absolute and relative Hbmass (absolute = 14.45 g LoA = -64.78 g - 93.67 g; relative = 0.17 g · kg^{-1}, LoA = -0.84 g·kg^{-1} – 1.19 g·kg^{-1}) and intravascular volumes (RBCV = 0.04 L, LoA = -0.20 L – 0.29 L; BV = -0.03 L, LoA = -0.87 L – 0.81 L; PV = -0.07 L, LoA = -0.80 L – 0.65 L). The data dispersion within these plots is consistent irrespective of the site average, except in PV where values have greater dispersion over 3.78 L, although it is unclear why this dispersion is greater above this volume.

3.3.2 Test-Retest Reliability

No significant differences were found in the change in mean (Test 2 – Test 1) between capillary and venous blood for Hbmass (-41.4 g vs -11.6 g, $p = .160$), relative Hbmass (-0.66 g·kg^{-1} vs -0.21 g·kg^{-1}, $p = .145$), ΔCOHb% (0.29 % vs 0.08 %, $p = .170$), RBCV (-1.24 L vs -0.04 L, $p = .173$), BV (-0.32 L vs -0.15 L, $p = .223$) or PV (-0.19 L vs 0.02 L, $p = .277$). Data for the TE% in each of the calculated variables are displayed in Table 3. Test-retest reliability, assessed through the intraclass correlation coefficient, other than COHb% before the inhalation of CO (BL and PRE) was strong in all analytes (Table 3). The Bland-Altman analyses (Fig. 4) revealed a lower bias and bias SD in Hbmass and PV values from venous blood (Hbmass = 11.64 ± 28.60 g; PV = -0.02 ± 0.22 L) than capillary blood (Hbmass = 41.37 ± 75.70 g; PV = 0.19 ± 0.70 L).

3.3.3 Postural Comparison

A one-way repeated measures ANOVA analysis revealed a significant main effect for time (V0, V1, and V2) in [Hb] ($p < .001$), COHb% ($p = .021$) and Hct ($p < .001$). Pairwise comparisons indicate a significant reduction in Hct between V0, V1 and V2 (V0 = 44.7 ± 2.1%, V1 = 43.4 ± 2.5%, V2 = 42.6 ± 2.3%) and reduced haemoglobin concentration [Hb] (V0 = 146 ± 7 g·L^{-1}, V1 = 141 ± 9 g·L^{-1}, V2 = 139 ± 8 g·L^{-1}). The pairwise comparisons identified significant differences in the COHb% between V1 and V2 (i.e., sitting and supine, V1 = 1.38 ± 0.09%, V2 = 1.46 ± 0.09%), however, there were no significant differences between V0 and V1 or between V0 and V2. Individual responses to the postural analysis are presented for each time point in Figure 5.

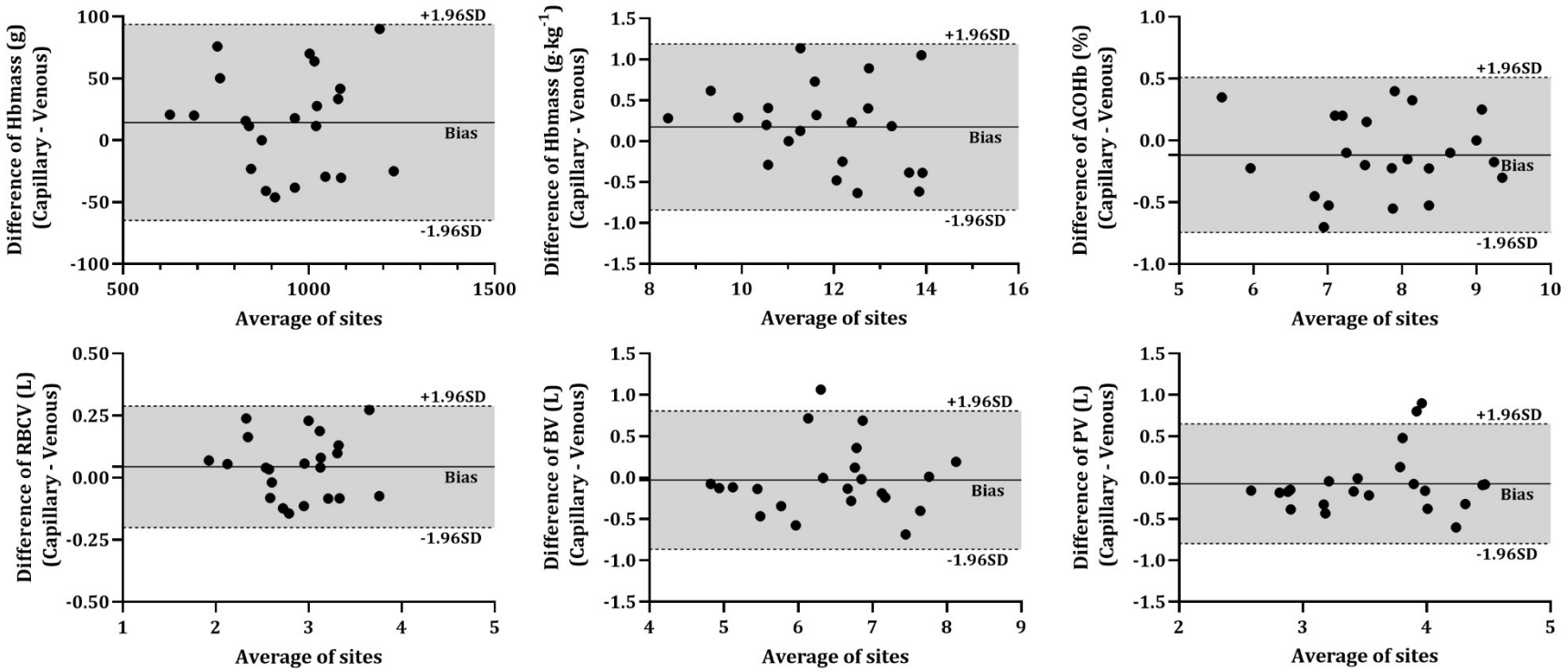

Figure 3.3: Difference in various analytes between capillary and venous blood after CO rebreathing. Horizontal lines indicate the mean bias (solid line) and the upper and lower 95% CI (dashed lines). Note: Hbmass – haemoglobin mass; ΔCOHb% - change in carboxyhaemoglobin percentage; RBCV – red blood cell volume; BV – blood volume; PV – plasma volume.

Table 3.2: Haematological variables determined from different sampling techniques (capillary and venous) during a CO rebreathing protocol.

Analyte	**Capillary**		**Venous**		***t*-test**	**Correlation**	
	Mean	**SD**	**Mean**	**SD**	***P***	***r***	***p***
BL Hct (%)	46.3	4.6	46.0	3.8	.572	.835	**<.001**
Pre Hct (%)	45.3	4.5	44.3	3.5	.058	.860	**<.001**
Post Hct (%)	45.6	4.0	44.3	4.0	**<.001**	.937	**<.001**
BL [Hb] ($g \cdot L^{-1}$)	151	15	150	13	.543	.831	**<.001**
Pre [Hb] ($g \cdot L^{-1}$)	148	15	145	11	.061	.863	**<.001**
Post [Hb] ($g.L^{-1}$)	149	13	144	13	**<.001**	.938	**<.001**
BL COHb (%)	1.0	0.3	1.3	0.2	**<.001**	.238	.143
Pre COHb (%)	0.8	0.2	1.2	0.2	**<.001**	.678	**<.001**
Post COHb (%)	8.5	1.0	9.0	1.0	**<.001**	.947	**<.001**
ΔCOHb (%)	7.7	1.0	7.8	1.0	.101	.950	**<.001**
Hbmass (g)	948.8	156.8	943.4	157.3	.108	.967	**<.001**
Hbmass ($g \cdot kg^{-1}$)	11.9	1.5	11.7	1.6	.131	.946	**<.001**
RBCV (L)	2.9	0.5	2.9	0.5	.109	.966	**<.001**
BV (L)	6.5	1.0	6.5	0.9	.752	.899	**<.001**
PV (L)	3.6	0.6	3.6	0.6	.360	.816	**<.001**

Note: BL = Baseline, Hct = Haematocrit, [Hb] = Haemoglobin concentration, COHb = Carboxyhaemoglobin, Hbmass = Haemoglobin mass, RBCV = Red Blood Cell Volume, BV = Blood Volume, PV = Plasma Volume, **Bold** = statistically significant ($p < .05$), r = Pearson's correlation coefficient.

Table 3.3: Reliability analysis of haematological variables determined from different sampling techniques (capillary and venous) during a CO rebreathing protocol.

Analyte	**Capillary**			**Venous**			**ICC**	
	Mean	**SD**	**TE%**	**Mean**	**SD**	**TE%**	**Capillary**	**Venous**
BL Hct (%)	47.9	4.4		47.1	3.8		**.888**	**.949**
Pre Hct (%)	46.6	4.7		45.0	3.9		**.843**	**.950**
Post Hct (%)	46.9	4.2		45.4	4.2		**.751**	**.948**
BL [Hb] ($g \cdot L^{-1}$)	156	15		154	13		**.891**	**.963**
Pre [Hb] ($g \cdot L^{-1}$)	152	16		147	13		**.842**	**.946**
Post [Hb] ($g.L^{-1}$)	153	14		148	14		**.752**	**.904**
BL COHb (%)	1.0	0.3		1.2	0.2		.606	-.196
Pre COHb (%)	0.8	0.3		1.2	0.3		-.623	-1.132
Post COHb (%)	8.6	1.1		9.1	1.1		**.976**	**.969**
ΔCOHb (%)	7.8	1.2	4.9	7.9	1.2	1.9	**.934**	**.991**
Hbmass (g)	967.6	186.1	5.5	952.4	172.5	2.1	**.949**	**.993**
Hbmass ($g \cdot kg^{-1}$)	11.6	1.7	7.3	11.4	1.6	2.7	**.827**	**.978**
RBCV (L)	3.0	0.6	5.5	2.9	0.5	2.1	**.949**	**.993**
BV (L)	6.4	1.2	9.4	6.5	1.0	2.8	**.844**	**.986**
PV (L)	3.5	0.7	14.2	3.6	0.6	4.5	**.732**	**.968**

Note: **Bold** = statistically significant ($p < .05$), TE% = Typical measurement error %, ICC = Intraclass correlation

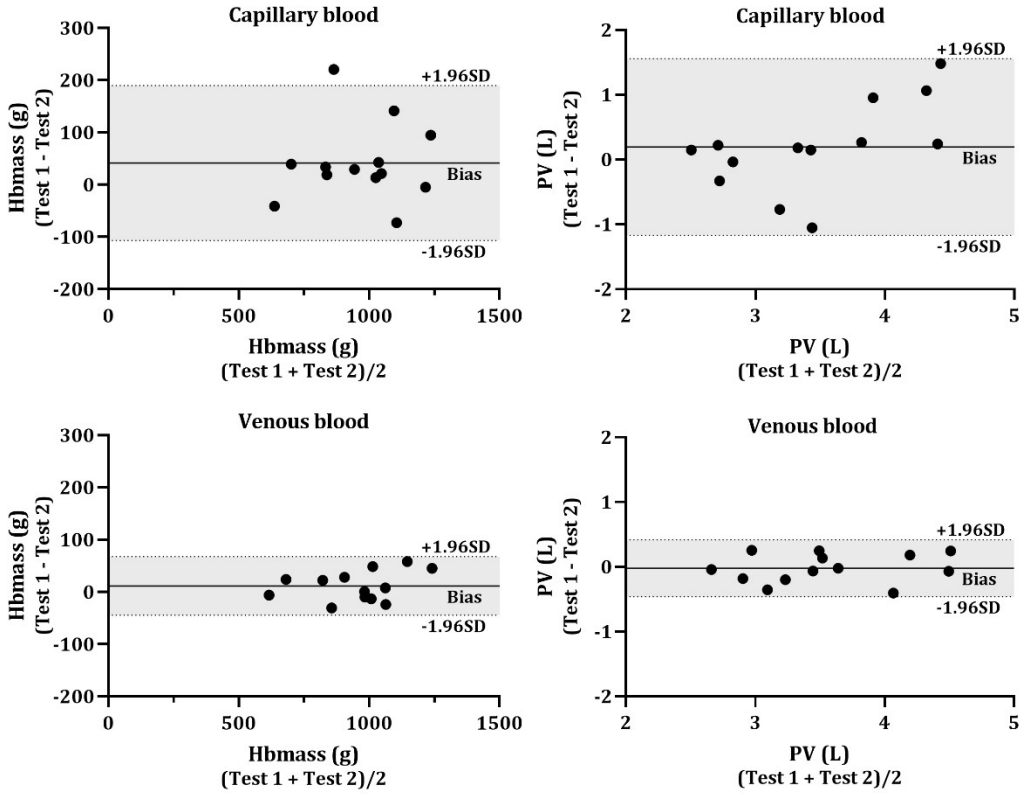

Figure 3.4: Bland-Altman plot for the test-retest reliability. Hbmass (left side) and plasma volume (PV; right side) were calculated from the capillary (top panels) and venous (bottom panels) blood. The individual data (N = 13) shows the difference between the Hbmass, and PV determined from two CO rebreathing procedures. Horizontal lines indicate the mean bias (solid line) and the upper and lower 95% CI (dashed lines).

