HYDROSTATIC AND THERMAL INFLUENCES ON INTRAVASCULAR VOLUME DETERMINATION DURING IMMERSION: QUANTIFICATION OF THE F-CELL RATIO

Submitted by

Christopher James Gordon, B.N.

A thesis submitted in total fulfilment of the requirement for the award of the degree

Master of Exercise Science

from

School of Exercise Science
Faculty of Health Sciences

Australian Catholic University
Research Services
Locked Bag 4115
Fitzroy, Victoria 3065
Australia

2001

This thesis contains no material published elsewhere or extracted in whole or in part from a thesis by which I have qualified for or been awarded another degree or diploma.

No other person's work has been used without due acknowledgment in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

All research procedures reported in the thesis received the approval of the relevant Ethics/Safety Committees (where required).

_____ Date: _____

Christopher James Gordon

HYDROSTATIC AND THERMAL INFLUENCES ON INTRAVASCULAR VOLUME DETERMINATION DURING IMMERSION: QUANTIFICATION OF THE F-CELL RATIO

Abstract

Previous data have shown that the most prevalent, indirect plasma volume (PV) measurement technique, which utilises changes in haematocrit (Hct) and haemoglobin concentration ([Hb]), underestimates actual PV changes during immersion, when compared to a direct tracer-dilution method. An increase in the F-cell ratio (whole-body haematocrit (Hct_w) to large-vessel haematocrit (Hct_v) ratio) has been purported as a possible explanation, probably due to hydrostatic and thermally-mediated changes during water immersion. Previous investigators have not quantified the F-cell ratio during immersion. Therefore, this study sought to determine the effect of the F-cell ratio on the indirect method during both, thermoneutral and cold-water immersions.

Seven healthy males were tested three times, seated upright in air (control: 21.2°C SD ±1.1), and during thermoneutral (34.5°C SD ±0.2) and cold-water immersion (18.6°C SD ±0.2), immersed to the third intercostal space for 60 min. Measurements during the immersion tests included PV (Evans blue dye column elution, Evans blue dye computer programme, and Hct [Hb]), red cell volume (RCV; sodium radiochromate), cardiac frequency (f_c) and rectal temperature (T_{re}).

Plasma volume during the control trial remained stable, and equivalent across the three tests. There was a hydrostatically-induced increase in PV during thermoneutral immersion, when determined by the Evans blue dye method (16.2%). However, the Hct/[Hb] calculation did not adequately reflect this change, and underestimated the relative PV change by 43%. In contrast, PV decreased during cold immersion when determined using the Evans blue dye method by 17.9% and the Hct/[Hb] calculation by 8.0%, respectively, representing a 52% underestimation by the latter method. There was a non-significant decline in RCV during both immersions. Furthermore, an increase (8.6%) and decrease (-14.4%) in blood volume (BV) was observed during thermoneutral and cold-water immersions, respectively. The decline in RCV during thermoneutral immersion attenuated the BV expansion. Despite the disparity between the PV methods, there was no increase in the F-cell ratio during either immersion. In contrast, there was a significant decline in the F-cell ratio during the control: air and thermoneutral immersion, which may indicate that other, undefined variables may impact on the stability of the red cell compartment.

The current study is the first to show that the Hct/[Hb] method clearly underestimates PV changes during both thermoneutral and cold-water immersion. Furthermore, RCV was shown, for the first time, to decline during both immersions. However, the changes in the F-cell ratio during this study, did not account for the underestimation of PV change using the Hct/[Hb] method.

Acknowledgements:

I would like to thank Dr Jodie Stocks for her patience, commitment, and foremost her unswerving encouragement. Without her expert guidance this project would not have been completed, and to this I owe her enormous gratitude. I would also like to thank Dr. Nigel Taylor for his superb supervision during the project and his excellent advice during the preparation of this manuscript. A special mention must also go to Dr. John Greenleaf, who provided expert information and unsurpassed opinion during various stages of the project.

I must also thank Karen and Annerieke for their valuable contributions to parts of this thesis. The person who I would like to thank the most on both a professional and personal level is Alison, for her enormous contribution to this project and her reluctance but resolute steadfastness in taking blood from an obstinate subject whom will remain nameless. In addition, I would like to thank my family for all their love and support throughout these years.