3.4 Discussion

The present study's primary aim was to assess the comparability of Hbmass and PV estimates derived from capillary and venous blood samples collected during a 10-minute supine CO rebreathing protocol. The data demonstrate that capillary blood sampling is an acceptable alternative to venous blood for the calculation of Hbmass and blood vascular volumes (RBCV, BV, and PV) in terms of accuracy, even with a single sample analysis. Secondly, this study addressed whether posture plays a role in altering the variables of interest for the calculation of Hbmass ([Hb], Hct and COHb%). There are significant reductions noted in the concentration of haemoglobin and haematocrit in a supine position compared to sitting. It is crucial to take into account the effect of posture and to interpret the data correctly based on the CO rebreathing protocol followed (Table 1). Finally, this study found reduced reliability and increased error compared to venous blood were present when using the capillary method, albeit limited. However, there is a methodological caveat that needs consideration, in particular, multiple replicate analyses of capillary blood samples, which were not carried out in the current study are a necessity in order to reduce the TE% from 5.5% to an acceptable level of 2%.

3.4.1 Validity

In contrast to previous research (Durussel et al., 2013), venous COHb% values were consistently larger and significantly different (Table 2) compared to those obtained from capillary blood across all time points. Hütler et al. (2001), suggested that blood oxygen saturation could be a significant contributing factor to the COHB% measurement. However, the ΔCOHb% obtained from capillary and venous blood is statistically indifferent in the present study and shares a strong positive correlation. The difference noted between the two sampling sites is consistent and therefore allows for the accurate calculation of ΔCOHb% from either. Furthermore, despite a larger TE%, the current findings are in line with the literature, where the use of capillary or venous blood did not have any significant effect on the consequential calculations of absolute or relative Hbmass (Garvican et al., 2010; Gore et al., 2006; Hütler et al., 2000; Prommer & Schmidt, 2007).

Intravascular volumes (RBCV, BV, and PV) are calculated using Hct and [Hb] values. In the present study, no significant differences were noted between the Hct and [Hb] values collected at the PRE time-point between sampling techniques, however, POST [Hb] and Hct were significantly larger in the venous sample compared to in the capillary sample (Table 2). However, one should bear in mind that these differences in POST values are of little consequence, as the calculation of Hbmass and PV only requires the PRE time-point values. Indifferent, higher, or lower [Hb] and Hct values between venous and capillary sampling have been reported (Daae et al., 1988; Fahey & Rolph, 1975; Hütler et al., 2000; Moe, 1970). Such differences have been partially attributed to the capillary puncture depth, vessel diameter, sampling technique and blood flow rate to the extremities (Daae et al., 1988).

While there is ample evidence to support the use of a capillary sample as a valid method of blood collection during CO rebreathing, most evidence to date has been collected in a seated position (Burge & Skinner, 1995; Garvican et al., 2010; Hütler et al., 2000; Prommer & Schmidt, 2007). The overall accuracy of the capillary sampling method is a result of replicate sample analysis in the range of 3-6 (Table 1). Although, there are some examples of publications with less replication (Durussel et al., 2013; Steiner & Wehrlin, 2011), no replication (Schmidt & Prommer, 2005), or simply do not state what type of analysis was conducted (Ashenden et al., 1999; Heinicke et al., 2001; Otto et al., 2017; Prommer & Schmidt, 2007). Finally, there is little evidence of the reliability of capillary sampling in a supine position during CO rebreathing (Kellenberger et al., 2022). The current study indicates that results obtained from a single capillary analysis were highly correlated with those from venous and that there were no significant differences between BL Hct and [Hb], ΔCOHb (%) or Hbmass calculated from venous or capillary samples. The capillary TE% is larger than typically noted in the literature, however, this error may most likely be a technical error associated with the ABL and can be confidently reduced through replicate analysis (Alexander et al., 2011). In doing so, it is certainly possible that the use of capillary sampling could be a valid tool/method for use in a supine CO rebreathing protocol.

3.4.2 Test-Retest Reliability

Intra-class correlation coefficients (ICC) for absolute and relative Hbmass from both blood sampling techniques indicated good to excellent reliability ($> .700$) (Koo & Li, 2016). The intravascular volumes calculated from venous blood all had excellent ICC, while the values calculated with capillary blood ranged from moderate to excellent ICC (Table 3). When comparing CAP to VEN, the data indicates less reliable absolute and relative Hbmass determinations and intravascular volumes when calculated using blood drawn from the capillary. Values from venous blood consistently displayed higher ICC, lower TE%, and

lower Bland-Altman bias (Table 3, Fig. 4) when compared to the capillary. The composition of the capillary blood sample and the technique used to collect it tend to result in a less reliable source of haematological variables. Capillary fingerstick samples often contain extracellular fluid which can potentially contaminate the blood sample readings. The smaller volume drawn through a single capillary sample both increases the impact of potential contamination and eliminates the option of duplicated analyses. Although as aforementioned, several authors have used other techniques than duplicate analysis, i.e., Durussel et al. (2013) collected duplicate samples and Steiner and Wehrlin (2011) collected triplicate from capillaries and averaged the singular analysis of those data to improve the accuracy of the method, while Hütler et al. (2000) averaged samples from separate time points. In other cases, increasing the size of the capillary tube may help. Gore et al. (2006) collected capillary samples up to 200 µL to allow for 5 replicate analyses of each time point. This would not be a suitable volume for quintuplet analyses with the ABL 80 which requires 85 µL per capillary sample.

Reliable capillary blood sampling is dependent on maintained blood flow to the sampling site (ear lobe or finger) which will reduce drop-to-drop variability and error (Bond & Richards-Kortum, 2015). Over manipulation or "milking" of the finger to extract blood from participants with a lower blood flow during the sampling time can increase the drop-to-drop variability and dilute the sample with interstitial fluid. Further consideration should be given to the effects of deep lancing and haemolysation of the sample (Cembrowski & Füzéry, 2017). Additionally, due to the participants' posture during the CO rebreathing protocol (supine, legs raised), the current researchers found blood flow was reduced in most cases, despite limb warming and the pre-test consumption of 500 mL of water. Some participants' capillary blood samples could not be included in the analysis, as the sample clotted in the capillary tube as a result of the blood sample collection taking excessive time. Thus, reinforcing the essential use of heparinised capillary tubes. In the future, the vasodilator ointments, such as Finalgon and Transvasin, should be used to assess whether they can improve the likelihood of collecting duplicate samples from fingertip capilleries.

Siebenmann et al. (2017) state that a TE% of less than 2.5% has been common in recent CO rebreathing studies and that experts with this method often report a TE% of 1.5-2%. The TE% of 2.1% reported from venous blood in the current study is comparable with the literature assessing CO rebreathing protocol reliability (Table 1 provides details of published TE% and CoV: (Burge & Skinner, 1995; Hütler et al., 2000; Otto et al., 2017; Schmidt & Prommer, 2005). However, the TE% of 5.5% for Hbmass calculated from capillary blood is larger than previously reported, although there are examples of similar or higher TE%. Oberholzer et al. (2020) reported (in venous) TE% of up to 14.1% caused by changes in CO dose, length of rebreathing, number of replicates and altitude at the protocol was carried out. In order to ensure complete mixing - of CO & blood - and thus tagging of the haemoglobin molecule with CO both Garvican et al. (2010) and Siebenmann et al. (2017) suggest that 10 min is an appropriate amount of time after the onset of CO rebreathing to draw the post sample. This is further substantiated by Kellenberger et al. (2022) who report similar COHb% values for capillary and venous samples at 8 and 10 min of rebreathing. Unfortunately, Kellenberger et al. (2022) did not conduct a similar repeatability analysis utilised in the current study of CAP and VEN values using the Siebenmann method.

3.4.3 Postural Comparison

A seated posture is often used in alternate versions of CO rebreathing protocols A seated posture is often used in alternate versions of CO rebreathing protocols (Durussel et al., 2013; Hütler et al., 2000; Prommer & Schmidt, 2007), however, the protocols described by

Siebenmann et al. (2017) and Burge and Skinner (1995) are conducted in the supine position, either with or without legs raised respectively. The change of posture from seated to supine allows the protocol to be used during bed rest interventions where participants are required to remain horizontal which is useful in both clinical and space life science settings. Raising the legs also increases whole-body blood circulation, reduces the risk of syncope and allows for thorough distribution of the CO bolus throughout the circulation. Further, performing the protocol in a seated position is reported to lead to under-estimations of Hbmass around 28 g (~3%) (Keiser et al., 2013; Siebenmann et al., 2017). Data from Experiment 2 (Fig. 5) highlights that there is a postural influence (seated vs supine with legs raised) on [Hb], Hct and COHb%; and caution should be exercised when calculating and comparing Hbmass between protocols. Further, Lima-Oliveira et al. (2017) report clinically significant shifts in [Hb], Hct and PV as a result of changing posture after 20 or 25 min in a seated or supine position. In the present study, participants lay supine with their legs raised for 20 min prior to the 4-minute O_2 pre-breathe. The purpose of this period was to allow for increased blood circulation from the lower to upper extremities in order to reduce blood pooling in the legs which may result in PV shifts (Diaz et al., 1979). Changing the participants' position from supine to standing results in significant BV redistribution and erroneous calculation of PV of up to 500 mL (Hagan et al., 1978).

In contrast with the current results (Fig. 5), Durussel et al. (2013) discovered no postural effect (seated vs supine) on the estimation of Hbmass, BV, RBCV or PV. However, it is important to note that the authors conducted only 10 minutes in the supine position followed by 10 minutes in the seated position when 20 minutes in one posture is suggested for adequate stabilisation (Smith et al., 1994; Tan et al., 1973). Therefore, the protocol used in their study neither waited long enough for vascular volumes to stabilise, nor counter the effect of the supine position on the seated position in order to draw the conclusion that posture does not affect Hbmass calculation. Finally, by obtaining blood samples from an antecubital vein and the saphena magma of the ankle, Keiser et al., (2013) found there to be an uneven distribution of CO in the blood circulation if the CO rebreathing protocol is performed in the seated position. This uneven distribution was ascribed to incomplete mixing of CO and may be negated by either gently exercising or by conducting the protocol in a supine position (Siebenmann et al., 2017).

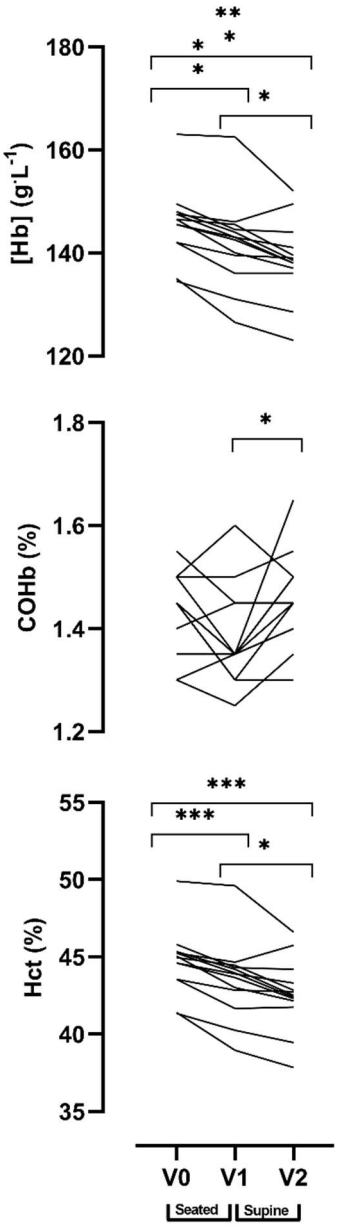

Figure 3.5: Effect of posture on venous blood values between three time points: Baseline (V0), 20 minutes seated (V1), and 20 minutes supine with legs elevated (V2). Note: alpha level signified as * <.05, ** <.01, *** <.001.

3.4.4 Practical Implications

Based on the current data, several recommendations may be put forward for consideration when using the current implemented rebreathing protocol. As aforementioned, a 20-minute rest in a supine position with legs raised is essential for conducting this protocol. Failure to allow for this period may result in significantly higher values of [Hb] and Hct in the Pre-rebreathing data than if there had been a stabilisation period following the posture change (Fig. 5).

Venous sampling is typically preferred when performing the methods described in the present study (Rønnestad et al., 2021; Siebenmann et al., 2017). However, in case a phlebotomist is unavailable, a capillary blood sample may be obtained instead. Previous evidence suggests that it is highly recommended to collect capillary samples large enough to be analysed in quadruplicate (Alexander et al., 2011; Gore et al., 2006). Failing that, several samples should be drawn with the singular analysis of each being averaged.

A capillary tube filling time of approximately 20 seconds has been reported (Durussel et al., 2013; Gore et al., 2006) and as previously mentioned, this was not the case in the current study and several samples clotted, requiring that they be discarded. As such it is highly recommended to use heparinised capillary tubes when conducting the study in a supine position to avoid any clotting-related issue prior to analysis and thus loss of data.

In the protocol described by Schmidt and Prommer (2005), CO in the lung pre- and post-rebreathing is calculated and accounted for. This correction means an increased precision in the measurement and reduction in potential typical error of measurement of ~4 g when assuming an absolute Hbmass of 950 g (<0.5%) (Prommer & Schmidt, 2007) and should be used, in combination with other mentioned factors. Further, it should be considered that with prolonged CO rebreathing there is an increase in the loss of CO from the vasculature, this CO may become bound to myoglobin. In order to reduce error by as much as possible, these corrections may be applied.

Finally, when using the capillary or venous sampling site for the estimation of Hbmass and blood volumes, the sampling sites must not be used interchangeably during testing. For example, if a venous line coagulates during the waiting period between samples, a new

venous line should be inserted or venepuncture performed rather than switching to drawing capillary blood, or vice versa. The reasoning for this suggestion is that there was a significant difference of 0.5% for the COHb% values between sampling sites. Failing to account for this consistent error between the methods would lead to an error of ~50 g based on an absolute Hbmass of 950 g as reported for the current participants.

3.4.5 Limitations

As noted in the Postural comparison section, the supine posture with elevated legs is the preferred position for measuring Hbmass. Blood collection from an antecubital vein was not affected whilst supine, however, in our laboratory, we found that fingertip capillary draws had reduced blood flow. Consequently, fingertip blood draws often took longer, clotted or were unsuccessful. The increased duration may also have affected drop-to-drop variation and ultimately the parameters of interest. As a result, large capillary samples were not obtained, and the results were analysed in singular. Assessing the capillary sample in duplicate may reduce the error by a factor of 2 (Schmidt & Prommer, 2005), a comparable reduction in error may be seen in our results (Table 3.2) between venous (2.1%) and capillary (4.9%) samples.

The main limitation in the comparison of capillary and venous blood data in the present study is the inability to collect sufficiently large capillary samples while the participant lay in the supine posture, despite taking several measures to increase the ease of the blood draw. To address issues that others may face in these circumstances, the following is recommended: i) 10 minutes of hand warming be conducted prior to capillary blood draws, ii) use of heparinised capillary tubes, iii) collect sufficiently large capillary blood samples that allow for the assessment of all samples in at least duplicate. Further, based on the literature presented in Table 3.1, replicate analysis in the range of 3 to 6 times is highly recommended.

Chapter 4

Hypoxic Acclimatisation in Competitive Swimmers?

Foreword

Chapter 2 investigated the heterogeneity in acclimatisation to hypoxia and highlighted potential issues with the measure of [Hb] and Hct for blood oxygen-carrying capacity. These issues were then addressed by the CO rebreathing protocol, which was evaluated in Chapter 3. The next step was to use the CO rebreathing method to compare haematological values between competitive swimmers, controls, and other athletic groups. These groups were selected for the following reasoning: the swimming group (training comprises high-intensity exercise and no breath hold training) was the group of main interest due to the context outlined in Chapter 1. The control group was selected due to being a group that took part in no form of organised exercise (no exercise and no breath-holding training), the apnoea diving group was selected due to their theoretical hypoxic stimulus as a direct result of their breath-holding training without the stimulus of high-intensity exercise (medium intensity exercise and breath hold training), and finally, cross-country skiers were selected as they were a group that perform whole body aerobic exercise like the swimmers, although without the requirement to breath-hold during their training (high-intensity exercise, no breath hold training).

Table 4.1: Summary of investigated training characteristics in subject groups.

Subject groups	Exercise intensity	Breath hold
Control	N/A	N/A
Swimmers	High	Moderate
Apnoea divers	Moderate	High
Cross country skiers	High	N/A

The findings to this chapter are currently in preparation for publication, under the working title “Breath-Holding in Swimming: Investigating Hypoxic Acclimatization and Physiological Responses Through Comparative and Seasonal Analysis” authored by Joshua T. Royal, Tinkara Mlinar, Jason T. Fisher, Jernej Kapus, Adam C. McDonnell and Igor B. Mekjavic.

4.1 Introduction

As mentioned in Chapter 1, the combination of reduced breathing frequency in order to gain an advantage in performance with high-intensity exercise causes significant increases in blood PCO_2 and decreases in PO_2 (Davies et al., 1995; Kapus et al., 2009; Miyasaka et al., 2002). In competitive swimming, reduced breathing frequency lends a biomechanical advantage. Breathing to the side in front crawl increases frontal drag which in turn slows the swimmer (McCabe et al., 2015). To maximise performance during sprint front crawl swimming, reduced breathing frequencies are preferred (Pedersen & Kjendlie, 2006). Theoretically, if a competitive swimmer, who trains >15 hours a week in the pool, is exposed to hypoxic stimuli through a reduced breathing frequency, there is the potential for hypoxic adaptation to take place.

In groups that experience large durations of breath-holding, it is hypothesised that reduced chemosensitivity will be present in the ventilatory responses to both hypoxia (HVR) and hypercapnia (HCVR). Several authors have researched this in groups of apnoea divers (Delapille et al., 2001; Florio et al., 1979; Foster & Sheel, 2005; Grassi et al., 1994; Masuda et al., 1981), underwater hockey players (Davis et al., 1987; Lemaitre et al., 2007), synchronised swimmers (Bjurstrom & Schoene, 1987), and submarine escape instructors (Schaefer, 1966). The results of these studies are varied and conflicting. In theory, blunting of the carbon dioxide (CO_2) chemosensitivity from repeated breath-holds allows divers to postpone their breath-hold breaking point and tolerate a higher pressure of CO_2 (Schagatay et al., 1999; Schagatay et al., 2000; Song et al., 1963), however, several studies report no differences in the HCVR of apnoea divers and control participants (Costalat et al., 2014; Masuda et al., 1981). The same is true in the research around HVR, however, the differences in many studies' protocols make this measure hard to compare across studies, mainly due to the control or lack thereof of CO_2 pressures. Few studies exist comparing HCVR and HVR of competitive swimmers to control participants. Rebuck and Read (1971) observed a higher HCVR in elite sprint swimmers compared to elite middle-distance and long-distance swimmers. Ohkuwa et al. (1980) however found no statistical significance between the HCVR values of swimmers and control participants or within the swimming group between long-distance swimmers and sprint swimmers. Further elucidation of the ventilatory responses to hypoxia and hypercapnia in swimmers compared to athletes from other breath-holding and non-breath-holding sports is warranted. As well as potential changes in chemosensitivity, hypoxic acclimatisation is typically reflected in increases in O_2 carrying capacity. This is reflected either indirectly in changes in aerobic capacity (VO_2 max) or directly through the calculation of the total mass of haemoglobin (Hbmass). Both VO_2 max and Hbmass have previously been shown to increase under hypoxic conditions when combined with aerobic training programs in normal populations (Shin et al., 2013), although the effectiveness of this in elite athletes is disputed (Levine, 2002; Millet & Brocherie, 2020; Morton & Cable, 2005). Typically, the effectiveness of hypoxic training strategies is reliant on the intensity and duration of the hypoxic stimuli (Czuba et al., 2018).

To assess whether swimmers undergo a degree of hypoxic acclimatisation due to the reduction of breathing frequency for performance gains, the present study compares swimmers to 3 other subject groups: 1) Control group. Sedentary subjects not participating in any form of organised sports. 2) Apnoea divers. Due to the nature of their training, apnoea divers exhibit differences in chemosensitivity and haematology compared to control participants, albeit without a high volume of endurance exercise training. 3) Cross-country skiers. This group was selected due to their high degree of endurance conditioning, however, without any restriction in their breathing frequency. It was hypothesised that the swimmers

would have an aerobic capacity similar to the cross-country skiers, and an adaptation to hypoxia and hypercapnia as observed in apnoea divers.

4.2 Methods

4.2.1 Ethical Approval

Participants' written informed consent was obtained prior to the study, and they were informed that they were free to withdraw their consent at any time. The procedures were approved by the Committee for Medical Ethics at the Ministry of Health (Republic of Slovenia; approval number: 0120-401/2020/8) and conformed to the standards set by the Declaration of Helsinki, except for registration in a database.

4.2.2 Study Design

To assess potential adaptation to a hypoxic stimulus in swimmers, four groups of participants were tested for their maximal aerobic capacity (VO2 max), ventilatory responses to hypoxia (HVR) and hypercapnia (HCVR), and haemoglobin mass (Hbmass). The tested groups were competitive swimmers (CS), apnoea divers (AD), cross-country skiers (XC), and control participants (CON). CS, AD, and XC were recruited from local clubs and were competing at national or international level competitions. CON participants were university students recruited through advertisements on the Slovenian national student work website.

4.2.2.1 Aerobic Capacity (VO_2 max)

Participants undertook an incremental step test on a cycle ergometer (ergo_bike premium8i, Daum Electronic, Furth, Germany) to volitional exhaustion to determine maximal oxygen uptake. This protocol started with a 5-minute seated rest on the ergometer, followed by a 3-minute warmup at an intensity of 50W. After the warmup, the exercise intensity was increased by 25W every minute, until volitional exhaustion. After completion of the maximal exercise test, participants continued to cycle on the bike for 5 minutes at 50W, before resting off the bike for a further 15 minutes. Participants warmed up on the bike once again at 50W before performing a verification effort at 105% of the maximum wattage achieved during the initial cycle ergometer test to volitional exhaustion.

The exercise modality selected for the protocol for VO_2 max assessment has a large consequence on the results obtained (Astrand & Saltin, 1961; Sousa et al., 2015). Holmér et al. (1974) evaluated VO_2 max values in elite male and female swimmers during swimming and running-based protocols, swimming VO_2 max was on average 6% lower than the running VO_2 max ($p < .01$). This is likely due to the greater reliance on arms for propulsion during front crawl swimming, despite the leg muscle usage involved in kicking, the energy efficiency of the legs for propulsion is far lesser than the arms (Deschodt et al., 1999). To avoid any potential effect of exercise familiarity on the data, cycle ergometry was therefore selected as the exercise modality.

4.2.2.2 Haemoglobin mass (Hbmass)

Hbmass and intravascular volumes were assessed using the same rebreathing protocol and equations previously described by Siebenmann et al. (2017). In brief, participants entered the laboratory in a hydrated state, >2 hours post-prandial where their height and weight

were immediately recorded. Participants consumed 500 mL of water before lying supine with their lower legs raised to facilitate adequate blood mixing, and to assist haemoglobin release from the spleen (Diaz et al., 1979), breathing ambient air for 20 minutes. During this period, a personalised dosage of 99.9% chemically pure CO (SIAD S.p.A., Bergamo, Italy) was measured based on the participant's weight (males: 1.5 mL.kg-1; females: 1.2 mL.kg-1) using a 140 mL syringe. Participants were then connected to an open-loop circuit, where they breathed 100% oxygen (O_2) for 4 minutes to initiate denitrogenation of the lungs and other tissues, before transitioning to the closed-loop circuit. Before changing circuits, simultaneous blood samples were drawn via venipuncture from an antecubital vein (4.5 mL) and via the fingerstick procedure from the middle finger (100 µL) and marked as pre-rebreathing (PRE). Following the blood draws, the participants fully exhaled and were switched to the closed-loop circuit by a sliding valve (Hans Rudolph Inc., Kansas, USA). Participants were now breathing via the closed-loop circuit filled with 100 % O_2, and after 30 seconds, their personalised dose of CO was introduced. Participants rebreathed this gas mixture for 10 minutes. During the rebreathing period, exhaled carbon dioxide (CO_2) was absorbed by soda lime in the circuit. Additional O_2 was added to the closed-circuit ad libitum depending on the participants' rate of O_2 consumption. At the end of the 10-minute rebreathing period, the participants again fully exhaled before being connected to the open-loop circuit, so that all unabsorbed CO in the participant's lungs was expired for use in the analysis. Immediately after moving the participant to the open circuit, a second set of blood draws was completed and labelled post-rebreathing (POST).

The volume of the remaining gas in the closed-loop circuit was assessed using a calibrated syringe (remaining gas + 1.28 L circuit dead space) and flow meter (S43OA Spirometric Module, KL Engineering Co., California, USA). CO concentration parts per million (ppm) were analysed using a CO analyser (DC710 flue gas analyser TPI Europe Ltd., West Sussex, UK). The remaining unabsorbed CO in the closed-loop circuit was then calculated as a percentage of the original dosage. All blood samples were analysed using an ABL80 FLEX CO-OX Blood gas analyser (Radiometer Medical, Brønshøj, Denmark).