Lastly, I would like to thank the subjects who participated in this project, for their commitment to science and their invaluable contribution to this research.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

CHAPTER ONE: INTRODUCTION AND HYPOTHESES

1. INTRODUCTION

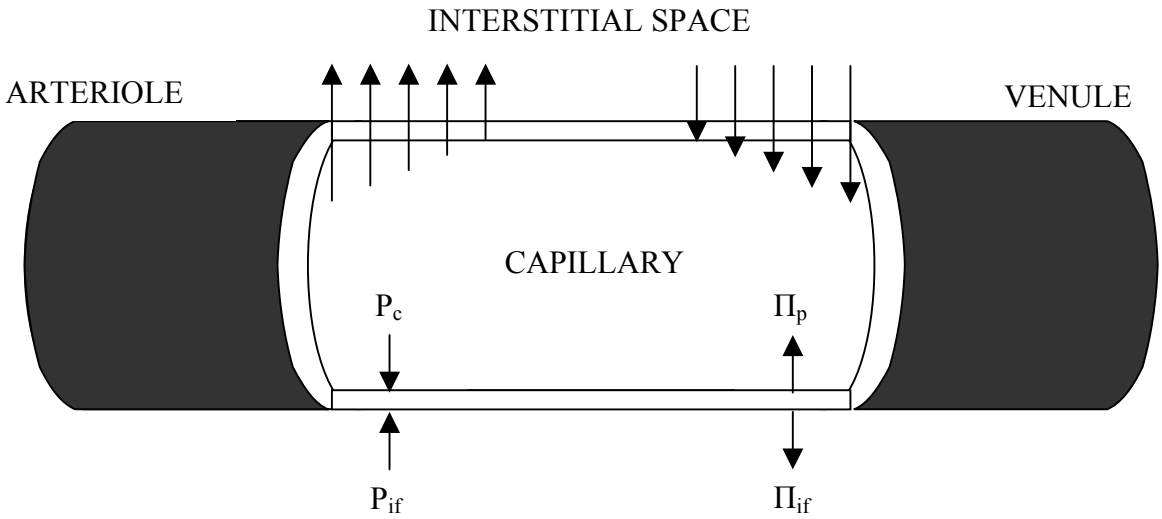
The intravascular volume, or blood volume (BV), accounts for approximately 8% of total body mass (Edelman and Leibman, 1959). This compartment is involved in a number of vital functions throughout the body, including electrolyte equilibration, haemostasis, nutrient and gas transportation, removal of metabolic waste and thermoregulation. The BV is comprised of extracellular (plasma volume) and intracellular (fluid in the erythrocytes) components, which approximate 70 $ml \cdot kg^{-1}$ and 65 $ml \cdot kg^{-1}$ in adult males and females, respectively (International Committee for Standardisation in Haematology, 1980). Of this volume, normal values for the red cell volume (RCV) are usually 30 $ml \cdot kg^{-1}$ and 25 $ml \cdot kg^{-1}$ for adult males and females, respectively, whereas the plasma volume (PV) accounts for 40 $ml \cdot kg^{-1}$ in both adult males and females (International Commission on Radiological Protection, 1975; International Committee for Standardisation in Haematology, 1980). The BV is a dynamic quantity, and depends on the equilibrium of fluid exchange between the vascular and the interstitial compartments (Gauer and Henry, 1963). This project investigates the separate thermal and hydrostatic factors upon human BV during water immersion, and how these factors influence the measurement of PV and RCV.

The regulation of the vascular volume is achieved by a number of integrated mechanisms which integrate to maintain vascular homeostasis. These include renal, neural, and humoral regulation, and microvascular fluid exchange. The renal control of BV occurs through the regulation of body sodium and water. Sodium is the principal ion of the extracellular fluid (PV and interstitial volumes), which chiefly determines extracellular fluid osmolality, and hence the volume of extracellular fluid. The kidney regulates extracellular fluid volume through the filtration and reabsorption of water and sodium. For instance, an increase in the plasma sodium concentration will increase the volume of water reabsorbed, and assist maintenance of normal BV. Conversely, an increase in extracellular fluid volume will cause the kidneys to excrete more water (Hainsworth and Drinkhill, 1995). Furthermore, the kidney regulates arterial pressure through a local renal haemodynamic response; for instance, with a reduction in arterial pressure, the outflow of sodium and water from the circulation is diminished, and the urine output is reduced. Consequently, BV is enhanced and arterial pressure is restored (Guyton, 1961).