4.2.2.3 Hypoxic Ventilatory Response (HVR)

The protocol used for the measurement of HVR is an adapted version of a method used by Richalet et al. (2012). Participants were seated on a cycle ergometer (ergo_bike premium8i, Daum Electronic, Furth, Germany) for 10 minutes whilst ventilatory values, Heart rate (HR), and oxygen saturation measured through pulse oximetry at the peripheral capillaries (S_pO_2) stabilised. Participants then cycled for six minutes breathing ambient air at an intensity that was equal to the exercise intensity at 30% of their VO_{2max} during the incremental exercise test, this was labelled as normoxic (NOR). This NOR period was then used as a baseline for three subsequent efforts at the same workload, however, the participants breathed a series of hypoxic gas mixtures (17%, 14%, and 11% O_2) in a randomised order. As the hypoxic gas mixture did not contain CO_2, during each cycling bout, CO_2 was introduced ad libitum into the gas mixture to keep $PETCO_2$ consistent with that measured in normoxic conditions.

Between each effort, the participant rested on the ergometer for six minutes while breathing ambient air. End-tidal CO_2 ($PETCO_2$) and O_2 ($PETO_2$) and minute ventilation (VE) are continuously measured via an online breath-by-breath gas analyser (Quark CPET, Cosmed, Rome, Italy). HR and SpO_2 were also measured using a Nonin WristOx 3100 (Nonin Medical Inc, Maine, USA).

From the results attained, HVR for each hypoxic gas mixture was calculated using the following method:

$$\text{Desaturation: } \Delta SpO_2(\%) = nSpO_2 - hSpO_2 \tag{4.1}$$

$$\text{Ventilation changes: } \Delta \dot{V}_E\ (L \cdot min^{-1}) = n\dot{V}_E - h\dot{V}_E \tag{4.2}$$

$$\text{Heart rate changes: } \Delta HR\ (beats \cdot min^{-1}) = nHR - hHR \tag{4.3}$$

$$HVR = \Delta \dot{V}_E \cdot \Delta {SpO_2}^{-1} \cdot BM^{-1} \tag{4.4}$$

$$HCR = \Delta HR \cdot \Delta {SpO_2}^{-1} \tag{4.5}$$

where,
prefix n – value collected during the normoxic condition
prefix h – value collected during the hypoxic condition
SpO_2 – Oxygen saturation measured through pulse oximetry
$\dot{V}_E$ – minute ventilation
HR – heart rate
HVR – hypoxic ventilatory response
HCR – hypoxic cardiac response
BM – body mass (kg)

4.2.2.4 Hypercapnic Ventilatory Response (HCVR)

The protocol used for the measurement of HCVR is an adapted version of a method used by Read and Leigh (1967). Participants entered the laboratory and had their height and weight measured. Forced vital capacity (FVC) was then measured to calculate the volume of gas to be placed in the rebreathing bag (Bag Volume = FVC + 1.5 L). The participant then sat and rested for 10 minutes as the rebreathing circuit was prepared. After this period the participant was connected to the circuit and online breath-by-breath gas analyser (Quark CPET, Cosmed, Rome, Italy) with an oronasal facemask and continued to breathe ambient air for a further four minutes. On four minutes of recorded baseline data, the participant performed a maximal exhalation and was immediately switched to the rebreathing bag which was filled with their individualised volume of 93% oxygen (O_2) and 7% carbon dioxide (CO_2). Participants breathed the gas mixture for four minutes before being switched back to ambient air for the final two minutes in order to let values return to baseline. Breath-by-breath ventilatory equivalents ($\dot{V}_E$) and end-tidal pressure of CO_2 ($PETCO_2$) were plotted against each other for the rebreathing period, the gradient of the line of relationship was determined as their sensitivity to CO_2 and therefore their HCVR. In the original method, Read and Leigh (1967) used 50% O_2 in their gas mixture, however, the higher concentration of O_2, as used in the present thesis, has proven to have no effect on ventilatory responses (Huggard et al., 2023).

4.2.3 Statistical Analysis

Data are presented as Mean ± SD unless otherwise indicated. All statistical tests were performed unblinded to experimental conditions with SPSS (v25, IBM, New York, USA). GraphPad Prism (v8.4.2, GraphPad Software, San Diego, USA) was used for data visualisation. The significance level on all tests was set as $p < .05$, *a priori*. One-way ANOVA was selected for the comparison of variables across groups, with the Bonferroni correction on subsequent post-hoc pairwise comparisons where the main effects were significant. Two-way mixed model ANOVA analyses to assess the effect of hypoxic gas mixture, athletic groups and gas*group interaction during the HVR test.

4.3 Results

Group information can be found below in Table 4.1. One-way ANOVA results showed significance in the age of participants (p <.001). Post hoc analysis showed apnoea divers were significantly older than the other 3 groups (controls $p = .014$, swimmers and apnoea divers p <.001), and that control participants were significantly older than cross-country skiers ($p = .041$). No significance was found between groups in height or weight.

Table 4.2: Participant characteristics from each of the groups.

	CS	**AD**	**XC**	**CON**
n	8	7	10	7
Height (cm)	179.6 ± 5.2	178.9 ± 7.6	180.5 ± 7.0	181.3 ± 7.7
Weight (kg)	70.0 ± 7.5	75.2 ± 7.1	73.3 ± 6.5	76.8 ± 13.8
Age (yrs)	19.6 ± 2.4	30.1 ± 6.5	18.3 ± 1.3	23.6 ± 3.3

4.3.1 Aerobic Capacity (VO_2 max)

Mean and individual data are represented in Figure 4.1. Absolute VO_2 max was statistically indifferent across groups ($p = .070$). Relative VO_2 max in control participants (42.19 ± 5.34 $mL \cdot Kg^{-1} \cdot min^{-1}$) was statistically lower than swimmers (55.74 ± 4.64 $mL \cdot Kg^{-1} \cdot min^{-1}$, $p = .002$), apnoea divers (53.92 ± 7.49 $mL \cdot Kg^{-1} \cdot min^{-1}$, $p = .013$) and cross-country skiers (57.87. ± 7.73 $mL \cdot Kg^{-1} \cdot min^{-1}$, p <.001).

The exercise load at VO_2 max was statistically indifferent between the four groups (controls – 285 ± 53W, swimmers – 322 ± 28W, apnoea divers – 325 ± 66W, cross country skiers – 350 ± 50W). Relative power at VO_2 max was significantly higher in swimmers (4.62 ± 0.41 $W \cdot Kg^{-1}$; $p = .008$) and cross-country skiers (4.73 ± 0.37 $W \cdot Kg^{-1}$; $p = .002$) than controls (3.74 ± 0.42 $W \cdot Kg^{-1}$). Apnoea divers were statistically indifferent from all groups (4.23 ± 0.75 $W \cdot Kg^{-1}$).

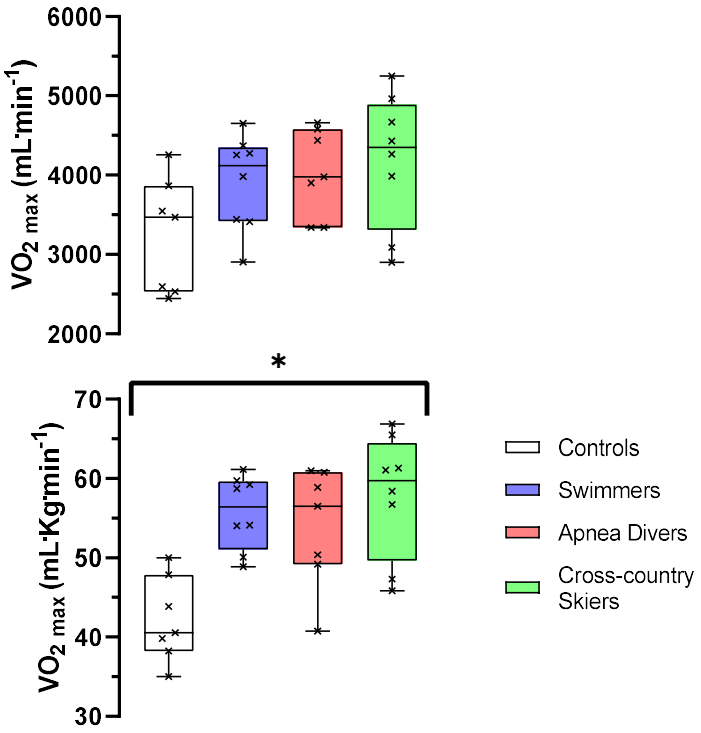

Figure 4.1: Absolute and relative VO_2 max values for participants from each group.

4.3.2 Haemoglobin Mass (Hbmass)

The results of the ANOVA indicated that there was no statistical difference in both absolute and relative Hbmass (corrected for body mass) between groups (absolute - $p = .969$; relative – $p = .406$), please see Figure 4.2 for individual and mean data.

In the intravascular volume data, no difference was detected in the total blood volume between groups ($p = .597$), however, plasma volume was statistically different between groups ($p = .016$), and post-hoc analysis showed that the apnoea divers had significantly higher PV than cross-country skiers ($p = .015$). Cross-country skiers had a significantly larger red blood cell volume than controls ($p = .003$), swimmers ($p = .001$), and apnoea divers ($p = .003$). The intravascular volumes are displayed as Mean ± SD in Table 4.2.

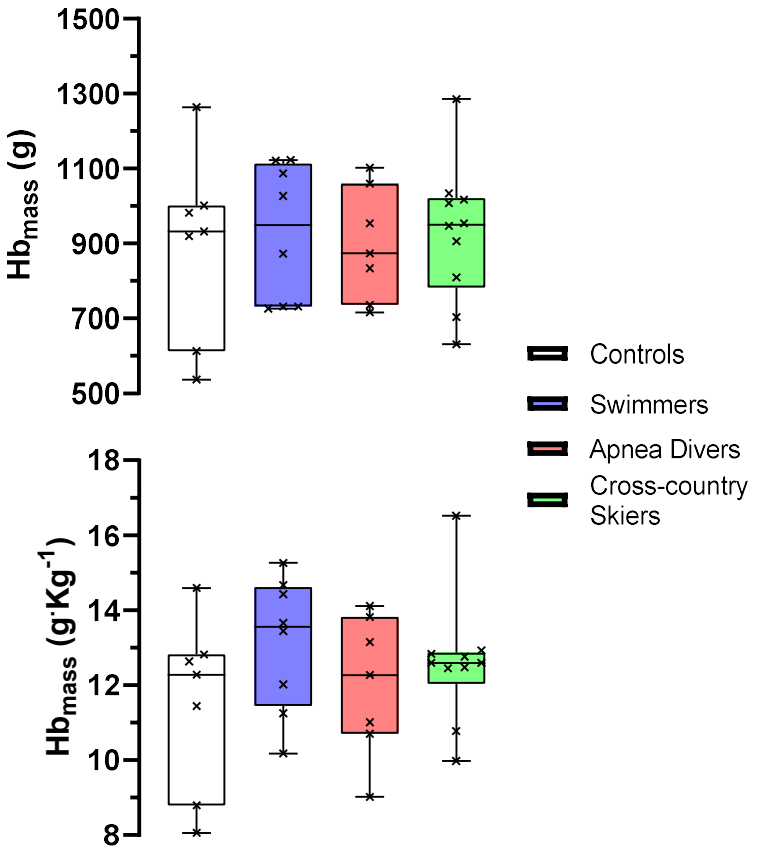

Figure 4.2: Absolute and relative Hbmass values for each group.

Table 4.3: Intravascular volumes for each group.

Variable	Controls	Swimmers	Apnoea Divers	Cross – Country Skiers
RBCV (L)	2.7 ± 0.7*	2.7 ± 0.7*	2.7 ± 0.5*	3.9 ± 0.5
BV (L)	6.2 ± 1.1	6.1 ± 1.1	6.5 ± 1.0	6.7 ± 1.0
PV (L)	3.4 ± 0.4	3.5 ± 0.6	3.8 ± 0.6*	2.9 ± 0.6
Hct (%)	43.5 ± 4.7	43.4 ± 5.4	42.2 ± 2.8	42.2 ± 2.4

Note: * denotes significantly different to cross-country skier data (p <.05)

4.3.3 Hypoxic Ventilatory Response (HVR)

Mean ± SD data for the change in SpO_2, HR, and VE as well as the resultant HCR and HVR values for each gas mixture are displayed in Figure 4.3. Two-way mixed model ANOVA results showed no significant effect of hypoxic gas (p = .873) or gas*group interaction (p = .202) in HVR data. One-way ANOVA data showed differences in the magnitude of SpO_2 desaturation between groups at 17% (p = .004), 14% (p = .001) and 11% O_2 (p = .040). Post-hoc analysis showed at 17% O_2, cross-country skiers had a

significantly larger desaturation than controls ($p = .027$) and swimmers ($p = .004$). At 14 %, apnoea divers and cross-country skiers had significantly higher degrees of desaturation than controls (vs divers $p = .007$, vs skiers $p = .039$) and swimmers (vs divers $p = .008$, vs skiers $p = .049$). At 11% O_2, despite a significant main effect, there was no significance between groups in the post-hoc analysis. There was no statistical difference in HR change (17% $p = .803$; 14% $p = .767$; 11% $p = .547$), V_E change (17% $p = .231$; 14% $p = .767$; 11% $p = .392$), HVR (17% $p = .351$; 14% $p = .533$; 11% $p = .447$), and HCR (17% $p = .341$; 14% $p = .203$; 11% $p = .053$) in any of the gas mixtures. Spearman's correlation analysis showed strong positive correlations between the chemosensitivity responses to 14% and 11% O_2 hypoxic gases in swimmers ($r = .812$; $p = .026$) and cross-country skiers ($r = .805$; $p = .029$). In all other gases for all groups no other correlations were found between response values ($p > .05$).