Additionally, substances such as vasopressin, renin-angiotension and atrial natriuretic peptide interact with the kidneys to regulate BV through negative feedback mechanisms. Vasopressin is formed in the hypothalamus and secreted by the neurohypophysis in response to changes in plasma osmolality, blood volume and pressure, whereby a decrease in BV stimulates vasopressin release and water reabsorption is increased (Berne and Levy, 1990). The renin-angiotension system responds to changes in renal arterial pressure and elevates blood pressure through arteriole vasoconstriction, water and sodium reabsorption. In contrast, atrial natriuretic peptide is released in response to atrial stretch, such as an increase in intrathoracic BV, and produces diuresis and natriuresis by the kidney and systemic vasodilatation (Stadaeger *et al.,* 1992). Several of these hormonal interactions have been shown to be modified by both thermoneutral and cold immersion (Greenleaf *et al.,* 1980; Greenleaf *et al.,* 1981), thus modifying fluid volume within the vascular compartment.

As well as the renal and humoral systems, short-term regulation of BV is mainly initiated by the neural system. Activation of volume and baroreceptors increases fluid excretion in response to an increase in blood pressure, produced by the increase in vascular volume. Similarly, a decrease in the vascular volume and blood pressure will initiate an uptake of fluid (Guyton, 1961). However, the stability of the BV is largely dependent on the microcirculation, and the combination of capillary and oncotic pressures across the capillary membrane in maintaining homeostasis (Starling, 1896; Landis and Pappenheimer, 1972).

At the microvascular level a dynamic equilibrium between the PV and interstitial space regulates BV continuously, with a two-way flux of fluid across the capillary wall (Starling, 1896; Gauer *et al.,* 1970; Michel, 1984). As blood enters the capillary, the hydrostatic pressure of the plasma, at the arterial end of the capillary, is greater than the oncotic pressure of the plasma, causing filtration of fluid from the plasma to the interstitial space. Accordingly, hydrostatic pressure of the plasma must be greater than the interstitial fluid pressure for plasma to flow out of the capillary. As blood flows through the capillary towards the venule, the hydrostatic pressure is reduced, and oncotic forces favour a reabsorption of fluid from the interstitium to the plasma (Figure 1.1). At rest, these forces are usually balanced, and the outflow of fluid equates with that which is reabsorbed, therefore maintaining normal BV (Levick, 1995). These factors regulating microvascular fluid shifts are commonly referred to as Starling forces, after the original investigator (Starling, 1896), and were confirmed experimentally by Landis (1927). Accordingly, the forces can be expressed in



Fluid movement = $k [(P_c - \Pi_p) - (P_{if} + \Pi_{if})]$

where: P_c = capillary hydrostatic pressure;

P_{if} = interstitial fluid pressure;

Π_p = plasma oncotic pressure;

Π_{if} = interstitial fluid oncotic pressure;

k = capillary filtration constant.

Figure 1.1: Schematic of the Starling forces acting across the capillary wall. The arrows represent the approximate magnitude and direction of fluid shift within the capillary, as blood flows from the arteriole to the venule. Abbreviations: P_c = capillary hydrostatic pressure; P_{if} = interstitial fluid pressure; Π_p = plasma oncotic pressure; Π_{if} = interstitial fluid oncotic pressure. (Adapted from Landis and Pappenheimer, 1972; Berne and Levy, 1990; Levick, 1995).

mathematical terms to determine the direction and magnitude of fluid movement (Landis and Pappenheimer, 1972). Thus, an increase in the interstitial fluid pressure, or a decrease in the capillary hydrostatic pressure, will negate the outflow of fluid from the capillary. This will result in an increase in intravascular fluid, due to the reabsorption of fluid. Conversely, a decrease in plasma oncotic pressure, or an increase in capillary hydrostatic pressure, will induce a local hypovolaemia, due to the excess outflow of fluid. It is the effect of immersion on these Starling forces which is the primary focus of this project, with particular interest upon whole-body and venous ratios of red cell concentrations relative to the PV.

The cellular content of the intravascular volume is controlled independently of the PV. The volume of erythrocytes is closely regulated by a feedback control mechanism, which governs the circulating hormone, erythropoietin (Ganong, 1979; Guyton, 1991). Briefly, conditions such as hypoxia, and local vascular changes that decrease oxygen transportation to the tissues increase the rate of erythrocyte production. Erythropoietin is inhibited when the circulating RCV becomes concentrated and there is greater risk of reduced blood flow .

This study focuses upon the determination of the intravascular volume, the difference in the methods used to make these measurements, and how environmental factors which affect these Starling forces may interact with these methods. In general terms, one may classify such measurement techniques as either direct or indirect methods. In the former case, an unknown volume can be determined by comparing the volume and concentration of a known marker (radioactive, thermal tracer or dye) before, and after, instillation into the unknown volume. However, the known marker must be uniformly distributed, and solely confined to the unknown volume to accurately determine that volume. In reality, the marker rarely stays within the target fluid compartment, and some loss occurs through diffusion, chemical combination or metabolic alteration, which is usually accounted for by a correction factor (Baker, 1963). Furthermore, the marker must not influence the volume measured, or be toxic to the individual (Chien and Gregersen, 1962). However, it is not always practical to undertake such direct measurements, and some indirect procedures have been developed (Strauss *et al.,* 1951; van Beaumont, 1972).

All indirect procedures are based on the key assumption that, when it is not possible or too difficult to actually measure changes in the target variable, one can very closely approximate modifications in one variable from observations of one, or more, related

variables, which can be more easily quantified. For such procedures to be valid and precise, it is assumed that the relationship between the target variable and the measured variable remains constant and proportional across the conditions of measurement. With respect to the vascular fluid compartment, one can measure changes in the Hct and [Hb] to approximate changes in the PV. These measures are based upon the assumptions that the volume of the individual erythrocytes (mean corpuscular volume) and the total RCV remain constant between experimental conditions (Harrison, 1985). While such indirect measures are more easily performed, they frequently suffer from limitations, such as the inability to detect absolute volume, to discriminate between PV and RCV directly, and to account for loss of fluid from the intravascular compartment. Nevertheless, and with a clear understanding of these limitations, such methods may be used to provide valuable insights into PV change before and after various interventions, such as exercise (Hayes *et al.,* 2000), heat acclimation (Sawka *et al.,* 1988) and water immersion (Greenleaf *et al.,* 1981). In this project, the focus is upon BV measurement, and how environment factors may modify the validity of indirect PV measures.

There are a variety of BV measurement techniques that have arisen, primarily from a clinical perspective, due to the need for accurate diagnostic determination. The only PV determination methods available to early researchers were direct tracer-dilution methods, such as Evans blue dye (Gregersen *et al.,* 1935). The advent of radioisotopes enabled total BV measurement, with the simultaneous determination of PV and RCV (Hahn *et al*., 1942; Gibson *et al.,* 1946). However, indirect methods of BV and PV determination, such as those utilising changes in haematocrit (Hct) and haemoglobin concentration ([Hb]; Strauss *et al.,* 1951) have been favoured by many researchers (Dill and Costill, 1974; Hagan *et al.,* 1978; Greenleaf *et al.,* 1979; Gaebelein and Senay, 1980; Ertl *et al.,* 1991). With this technique, the changes in BV and PV are derived from relative changes in Hct and [Hb] (*i.e.* Hct/[Hb]). The Hct/[Hb] calculation has been extensively applied, due to the ease of the measurement technique, and the fact that relative BV and PV change are assessed, rather than an absolute determination, which is often more pertinent to the experimental condition (Harrison, 1985). However, various environmental factors have been shown to interact with the PV, RCV and hence BV, thus affecting both the data and its interpretation.

Intravascular volume alterations have been demonstrated during environmental and physiological stress, such as altitude (Pugh, 1964), exercise (Green *et al.,* 1991), postural change (Maw, 1994) and heat acclimation (Patterson, 1999). These intravascular alterations

are brought about by changes in the magnitude and direction of the hydrostatic and oncotic pressures at the capillary. During upright-seated water immersion, the magnitude of the BV, and concomitant cardiovascular changes, are greater than those assumed after horizontal or head-down bed rest in air (Greenleaf, 1984). Moreover, the magnitude, and time course, of these physiological responses are proportional to the depth of immersion (Lange *et al.,* 1974; Risch *et al.,* 1978b).

There are two main mechanisms that occur during short-term (<2hr) thermoneutral-water (34.5ºC) immersion. There is an increase in the intrathoracic blood volume, as blood is shunted from the peripheral vasculature and into to the thorax (Arborelius *et al.,* 1972; Echt *et al.,* 1974; Risch *et al.,* 1978a). There is also an increase in PV due to increased tissue pressure from the external hydrostatic water pressure (McCally, 1964; Khosla and DuBois, 1979; Greenleaf *et al.,* 1983; Miki *et al.,* 1989; Regan, 1998). Therefore, the external hydrostatic pressure of the water during immersion evokes BV changes during a thermoneutral immersion. However, water immersion has been used to investigate the effects of thermal strain, as heat conduction in water is 25 times greater than air (Toner and McArdle, 1996). Thus, cold water, relative to air at the same temperature, can impose a significant thermal stress on the body that requires substantial thermoregulatory responses (Craig and Dvorak, 1966; Regan, 1998). These responses are associated with a generalised cutaneous veno- and vasoconstriction (Arborelius *et al.,* 1972), which further increase the central BV and markedly modify the Starling forces. Accordingly, there are differences in the magnitude and direction of PV changes during cold-water immersion, despite methodological factors remaining consistent with thermoneutral studies (Knight *et al.,* 1986; Young *et al.,* 1987). Hence, the upright body position in water immersion at different water temperatures provides an appropriate environment to assess the hydrostatic and thermal influences on intravascular volume change.