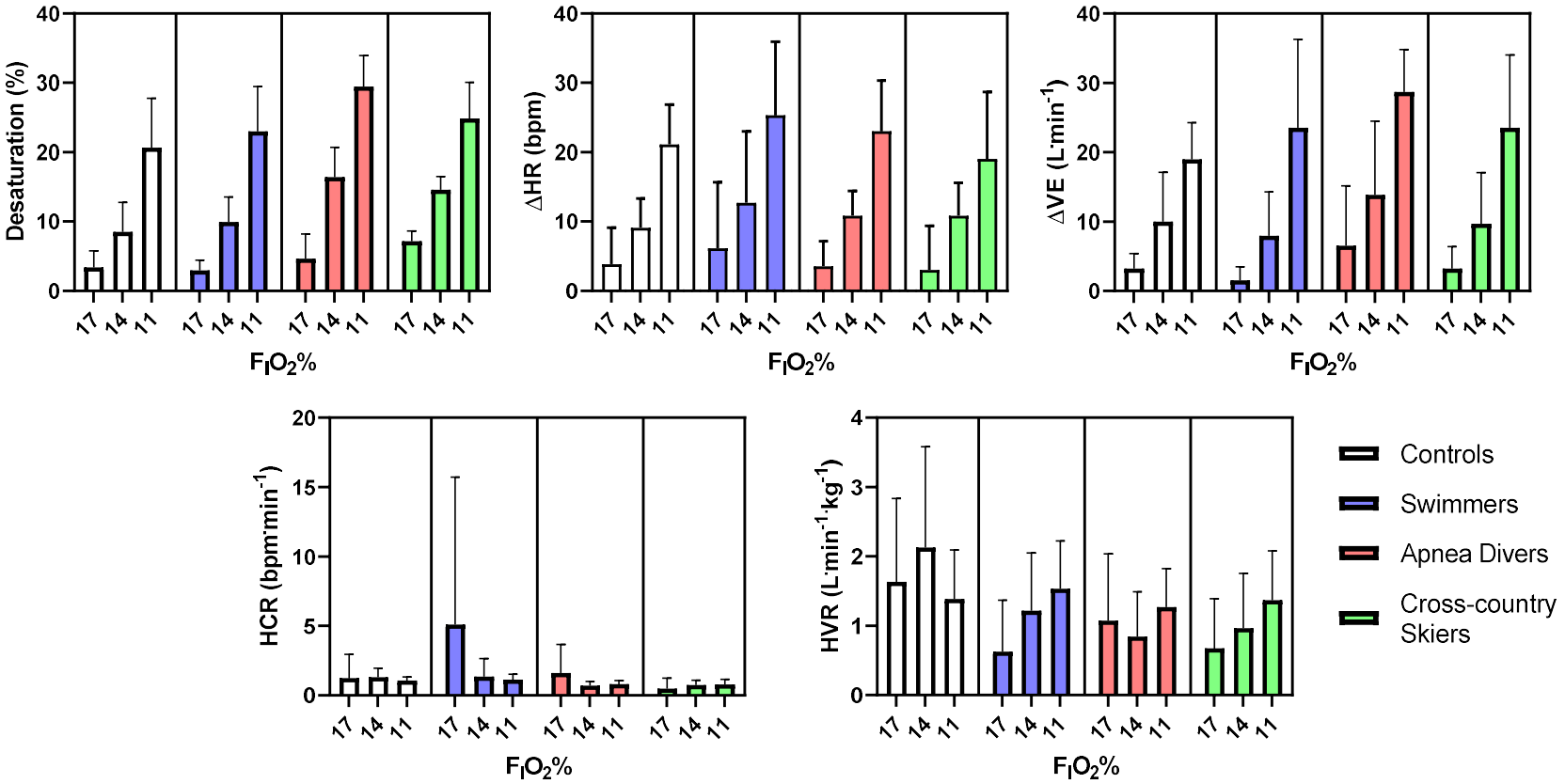

Figure 4.3: Outcome variables from the HVR testing for each group and gas mixture. Note: HR = Heart Rate, VE = Ventilatory Equivalents, HCR = Hypoxic Cardiac Response, HVR = Hypoxic Ventilatory Response, F_IO_2% = Fraction of Inspired Oxygen.

4.3.4 Hypercapnic Ventilatory Response (HCVR)

The results of the one-way ANOVA analysis indicate that there were no significant differences in the $PETCO_2$ break point for each group ($p = .188$). No significant differences were present between the groups' HCVR ($p = .619$). Mean and individual data for HCVR and the CO_2 breakpoint are displayed in the box and whisker plot in Figure 4.4.

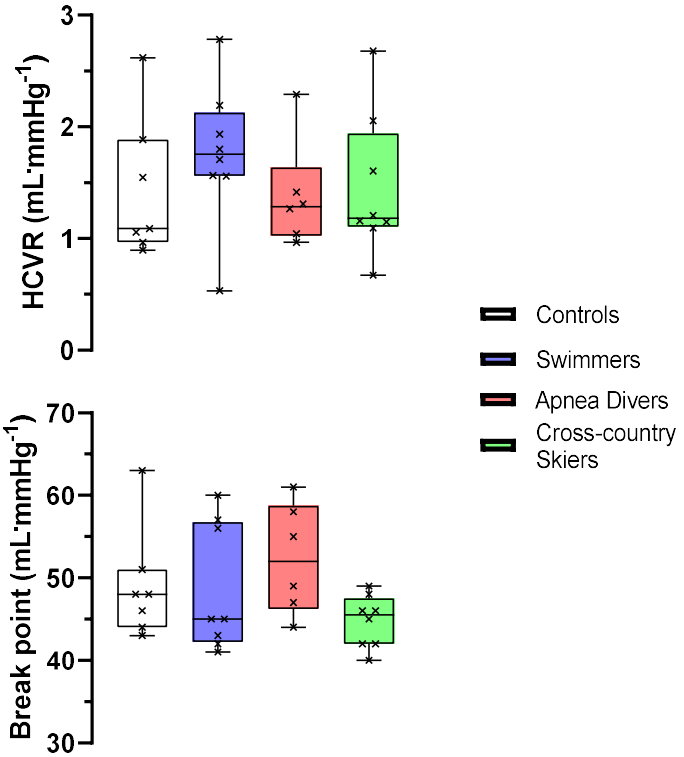

Figure 4.4: HCVR and CO_2 breakpoint for each group.

4.4 Discussion

The primary aim of this study was to compare markers of hypoxic acclimatisation, namely ventilatory and haematological variables in swimmers and other athletic and non-athletic groups. The main finding from the data in the present study was that despite differences in VO_2 max between all athletic groups and the control group, no differences were found in the haemoglobin mass or chemosensitivity to hypoxia and hypercapnia. RBC was significantly higher in cross-country skiers than in all other groups. PV was also significantly higher in apnoea divers compared to cross-country skiers.

4.4.1 Comparison of VO_2 max Across Groups

The results of the present study indicate that while the absolute VO_2max did not differ significantly across groups ($p = .070$), the relative VO_2max was significantly lower in control participants compared to swimmers ($p = .002$), apnoea divers ($p = .013$), and cross-country skiers ($p < .001$), as shown in Figure 4.1. This suggests that, when considering

body mass, the capacity to transport and utilize oxygen is lower in control participants compared to the athletic groups. This finding is consistent with previous studies that have shown higher relative VO_2 max in athletes compared to non-athletes (Sotiridis et al., 2020; Sotiridis et al., 2018), and reflects typical VO_2 max values seen in the groups evaluated (Buttar et al., 2022; Fernández et al., 2017; Holmer, 1972; Lavoie & Montpetit, 1986). It is well established that VO_2 max can be increased through endurance exercise, hypoxic exposure, or a combination of both. Therefore, it is unsurprising that the relative $VO_{2\,max}$ values in athletic groups are higher than that of control subjects.

The power output at VO_2 max was statistically indifferent between the four groups. This suggests that the maximum power output achieved at VO_2max does not differ significantly between controls, swimmers, apnoea divers, and cross-country skiers. This finding is somewhat surprising, as one might expect that the power output at VO_2max would be higher in athletes, however, cycling was the chosen exercise mode due to being equal familiarity among participants. If this test were performed in a swimming flume or on a cross-country skiing treadmill, it would be expected that athletes familiar with those sports would have a greater power/velocity at VO_2 max (Kenney et al., 2015). Despite no differences in absolute power at VO_2 max, when accounting for body mass, athletes from sports with greater training load (swimmers and cross-country skiers) had a higher relative power at VO_2 max.

4.4.2 Comparison of Hbmass Across Groups

Despite the significant statistical difference in the relative VO_2max between the control participants and those in athletic groups, no such statistical significance was found in relative Hbmass data. This result is not in line with the majority of literature in this area that suggests athletes who participate in endurance sports training should have an elevated Hbmass compared to non-endurance-trained athletes (Heinicke et al., 2001; Kjellberg et al., 1949; Ulrich et al., 2011). There are numerous factors that might have led to this result. For instance, the physiological attributes of the participants, including the training history of the control group or genetic predispositions, might have played a role in determining their Hbmass levels.

Saunders et al. (2013), reported a strong relationship between absolute Hbmass and VO_2 max ($r = .75$) in elite endurance athletes. This association was also reported in various studies involving a range of training modalities and fitness levels (Heinicke et al., 2001; Schmidt & Prommer, 2008; Schmidt & Prommer, 2010). In the present study, the relationship between absolute Hbmass (g) and VO_2max in all participants combined was considered strong ($r = .73$, $p < .001$), although when each group is analysed individually the relationship is stronger in athletes than in the control population (controls $r = .72$, swimmers $r = .92$, divers $r = .92$, and skiers $r = .94$). The slope for all participants combined was ~ 4.3 $mL \cdot min^{-1}$ when then slope was constrained through the origin which fits with other data of this type. It is unlikely that the standard of athletes in the sporting groups is the reason for this lack of difference, as the athletes selected for the present study are of a national and in some cases international standard. A possible cause of the lack of difference between groups may be the units used to measure Hbmass. Goodrich et al. (2020) found that in both athletic and nonathletic groups Hbmass was more closely related to lean body mass than total body mass (total mass $R^2 = .69$, lean mass $R^2 = .90$). Lean body mass is a better determinant of energy expenditure due to its higher metabolic activity than fat mass (Cunningham, 1991; Gersh & Still 1945). Increases in Hbmass are speculated to be due to increases in lean body mass and therefore increased overall O_2 demand. Comparing the same groups using lean body mass as a relative measure may have provided

greater insight into oxygen-carrying capacity differences as there were no statistical differences in the participants' mass.

4.4.3 Comparison of Chemosensitivity Tests Across Groups

Involuntary breathing control is regulated by the respiratory centres in the medulla and pons of the brain stem. Centres of respiration regulate the rate and depth of breathing based on signals from the central and peripheral chemoreceptors, proprioceptors, and stretch receptors (Hudson et al., 2011). Peripheral chemoreceptors, such as the carotid bodies, primarily detect reductions in the oxygen pressure in the blood. Feedback from peripheral chemoreceptors is sent to the cardiorespiratory centres, which in turn increases V_E (Foresman et al., 2000). The degree of sensitivity to hypoxia can therefore be deemed by the increase in ventilation per decrease in the O_2 saturation of blood. In the present study, there was no difference between any of the groups' chemosensitivity to decreases in SpO_2 to any of the 3 tested gases, despite significant differences in the degree of blood oxygen desaturation (Figure 4.3). As shown in the results of cross-country skiers in the present study, endurance normoxic exercise appears to have no impact on HVR. Research in this area appears to support this claim (Levine et al., 1992; Townsend et al., 2002). Literature on the combined effects of intermittent hypoxia and exercise found that HVR is either unchanged or increases mildly (Katayama et al., 1999; Levine et al., 1992) found that individuals who performed an intermittent hypoxic endurance training intervention had unchanged hypoxic ventilatory responses post-intervention. Chronic hypoxic exposure and acclimatisation are suggested to be required to induce changes in hypoxic ventilatory response (Weil et al., 1971). Previous research shows a variety of HVR magnitudes compared to controls in apnoea divers (Costalat et al., 2014; Grassi et al., 1994; Masuda et al., 1981). One potential discrepancy that may affect these data is the discipline that they compete and train for. Divers who train for depth-based competitions may not experience hypoxia when they dive due to the increase in hydrostatic pressure at depth. This pressure increase means that these divers experience hyperoxic hypercapnia for a major portion of their dives (Ferretti, 2001). Weil (2003) concluded after a literature review of relevant research that there is a strong influence of genetics on HVR, considerably more than on HCVR, suggesting the link is based on the hereditary physiological structure of peripheral chemosensitivity. Family members of endurance runners had near identical HVR magnitude to the athletes independent of the relatives' sex, age and degree of physical conditioning (Scoggin et al., 1978). As the familial history of participants was not collected in the present study, it is not possible to know the potential impact of hereditary chemoreceptor traits.