The current project relates exclusively to human water immersion, but more particularly to BV changes accompanying cold-water immersion. To date, the vast majority of studies have concentrated on examining the thermoneutral environment (Khosla and DuBois, 1979; Greenleaf *et al.* 1983), while there is a small volume of literature in relation to BV changes accompanying cold-water immersion (Rochelle and Horvath, 1978; Young *et al.,* 1987; Deuster *et al.,* 1989). Generally, thermoneutral immersion elicits a relative hypervolaemia (McCally, 1964; Knight *et al.,* 1986), due primarily to hydrostatic water

pressure, whereas cold-water exposure decreases PV (Rochelle and Horvath, 1978; Young *et al.,* 1987; Deuster *et al.,* 1989). However, there is a lack of data regarding the differentiation between the hydrostatic and thermal influences of cold-water immersion. Furthermore, the most prevalent measurement of BV has been determined indirectly, and thus the accuracy of these measures have not been compared to direct tracer methods.

Despite broad agreement on the PV change during thermoneutral and cold-water immersion, discrepancies remain over the magnitude of the shift. The majority of these studies utilised the Hct/[Hb] calculation for computation of the PV and BV (McCally, 1964; Khosla and DuBois, 1979; Knight *et al.,* 1986; Deuster *et al.,* 1989). However, previous investigators have suggested the indirect calculation remains accurate only if the RCV and the F-cell ratio, the ratio between whole body haematocrit (Hct_w) and large-vessel haematocrit (Hct_v), where the Hct_w is derived from the PV and RCV, remain constant (Harrison *et al.*, 1982; Greenleaf and Hinghofer-Szalkay, 1985; Harrison, 1985). To date, no attempt has been made to quantify the RCV and the F-cell ratio during water immersion at any temperature. Therefore, the impact of the RCV and F-cell ratio will be determined during this study, to examine the affect on the indirect calculation of PV change during thermoneutral and cold-water immersion.

It is clear that discrepancies and uncertainties regarding the PV response to immersion are present in the literature. The literature primarily points to alterations in the F-cell ratio as the explanation, but this remains speculative. Furthermore, to our knowledge, the RCV has never been quantified with a direct tracer-dilution technique during water immersion in humans. The simultaneous determination of PV and RCV directly with tracer-dilution measurements eliminates some of the possible errors associated with the Hct/[Hb] calculation. The indirect PV measurement has been compared with Evans blue dye dilution methods and found to underestimate PV in thermoneutral water (Greenleaf *et al.,* 1983; Johansen *et al.,* 1992; Johansen *et al.,* 1995). Therefore, data are needed to determine the influence of the F-cell ratio on PV changes in both thermoneutral and cold-water environments. This can be adequately achieved by measuring both the PV and RCV concurrently with direct tracer-dilution methods.

1.1 AIMS AND HYPOTHESES

It was the purpose of this study to determine the PV, RCV and hence BV, using direct-tracer dilution methods, and to compare changes observed during control (air), thermoneutral and cold-water immersions. Furthermore, the change in PV during these conditions was also determined using an indirect method. Thereafter, the F-cell ratio was quantified during control (air), thermoneutral and cold-water immersions, from the measurement of peripheral Hct and the whole-body Hct, determined from the direct measurement of RCV and BV. The influence of the F-cell ratio on the different methods was assessed.

It was hypothesised that:

1.1.1 During short-term thermoneutral-water immersion, the plasma volume, measured using a direct tracer-dilution method would increase due to the external hydrostatic pressure.

1.1.2 During short-term thermoneutral-water immersion, the plasma volume change, measured using an indirect method, would increase due to the external hydrostatic pressure, however the magnitude of increase would be less than observed via the direct method.

1.1.3 During short-term cold-water immersion, the plasma volume, measured using a direct tracer-dilution method, would decrease primarily due to the combination of external hydrostatic pressure and the thermal stress of the cold water.

1.1.4 During short-term cold-water immersion, the plasma volume change, measured using an indirect method, would decrease due to the combination of external hydrostatic pressure and the thermal stress of the cold water, however the magnitude of decrease would be less than observed via the direct method.

1.1.5 During short-term thermoneutral-water immersion, the F-cell ratio would increase, due to the compression of the peripheral tissues by the external water pressure, thereby producing a relatively larger decrease in the peripheral vessel haematocrit, compared to that of the whole-body haematocrit.

1.1.6 During short-term cold-water immersion, the F-cell ratio increase would be larger than that during thermoneutral immersion, due to combined effects of external water pressure and thermal stress that arises during cold immersion.

CHAPTER TWO: LITERATURE REVIEW

2. INTRODUCTION

The regulation of body-fluid distribution occurs through the interaction of several forces that attempt to maintain homeostasis. However, alterations in the magnitude of the hydrostatic and oncotic pressures induced by conditions such as environmental temperature, exercise, posture change, and water immersion can change the fluid equilibrium and increase or decrease blood volume (BV). Water immersion is known to alter intravascular volume, primarily through the interaction of hydrostatic pressure and thermal stress. Generally, plasma volume (PV) increases during thermoneutral-water immersion, and decreases during cold-water immersion (Khosla and DuBois, 1979; Greenleaf *et al.,* 1983; Young *et al.,* 1987; Deuster *et al.,* 1989). However, discrepancies have been demonstrated in the determination of PV using an indirect method that measures haematocrit (Hct) and haemoglobin concentration ([Hb]) change (Johansen *et al.,* 1992). Alterations in the ratio of whole-body to peripheral Hct (F-cell ratio) have been implicated as a potential source of error when calculating PV using the Hct/[Hb] method (Harrison *et al.,* 1982; Young *et al.,* 1987; Johansen *et al.,* 1992), yet direct measurement of the ratio has not occurred during water immersion. Therefore, this project sought to measure BV during thermoneutral- and cold-water immersions, and to quantify PV change using both direct and indirect methods, and hence, determine the influence the F-cell ratio has on the indirect BV measurements.

The effectiveness of method-based investigations is dependent on the reliability and validity of the techniques utilised. The validity of a measurement designates that the test actually measures the target variable, and gives reliable results that are consistent and reproducible (MacDougall and Wenger, 1982). Accordingly, a method-based investigation of the intravascular volume requires the independent determination of PV and red cell volume (RCV), rather than calculating either variable from measurements made on other variables. However, RCV has been calculated from the measurement of PV (Muldowney, 1957; Greenleaf *et al.,* 1979; Convertino *et al.,* 1980; Jain *et al.,* 1980). This indirect method can introduce significant error, and contradict the assumptions of validity inherent in a method-based investigation. Accordingly, this study will simultaneously determine PV, using different methods and RCV. This facilitates comparison of these different methods to possibly explain why differences occur in the indirect determination of intravascular fluid volumes during various environmental exposures.

2.1 Body-fluid compartments

The total volume of water in the body accounts for approximately 50% to 70% of the total adult body mass (Sheung and Huggins, 1979). Water is distributed throughout the body, and can be divided into two main fluid compartment volumes: the intracellular and extracellular. Of the total body water, approximately 55% is distributed within the intracellular space and 45% is contained in the extracellular space (Figure 2.1). The intracellular space encompasses the RCV, which accounts for 5% of total body water, while the volume of water in the extracellular space is divided between the interstitial (38%) and plasma volumes (PV: 7%). Therefore, the intravascular volume is distributed between the intracellular and extracellular compartments and contains approximately 12% of the total body water (International Commission on Radiological Protection, 1975; International Committee for Standardisation in Haematology, 1980). The intravascular space continually interacts with the interstitium moving fluid into and out of the vasculature, and is regulated by forces that attempt to maintain these volumes within the different compartments (Gauer and Henry, 1963).

The movement of fluid between the compartments is regulated by a combination of hydrostatic, oncotic, and osmotic forces interacting on the cellular and vascular membranes (Starling, 1896; Gauer *et al.,* 1970): the Starling forces. The intracellular and extracellular fluids are demarcated by the cell membrane which, despite being freely permeable to water, is regulated by the osmotic forces exerted by the intracellular and extracellular ions. The cell membrane is not readily permeable to ions and, as such, the volume of fluid within the cell is regulated by osmosis. For instance, a cell that has a higher ionic concentration compared with the surrounding fluid, will permit the movement of fluid into the cell, thus maintaining equilibrium with the extracellular fluid. However, the capillary wall is freely permeable to ions, yet plasma proteins are not. Thus, the boundary between the intravascular and interstitial compartments is influenced by the combination of hydrostatic and oncotic forces on either side of the capillary wall (Landis and Pappenheimer, 1972).