Central chemoreceptors provide signals for respiratory control for responses to changes in blood CO_2. The present study found no differences in HCVR or CO_2 break point between all 4 groups. All the values presented fit within previous values for non-athletes and athletes (Mann et al., 2022; McGurk et al., 1995). HCVR in previous research is significantly blunted in endurance-based athletes compared to untrained controls (Byrne-Quinn et al., 1971; Miyamura et al., 1976; Scoggin et al., 1978). Saunders et al. (1976) noted a low HCVR in 1 swimmer who later achieved international-level competition in endurance front crawl events, the authors declared that blunted HCVR potentially could be an important characteristic of high-level endurance athletic performance. In the present study, when observing individual HCVR responses (Figure 4.3), despite similar ranges, swimmers' individual data is mainly distributed on the higher end of the spectrum compared to the other groups. The individual with the highest HCVR response in this group and overall (2.78 $mL{\cdot}mmHg^{-1}$) was objectively the most successful athlete out of all tested, having competed in multiple World and European level competitions, specialising in 200m events.

Sprint swimmers and athletes, in general, are typically reported as having higher HCVR than athletes of longer disciplines in their sport (Ohkuwa et al., 1980; Rebuck & Read, 1971). In the present study, all of the 8 tested competed and trained for distances of 400 metres and shorter, with most specialising in events 200 metres or less.

The results of the present study therefore indicate that the swimmers and apnoea divers selected did not exhibit a blunting of their chemoreceptive responses to hypoxia or hypercapnia. This suggests that the breath-holding periods undertaken during their training may not have provided sufficient stimuli to induce significant changes in their chemosensitivity. Despite previous research suggesting a relationship between exercise and hypoxic and hypercapnic ventilatory responses, our findings do not support this association in the context of the athletes studied. This unexpected result raises questions about the specific conditions under which exercise and breath-holding may impact chemoreceptive responses and suggests a need for further research to better understand the underlying mechanisms.

4.4.4 Limitations

The present study's testing phase was from September 2021 to September 2022. In early 2020, the world was struck by an influx of cases of the virus SARS-COV-2, leading to widespread lockdowns and affecting the training regimes of many athletes, including the participants in this study. Additionally, the study faced several other limitations. First, the number of participants was limited (7 or 8 per group), which may result in the study being underpowered. Second, the timing of the testing relative to the participants' training schedules varied and may influence the results. Lastly, no measure of lean body mass was included in the study, lean body mass is a significant determinant of both oxygen consumption and haemoglobin mass. Therefore, not including a measure of lean body mass may affect the interpretation of the VO_2 max and Hbmass results. These limitations should be considered when interpreting the findings of this study and may affect the generalizability of the results.

Chapter 5

Seasonal Variation in Competitive Swimmers' Haematology, Ventilatory, and Performance Parameters

Foreword

The previous chapter (4) compared markers (haematological and ventilatory) of hypoxic adaptation between a control group and three athletic groups (swimmers, apnoea divers, and cross-country skiers). One potential flaw with that study is that the swimmer and control groups were assessed from October to November 2021. As the swimming season begins in September, most swimmers have undergone only 4 to 6 weeks of training. To assess the effect of training status and periodisation, the full battery of testing protocols previously described was repeated on a select number of individual swimmers and control participants on two further occasions (January to February & May to June 2022). Therefore, the following chapter will discuss the results of the repeated assessment of these swimmers and control participants over the course of a swimming season.

The findings to this chapter are currently in preparation for publication, under the working title "Breath-Holding in Swimming: Investigating Hypoxic Acclimatization and Physiological Responses Through Comparative and Seasonal Analysis" authored by Joshua T. Royal, Tinkara Mlinar, Jason T. Fisher, Jernej Kapus, Adam C. McDonnell and Igor B. Mekjavic.

5.1 Introduction

Periodisation plays a crucial role in optimising athletic performance by strategically manipulating training variables throughout a season or training cycle, ensuring athletes peak at the right moments and prevent overtraining (Issurin, 2010; Smith, 2003). There are multiple models for periodisation in athletes (traditional, block, reverse linear), the most commonly used in swimming is the traditional pyramidal model (Hermosilla et al., 2021), first introduced by Matveyev to ensure USSR athletes were achieving their peak performances of the season during the summer Olympic games (Krüger, 2016). This model reduces training volume throughout each macrocycle, with the first macrocycle containing 80% of the total training volume and the remaining macrocycles containing the other 20% (Seiler, 2010).

The swimming season for most athletes is typically divided into two to three macrocycles, which is the longest measure of a training cycle and typically ranges from several months to a year. Each containing around 3 mesocycles (several weeks to months), which in turn contain several microcycles (typically a week of training). This structuring of training allows coaches and athletes to manipulate training variables, such as volume, frequency, and intensity for performance and fatigue management (Issurin, 2010). As swimmers navigate the periodisation phases, they experience targeted adaptations aligned with particular performance goals. These include cardiovascular (Aspenes & Karlsen, 2012), metabolic (Mujika et al., 1995), respiratory (Päivinen et al., 2021), musculoskeletal (Toussaint & Vervoorn, 1990), haematological (González-Ravé et al., 2021), and psychological (Hooper et al., 1998) adaptations. The first macrocycle of the season has the goal of improving athletes' aerobic endurance through swimming longer distances at an intensity below that of their lactate threshold. In the second macrocycle, training is based on developing athletes' speed, power, and anaerobic capacity. This is often achieved by higher intensity swimming sets, comprised of sprints or near maximal efforts at the swimmers' event distance or less. The third macrocycle is typically only included in swimmers schedule if they are focused on tapering for competitions in the summer months (Hermosilla et al., 2021). Tapering phase duration can vary depending on the experience level and preferences of the swimmer and their competition schedules, typically however at a duration between one to three weeks. Training comprises a reduced overall training volume, while training intensity is maintained or even increased (Hellard et al., 2017). The goal of this phase is to allow the athlete to recover from the accumulated fatigue from the previous macrocycle and adapt to the training stimulus, resulting in improvements in performance (Costill et al., 1991).

Intentional hypoventilation, characterized by breath-holding or reduced breathing frequency, has been widely employed by competitive swimmers to minimise frontal drag for swimmers that compete in freestyle events that are 200 metres and less in distance (McCabe et al., 2015; Seifert et al., 2005). In the training programmes of these athletes, the prevalent use of intentional hypoventilation techniques, including short apnoeic episodes or reduced breathing, is well-documented, primarily focusing on their short-term benefits (Woorons et al., 2014; Woorons et al., 2016). Despite this, information on the understanding of its long-term effects on swimmers' haematological and ventilatory parameters remains limited. If there is sufficient hypoxemic stimulus from repeated periods of hypoventilation to cause adaptation to tissue hypoxia then it would be plausible that this degree of adaptation would change during each macrocycle. In the first macrocycle, there is typically a far larger volume of training therefore could be argued that this block would induce the highest hypoxic stimulus, however, the majority of training is performed at an aerobic intensity and does not require voluntary hypoventilation. In the second and

third macrocycles, the intensity of training increases to a point where hypoventilation occurs regularly in training in preparation for freestyle sprint events, however, the overall training volume is far less. It is therefore important to measure these potential changes across the swimming season to check their longitudinal variation.

The previous chapter investigated the ventilatory and haematological parameters of swimmers against other athletes and a control population. However, these tests were conducted on swimmers and control participants from October to November 2021, within 1 to 2 months of the start of the swimming season. At this point in the season, swimmers should not have gained the adaptive effects from their training stimulus. To better assess swimmers' relationship between training volume, fitness level and the measured values, assessment across the entire season, throughout periodisation is preferable.

Therefore, the primary objective of this study was to investigate the seasonal variations in haematological and ventilatory parameters among competitive swimmers as they progress through different training macrocycles. Due to the nature of the testing and at the request of the coaches, test timing was scheduled so it did not interfere with any of the major swimming competitions during the year. These would be compared to those of control subjects over the same period to ascertain differences in typical biological variation and impacts from the training phase. By elucidating the relationships between training periodisation and physiological adaptations, the findings of this study are expected to enhance our current understanding of the role of periodised training in shaping the physiological responses of swimmers and optimising their performance potential.

5.2 Methods

5.2.1 Ethical Approval

Subjects' written informed consent was obtained prior to the study, and they were informed that they were free to withdraw their consent at any time. The procedures were approved by the Committee for Medical Ethics at the Ministry of Health (Republic of Slovenia; approval number: 0120-401/2020/8) and conformed to the standards set by the Declaration of Helsinki, except for registration in a database.

5.2.2 Study Design

A total of 13 swimmers and 8 control participants were originally recruited for the 9-month-long study. As the participants recruited in the present study were all partaking in either high school or university level education, throughout the year, there was a number of participants that dropped out of the study due to their academic responsibilities. Therefore, only a selection of swimmers ($n = 4$) and control participants ($n = 5$) completed the full set of haematological (Hbmass) and ventilatory tests (HVR, HCVR, VO_2 max), described previously in Chapter 4.2.2, on 3 occasions over the course of a competitive swimming season (October/November 2021, January/February 2022, and April/May 2022). Throughout the season, training was administered for the swimmers by the coaches of their teams. 3 of the 4 swimmers followed the same seasonal race timeline: August – Off Season, December – Winter Nationals (Short Course), and July – Summer Nationals (Long Course). The final swimmer had an alternate competition schedule due to competing in European and World level competitions at the beginning of the season (November), therefore this swimmer had not taken the typical break between seasons.

5.2.3 Statistical Analysis

Due to the low participant numbers in these analyses, no cohort comparison analysis was performed to prevent the presentation of conclusions based on results with inadequate sample sizing.

Data are presented as Mean ± SD unless otherwise indicated. All statistical tests were performed unblinded to experimental conditions with SPSS (v25, IBM, New York, USA). GraphPad Prism (v8.4.2, GraphPad Software, San Diego, USA) was used for data visualisation. The significance level on all tests was set as $p < .05$, a priori. Pearson's correlation analysis was used in order to identify potential relationships between changes in variables.

5.3 Results

A total of 9 participants completed the present study, evenly divided into two primary groups: swimmers (n=4) and controls (n=5). Each group contained a single female participant. Participant anthropometric details are described in Table 5.1 per group.

Table 5.1: Age and body mass data for control and swimmer participants.

		Controls (n = 5)	**Swimmers (n = 4)**
Height (cm)		181.9 ± 8.8	182.9 ± 2.2
Age (yrs)		22 ± 2.3	20.3 ± 3.3
Body Mass (Kg)	**Oct/Nov**	72.5 ± 10.7	72.3 ± 11.1
	Jan/Feb	74.0 ± 11.7	74.5 ± 12.9
	May/Jun	74.2 ± 12.0	71.9 ± 12.0

All swimmers' data displayed a reduction in VO_2max values from Oct/Nov to Jan/Feb (-9.5 ± 4.0%), whereas control participants' data displayed decreases of a lesser degree and in two cases unchanged values (-5.9 ± 7.7%). From Jan/Feb to May/Jun, the variability in VO_2max values was greater for both groups than in the previous period (controls = 0.8 ± 8.3%, swimmers = 2.3 ± 8.1%). In some individuals, VO_2max values increased, whereas in others they remained unchanged, or decreased.

In terms of Hbmass, controls' and swimmers' values displayed a mean decrease from Oct/Nov to Jan/Feb (controls = -2.8 ± 5.0%, swimmers = -6.3 ± 3.5%), and from Jan/Feb to May/Jun (controls = -1.4 ± 5.8%, swimmers = -1.0 ± 3.4%). Pearson's correlation analysis identified a strong positive relationship between the change in relative VO_2max and the change in relative Hbmass in swimmers ($r = .78$, $p = .020$). However, the same was not found in control participants where there was no relationship ($r = .07$, $p = .838$).

Changes in VO_2max ($r = -.78$, $p = .021$) and Hbmass ($r = -.75$, $p = .031$) were significantly correlated with changes in body mass in swimmers. In controls, changes in body mass were only correlated with changes in VO_2max ($r = -.69$, $p = .027$) and not with Hbmass ($r = .33$, $p = .346$). Individual data for relative Hbmass and VO_2max, HCVR, and HVR are presented in Figure 5.1.

The results derived from chemosensitivity tests revealed significant intra- and inter-group variations as represented in Figure 5.1. For the Oct/Nov testing phase, HVR at 11% O_2 for controls ranged between 0.663 to 2.418 and for swimmers, it ranged between 1.491 to 5.320. The corresponding HCVR values for controls were between 0.897 to 2.619, while for swimmers, it varied from 1.530 to 4.610. The subsequent Jan/Feb test results showed that the HVR at 11% O_2 for controls ranged from 0.790 to 2.511, and for swimmers, it spanned from 0.954 to 4.185. The respective HCVR values for controls were between 0.662 to 3.930, and for swimmers, they ranged from 0.964 to 2.233. By the final testing period in May/June, controls' HVR values at 11% O_2 were between 0.834 to 2.854, whereas those for swimmers ranged from 0.847 to 5.198. The HCVR values for controls during this period were between 0.702 to 1.715, and for swimmers, they spanned from 0.642 to 3.895. A notable observation from the data was the heightened variability in swimmers' responses across the season as opposed to the relatively consistent data from the control subjects.

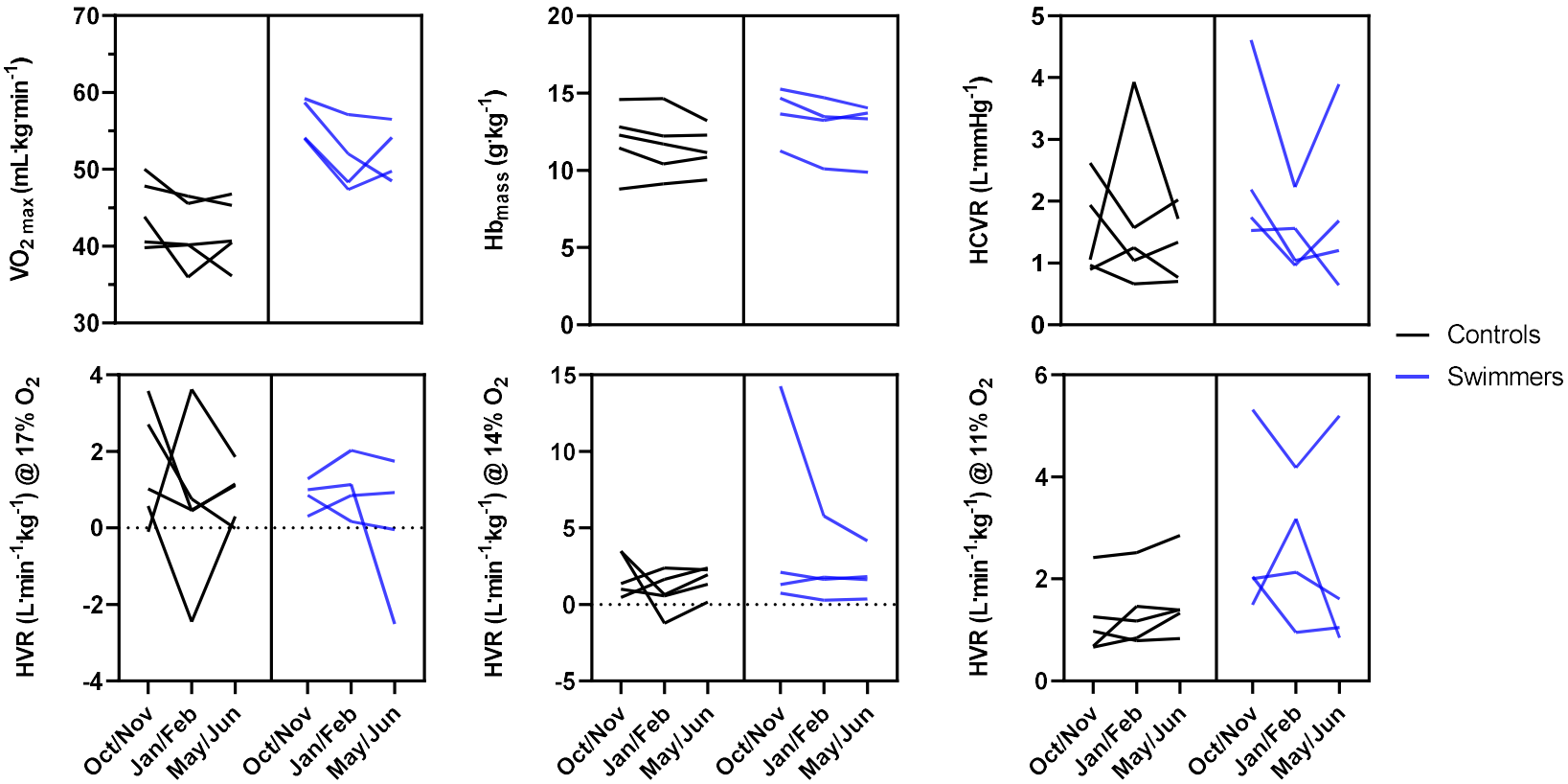

Figure 5.1: Individual values from the physiological tests performed by participants.

5.4 Discussion

The present study's aim was to investigate the longitudinal variations in haematological, ventilatory, and cardiovascular values among competitive swimmers across a training season and compare them to control subjects. The study findings would suggest trends in the physiological parameters of both the control participants and swimmers are similar over this period. Therefore, changes in training volume, intensity, and frequency over the course of a season have no effect on the values measured in the few individuals tested in the present study.

5.4.1 Longitudinal Variations in VO_2 max

The selected swimmers demonstrated a larger mean reduction in VO_2 max values from Oct/Nov to Jan/Feb (Figure 5.1), compared to control participants, who showed a smaller reduction, albeit with a larger variation. Two of the control subjects had unchanged VO_2max values over this period. The greater reduction in swimmers' VO_2max values could possibly be attributed to the intense training schedules that swimmers adhere to during the first macrocycle. Increased training load and intensity could have led to transient decreases in VO_2max, as athletes might be in a phase of overreaching or short-term overtraining (Hedelin et al., 2000). Biological factors such as nutritional status (Buzina et al., 1982) or sleep quality (Castelli et al., 2022), which were not controlled for or measured in this study, could also contribute to the changes observed in VO_2max. From Jan/Feb to May/June, both swimmers and control groups demonstrated variability in VO_2max responses, including increases, decreases, or no changes. This could potentially be related to individual differences in training adaptations or variations in training loads when tapering for their respective events at the end of the season. However, the underlying mechanisms are complex and may need further investigation.

5.4.2 Longitudinal Variations in Haematological Values

Regarding Hbmass, both control participants and swimmers showed a mean decrease from Oct/Nov to Jan/Feb and from Jan/Feb to May/Jun. Interestingly, there was a strong positive relationship between changes in VO_2max and changes in Hbmass in swimmers, a correlation that was not observed in the control group. This suggests that the swimmers' VO_2max and their Hbmass are coupled, possibly reflecting the athletes' adaptation to their training regimes (Saunders et al., 2013). Eastwood et al. (2009) compared a group of adolescent cyclists against a group of adolescent controls over 12 months and found that there was no correlation in the change of VO_2max and change in Hbmass, despite a significant correlation in both groups' PRE VO_2max with PRE Hbmass, and POST VO_2max with POST Hbmass. The authors, therefore, suggested that any improvements in VO_2 max in the cyclists were due to other factors that influence VO_2 max than improvements in O_2 carrying capacity of the blood. Previous literature also suggests that Hbmass values do not change greatly during training interventions in already highly trained athletes (Glass et al., 1969; Gore et al., 1997; Saunders et al., 2013). Recent research has also indicated a lack of difference in Hbmass in swimmers of different ability levels or swimming event distances (Mujika et al., 2023).

Furthermore, the changes in VO_2max and Hbmass in the present study were significantly correlated with changes in body mass in swimmers. This underlines the interconnectedness of these parameters and raises intriguing questions about the role body mass plays in influencing these adaptations. In contrast, in controls, changes in body mass

correlated only with changes in VO_2max, and not with Hbmass. While the mean body mass values remained consistent, several participants experienced substantial changes in body mass - as large as >7% of their initial (Oct/Nov) body mass. This suggests a high degree of individual variability that warrants further investigation. Meleski and Malina (1985) observed changes in the body composition of 15 collegiate-level swimmers over the course of a swimming season (October – December – March). Body mass significantly decreased in the initial 10 weeks of training, succeeded by a significant increase in the following 14 weeks. In this same period, fat mass significantly decreased as lean body mass significantly increased. Decreases in fat mass have also been seen over the course of a swimming season in former research (Wade, 1976). Skeletal muscle mass is positively correlated with swimming performance in male and female elite international swimmers, whereas fat mass is only negatively correlated in female athletes (Dopsaj et al., 2020).

5.4.3 Longitudinal Variations in Chemosensitivity

The results from the chemosensitivity tests show considerable variation both within and between the groups (Figure 5.1). For both controls and swimmers, the HVR and HCVR values displayed notable fluctuations across the season. The HVR values at 17% fluctuated to a similar amount in both groups. In retrospect, a HVR test at 17% O_2 was an unreliable variable to track longitudinally, due to there being such a low change in oxygen saturation measured using pulse oximetry (SpO_2). The manner in which the determinations for HVR are calculated relies upon using changes in SpO_2 and changes in V_E. As the desaturation was consistently between 3 to 6%, this meant that even minor changes in V_E had extreme effects on the eventual HVR value. HVR to 14% O_2 in controls and swimmers remained consistent across the season other than in one swimmer. It is unknown as to the exact reason why this individual exhibited such a large ventilatory response (+15 $L \cdot min^{-1}$) despite such a small degree of desaturation (-1.9 %). Possibly it was due to a lack of familiarity with the testing protocol as this was the participant's first visit to the laboratory to perform this test, and due to the randomised design of the HVR protocol, the first hypoxic gas mixture they experienced. What is apparent from the data is that the variability in swimmers' responses over the course of the season was larger than that of the control subjects which remained relatively consistent. These data again indicate significant individual variation and potentially the effects of individualised training on chemosensitivity. Hiruta et al. (1990) found no significant differences in hypercapnic ventilatory responses in 10 males across a year of testing.

These substantial changes in HVR at 11% O_2 values and HCVR values highlight the potential for individual physiological adaptations in response to hypoxic stimuli, which may be of particular relevance in training programme design and periodisation. These findings, in conjunction with the significant correlation between changes in body mass, VO_2max, and Hbmass, suggest that individual variation and seasonal changes should be taken into account in swimmer training programmes (Mujika et al., 2018). Moreover, the wide range of chemosensitivity responses observed in our study supports the need for further research into the physiological underpinnings of these responses and their relationship to performance outcomes in swimming.

5.4.4 Limitations

These findings need to be understood in light of their inherent limitations. For example, while our sample was representative of a diverse range of swimming disciplines, the ideal study participants would have been those specializing in front crawl for distances where breath-holding is beneficial (<200 m). Unfortunately, the number of athlete participants,

who competed at a national and international level, and were an appropriate age was low. Therefore, swimmers who specialised in other events were included. In this study, only one out of four swimmers specialized in 50m front crawl events, while two specialized in breaststroke events, and one in backstroke events. This participant selection is not ideal as it is common for swimmers to undergo specialized training tailored to their preferred stroke and event distance. Consequently, the swimmers in this study may not have been exposed to the same level of hypoxic stimulus as a group exclusively composed of front crawl sprinters would have been. This discrepancy is a limitation that could potentially influence the interpretation of the results and should be considered when comparing this study to others that focus solely on front-crawl sprinters. This mismatch between ideal and actual participants might have influenced the results. Another limitation of this study is that the exact training regimes of athletes or controls were unknown. The researchers understood the general periodisation of the swimmers from communication with the swimmers and their coaches however were not privy to the exact details of each athlete's training load. Future research should strive to recruit participants that align more closely with the study's primary focus to mitigate potential confounding influences and maintain a more accurate record of those athletes' training. Moreover, future studies could also explore the impact of other potential confounding factors such as nutritional status, sleep quality, and mental health, which were not controlled for in the present study but could play a significant role in the physiological adaptations observed.