Fluid movement across a capillary wall is dependent on the balance between Starling forces on either side of the vascular wall. Capillary hydrostatic pressure usually exceeds the interstitial fluid pressure at the arteriole end, thus shifting fluid out of the capillary into the interstitium. As blood courses the capillary, hydrostatic pressure falls, and the oncotic pressure of the plasma proteins within the capillary reabsorb fluid into the

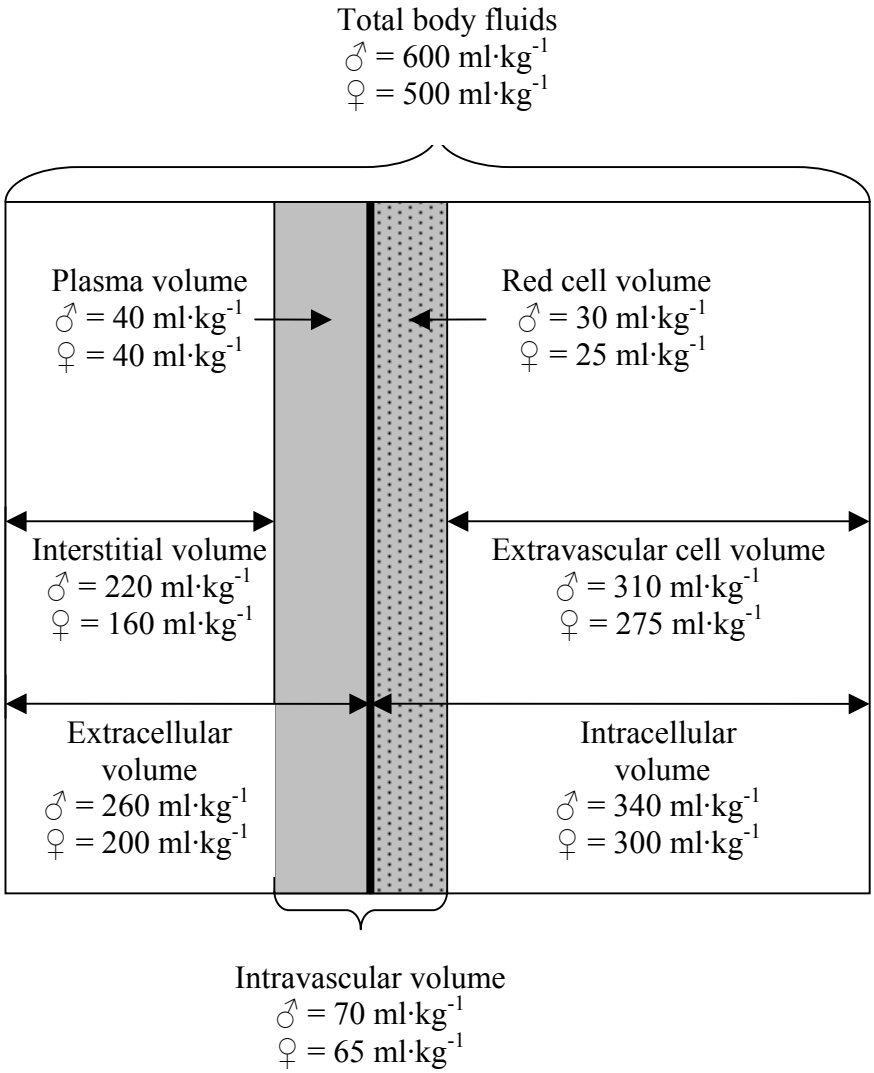


Figure 2.1: Schematic of the distribution and size of normal body-fluid volumes within adult males (♂) and females (♀: modified from International Commission on Radiological Protection, 1975; International Committee for Standardisation in Haematology, 1980; Guyton, 1991).

capillary and thus the volume of fluid remains relatively stable under resting conditions. However, the magnitude and direction of body-fluid forces may be altered when exposed to a wide variety of factors producing changes in the distribution of the body-fluid volumes.