Chapter 6

Voluntary Hypoventilation in Swimming

We would be remiss to discuss a hypoxic stimulus in swimming exercise without mentioning the series of work from Woorons and colleagues (2018; 2018; 2010; 2017; 2021; 2011; 2010; 2019; 2014; 2021; 2019; 2007, 2008; 2017; 2016). Where our research is based on monitoring swimmers to assess whether a sufficient hypoxic stimulus exists in their regular training, the work of Woorons and colleagues centres around inducing a hypoxic stimulus during training through sprint hypoventilation interventions. These interventions follow a specific breathing pattern called "end-expiratory breath-holding" (EEBH), this breathing technique entails breath-holding after exhalation to near the functional reserve capacity of the lungs and then exhaling again to clear the remaining air prior to inhalation (Figure 6.1). With a pulse oximeter sensor attached to the forehead of a swimmer, using EEBH, performing ten consecutive 50-meter front crawl efforts at 95% of 400-metre pace, oxygen saturation measured using pulse oximetry (SpO_2) was significantly lower at lower pulmonary volumes as opposed to both high volume breath-holds and spontaneous breathing (Trincat et al., 2017; Woorons et al., 2014; Woorons et al., 2016). This method was further validated with other modes of exercise such as running (Fornasier-Santos et al., 2018; Woorons et al., 2021; Woorons et al., 2019; Woorons et al., 2008) and cycling (Woorons et al., 2010; Woorons et al., 2021; Woorons et al., 2019; Woorons et al., 2007; Woorons et al., 2017).

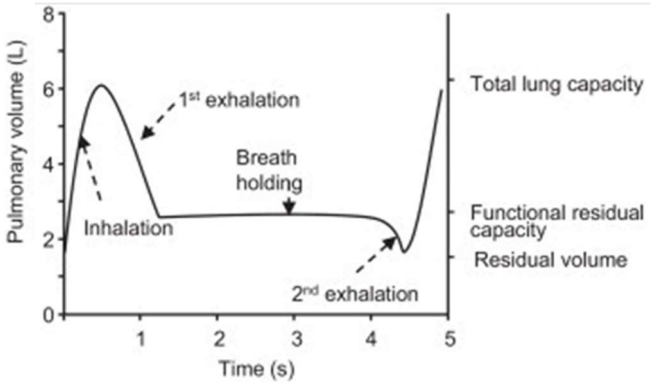

Figure 6.1: Description of voluntary hypoventilation at a low pulmonary volume.

This breathing method during sprint exercise has shown acute effects, mainly on sprint performance (Fornasier-Santos et al., 2018) and repeated sprint ability (Trincat et al., 2017) compared to groups performing the same sprinting interventions without breath-

holding. His research shows that swimmers who performed voluntary hypoventilation exercises for six weeks improved their 400-metre freestyle time by 2.3 seconds compared to the control group (Woorons et al., 2007). Another study found that swimmers who practiced voluntary hypoventilation for eight weeks had increased anaerobic capacity and improved their 200-metre freestyle time by 1.4 seconds, compared to the control group (Woorons et al., 2013). Their research has also concentrated on discovering the physiological mechanisms that underlie the effects of voluntary hypoventilation on athletic performance. They observed that the increase in blood CO2 concentration during voluntary hypoventilation stimulates red blood cell production. It was also observed that voluntary hypoventilation can increase the buffering capacity of the blood, which helps to prevent the build-up of lactic acid in the muscles (Woorons et al., 2011; Woorons et al., 2013).

Despite these results, no study yet exists investigating the long-term implications of this type of additional training intervention on haematological or chemosensitivity measures. As these interventions are typically less than 20 minutes in total length, with less than half of this time spent at a SpO_2 lower than 90% (Trincat et al., 2017), it is suspected that much like in the present study, the hypoxic stimulus may not be sufficient to undergo physiological adaptation.

In summary, the work of Woorons and colleagues in the field of voluntary hypoventilation in swimming has shown the potential to induce a hypoxic stimulus during training through sprint hypoventilation interventions using the EEBH method. While acute effects on sprint performance and repeated sprint ability have been observed, further research is needed to investigate the long-term implications of this type of training intervention on physiological adaptation.

Chapter 7

Conclusions

Through a series of studies, this thesis evaluated the concept of potential hypoxic adaptation in swimmers. Firstly, by assessing variability in individuals' responses to hypoxia and inactivity to gain greater insight into hypoxic adaptation beyond that of the group (Study I, Chapter 2). From this study, it was decided that an alternate method was required other than just a measurement of haemoglobin concentration and haematocrit, due to the potential influence of plasma volume changes. Thus, a haemoglobin mass protocol was implemented, and the validity and reliability of our laboratory in conducting these methods were assessed in Study II (Chapter 3). Once this method had been employed, the assessment of swimmers for markers of hypoxic acclimation could take place (Study III, Chapter 4). Of the swimmers and control participants selected, some returned to the laboratory on two further occasions throughout the swimming season to assess the influence of the training period (Study IV, Chapter 5). The main findings of these three studies are summarised below:

7.1 Study I

Typically, the results of a group of individuals' responses to a stimulus or intervention are reported as means and standard deviations. The current spike of interest in individual variation noted in recent years demonstrates that reporting only the group's mean and standard deviation may mask the true range of individual responses, which potentially leads researchers to draw inappropriate inferences. Acknowledgement of this variability is essential to optimize personal future medical and physiological interventions. The current investigation (Study I) of the haematological responses of inactive males and females exposed to the same magnitude and duration of a hypoxic stimulus, demonstrated the substantial heterogeneity in the cascade of responses from arterial O_2 saturation to RBC production. The individual variability in the EPO response to NBR and HBR in females appears to be considerably larger than in males, and the intervention duration appeared to have no impact on the heterogeneity of the haematological responses. Our findings suggest that relative EPO responses are not sufficient indicators of the resultant increased production of Rcts and RBCs. The data would suggest that the majority of the variability seen in HBR is due to mechanisms responding to hypoxia rather than severe immobilisation and inactivity. The significance of the current data is the identification and acknowledgement of large individual variability within the mechanistic response to hypoxia, thus creating justification to further investigate the sources and moderating factors of such variability.

7.2 Study II

The calculated measures of absolute and relative Hbmass, and intravascular volumes (Study II), are considered valid when determined using capillary-drawn blood due to the statistical indifference and high correlation with values calculated from venous-drawn blood. Reliability analysis showed ICC and change of mean values are deemed acceptable for capillary blood use. Higher TE% indicates a certain level of caution should be applied when using capillary sampling analysed in the singular for research or medical purposes. Significant differences between seated and supine (V0, V1, and V2) blood values indicate the importance of a 20-minute supine rest with legs raised before the collection of the PRE sample.

7.3 Study III

The ventilatory and haematological markers of hypoxic acclimatisation tested in swimmers compared against controls, apnoea divers, and cross-country skiers (Study III) indicate that reduced breathing frequency in combination with exercise does not create a strong enough hypoxic stimulus to initiate the acclimation. Longitudinal analyses (Study IV) in the measured variables between controls and swimmers also showed no large changes in individuals' changes or between the groups. The present results do not allow us to state that the changes in haematological and ventilatory parameters are from anything besides the typical effects of aerobic training programming. This potentially may be due to one of several reasons: I) The amount of time during a training session where an athlete is intentionally hypoventilating for drag reduction is too small, II) The amount of time in a day swimmers spend in training sessions may not be enough to invoke adaptation, III) The original assessment of hypoxic stimulus from the measurement from the hand during swimming could have been inaccurate due to motion artefacts, IV) The athletes selected for the present study (not all front crawl sprint swimmers), did not experience such a stimulus due to their specialisations in other strokes causing their training to not be front crawl centric.

7.4 Future Research

Future research should investigate the effects of voluntary hypoventilation interventions, such as the work of Woorons (Chapter 6), on hypoxic adaptation markers. The pilot testing of pulse oximeters in the introduction (1.3.2) highlighted the need for clarification on the hypoxic stimulus experienced commonly in a swimmer's training schedule. Based on the findings in Chapter 1, due to the difficulty to obtain adequate fingertip SpO_2 data during swimming, it is proposed that in future research, a vasodilator ointment should be used prior to sampling to increase the chance of sampling. Vasodilator ointments are commonplace when taking blood samples from the earlobe to increase blood flow and therefore may have a desirable effect on pulse oximetry practices.

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Appendix A

Ethics Approval

REPUBLIKA SLOVENIJA
MINISTRSTVO ZA ZDRAVJE
Komisija Republike Slovenije za medicinsko etiko

Štefanova ulica 5, 1000 Ljubljana

T: 01 478 69 06, 01 478 69 20
F: 01 251 77 55
E: gp.mz@gov.si, kme.mz@gov.si
www.mz.gov.si

mag. Joshua Toby Royal
Institut Jožef Stefan
Odsek za avtomatiko, biokibernetiko in robotiko
Jamova cesta 39
1000 Ljubljana

Številka: 0120-401/2020/8
Datum: 24. 3. 2021

Zadeva: **Ocena etičnosti predložene raziskave**
Zveza: vaša vloga z dne 2. 9. 2020 in dopolnjena vloga z dne 22. 2. 2021

Komisija Republike Slovenije za medicinsko etiko (KME RS) je 2. 9. 2020 prejela vlogo za oceno etičnosti raziskave z naslovom »Hipoksija pri vodnih športih«.

Raziskovalno delo se bo izvajalo v okviru raziskovalnega programa Heat Shield št. PR-06793.
Cilj pričujoče raziskave je oceniti stopnjo hipoksičnega dražljaja v izbranih vodnih športih ter ugotoviti, ali je dražljaj dovolj močan, da izzove določene hipoksične prilagoditve na fiziološki ravni.

KME RS je na seji 20. oktobra 2020[1] obravnavala prejeto vlogo in ugotovila, da vloga ni popolna zato vas je v pozivu k dopolnitvi vloge št. 0120-592/2020/4 z dne 25. 11. 2020 pozvala, da metode meritev, protokol, informacije za prostovoljce in pojasnilo raziskave k udeležbi posameznika v raziskavi prevedete v slovenski jezik. V pozivu vas je tudi opozorila, da v vlogi ni zapisano, kako boste pridobili prostovoljce.

22. 2. 2021 je KME RS prejela dopolnitev vloge.

KME RS je na videokonferenčni seji 16. marca 2021[2] obravnavala dopolnjeno vlogo in ugotovila, da je vloga sedaj popolna ter ocenila, da je raziskava etično sprejemljiva. S tem vam za njeno izvedbo izdaja svoje soglasje.

[1] Seznam članov KME RS , ki so odločali o vlogi, in izjava, da KME RS deluje v skladu z zadevnimi zakoni in priporočili, sta na voljo na spletni strani MZ (zavihek "O Ministrstvu – Komisija Republike Slovenije za medicinsko etiko", rubrika "Seje Komisije").
[2] Seznam članov KME RS , ki so odločali o vlogi, in izjava, da KME RS deluje v skladu z zadevnimi zakoni in priporočili, sta na voljo na spletni strani MZ (zavihek "O Ministrstvu – Komisija Republike Slovenije za medicinsko etiko", rubrika "Seje Komisije").

Bibliography

Publications Related to the Thesis

This thesis is based in the following papers:

Thesis Publications

Royal, J.T., Eiken, O., Keramidas, M.E., McDonnell, A.C. and Mekjavic, I.B., 2021. Heterogeneity of Hematological Response to Hypoxia and Short-Term or Medium-Term Bed Rest. *Frontiers in Physiology*, 12.

Royal, J.T., Mlinar, T., Fisher, J.T., Mekjavic, I.B. and McDonnell, A.C., 2022. Validity and reliability of capillary blood vs venous blood for the assessment of haemoglobin mass and intravascular volumes. *Frontiers in Physiology*, 13.

Royal, J.T., Kapus J., Mlinar, T., Fisher, J.T., McDonnell, A.C., Mekjavic, I.B. 2024. Breath-Holding in Swimming: Investigating Hypoxic Acclimatization and Physiological Responses Through Comparative and Seasonal Analysis *In preparation.*

Royal, J.T., Kapus J., McDonnell, A.C. and Mekjavic, I.B. In-ear oximetry for aquatic sports. *In preparation.*

Other Publications

Lomax, M., **Royal, J.T.**, Kapus, J., Massey, H. and Saynor, Z., 2022. Oxygen uptake kinetics and ventilatory and metabolic parameters do not differ between moderate-intensity front crawl and breaststroke swimming. *Physiological Reports*, 10(12).

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Biography

Joshua Royal is a PhD candidate at the Jožef Stefan International Postgraduate School and a member of the Environmental Physiology and Ergonomics Laboratory at the Jožef Stefan Institute. His research focuses on cardiovascular and haematological adaptation to hypoxia, with particular relevance to adaptation during exercise. His supervisor for his doctoral dissertation was Professor Igor B. Mekjavic (co-supervisors: Dr. Adam McDonnell and Associate Professor Jernej Kapus).

In 2017, Josh completed his BSc (Hons) in Sport and Exercise Science at the University of Portsmouth. The title of his undergraduate dissertation was: "Characterising the Pulmonary Oxygen Uptake Kinetic Responses in Swimming Above and Below Critical Velocity" mentored by Dr. Mitch Lomax, Dr. Zoe Saynor, and Dr. Heather Massey.

In 2018, he completed his MSc in Sports Performance, also at the University of Portsmouth. The title of his masters' dissertation was: "Pulmonary Oxygen Uptake Kinetic Responses During Relative Moderate Intensity Front Crawl and Breaststroke Swimming" completed under the supervision of Dr. Mitch Lomax, Dr. Zoe Saynor, and Dr. Heather Massey.