When humans are exposed to stress, such as altitude, postural manipulation, exercise, and environmental temperature, maintenance of the normal body-fluid distribution is challenged. These stresses may alter both the magnitude and direction of the hydrostatic, osmotic, and oncotic forces, such that the distribution of the body-fluid compartment volumes are changed. The regulation of body-fluid volumes is essential for human thermoregulation, and the distribution of these volumes will change during different environmental temperatures. For instance, it has been demonstrated in resting males, that intravascular fluid volumes are dependent on environmental temperature producing changes in skin temperature with minimal alteration to core temperature (Maw *et al.,* 2000). As a result, the distribution of body-fluids, in particular PV, and to a lesser extent RCV, have been investigated during a wide range of stresses (Table 2.1). During this study, the effect of hydrostatic pressure and water temperature upon PV, measured using direct and indirect methods, and RCV will be assessed, as well as the influence of changes in the peripheral and whole-body haematocrit (Hct_w).

2.2 Direct and indirect body-fluid measurement

The body-fluid compartment volumes can be determined using a variety of techniques, such as bioelectrical impedance, carbon monoxide gas-rebreathing, changes in Hct and [Hb], various dyes, radioisotopes, and thermodilution methods. Direct quantification of these volumes, in particular the intravascular volume, has been achieved with the use of dyes and radioisotopes. Direct tracer-dilution techniques follow the general dilution principle, whereby an unknown volume can be determined from a tracer with a known concentration and volume. This tracer is introduced and allowed to circulate throughout the unknown compartment. Thereafter, a sample is acquired from the unknown volume, and the concentration of the tracer is determined and compared with the concentration and volume before administration. The unknown volume can be calculated from (after Chien and Gregersen, 1962):

$$V_U = \frac{V_T \times C_T}{C_U}$$

Table 2.1: Review of body-fluid studies during different conditions (males)

Study	Exposure	ΔPV	ΔRCV	Method
Alexander *et al.,* 1967	Altitude: < 4000 m	↓ 13%	—	Evans blue dye
Dill *et al.,* 1969	Altitude: < 4000 m	↓ 20%	—	[Hb] changes
Jain *et al.,* 1980	Altitude: < 4000 m	↓16.8%	↑ 2.8%	^{125}I albumin / indirect
Convertino *et al.,* 1980	Cycle ergometry (8 days: 60-65% $VO_{2\ max}$)	↑12%	—	Evans blue dye
Green *et al.* 1991	Cycle ergometry (4 wks: 62% $\dot{V}O_{2max}$)	↑13.8%	↓2.6%	^{125}I albumin / ^{51}Cr
Åstrand & Saltin, 1964	85 km cross-country ski	↑11%	↓3.2%	Evans blue dye / ^{51}Cr
Röcker *et al.,* 1990	Marathon running	↓10.3%	—	Hct/[Hb] calc.
Hagan *et al.,* 1978	Posture: supine to erect (35min)	↓11.7%	—	Hct/[Hb] calc.
Maw, 1994	Posture: supine to erect (30min)	↓ 6%	↓ 5.7%	^{125}I albumin / ^{51}Cr
Johansen *et al.,* 1998	Posture: supine to 6ºHDT(60 min)	↓ 9.3%	—	Evans blue dye
Wyndham *et al.,* 1968	Heat acclimation: (40ºC 17-d / 4 h bench stepping)	↑ 4.8%	—	^{131}I albumin
Senay *et al.* 1976	Heat acclimation: (45ºC 10-d / 4 h cycle)	↑ 9%	—	Hct changes
Patterson, 1999	Heat acclimation: (40ºC 22-d / 90 min cycle)	↑15.1%	↑ 4.5%	Evans blue dye/ ^{51}Cr
Bass & Henschel, 1956	Cold–air: 120 min (15.5ºC)	↓ 7%	—	Evans blue dye
Vogelaere *et al.* 1992	Cold–air: 120 min (1ºC)	↓ 15%	—	Hct/[Hb] calc.
Maw, 1994	Cold-air: 30 min (14ºC)	↓ 6.2%	↓ 3.5%	^{125}I albumin / ^{51}Cr
Yoshida *et al.,* 1999	Cold-air: 60 min (15ºC)	↓ 5.6%	—	Hct/[Hb] calc.
McCally, 1964	Immersion: 25 min (33ºC)	↑ 9 %	—	Hct/[Hb] calc.
Johansen *et al.* 1992	Immersion: 30 min (34.5ºC)	↑16%	—	Evans blue dye
Regan, 1998	Immersion: 60 min (33.1ºC)	↑12%	—	Hct/[Hb] calc.
Rochelle & Horvath, 1978	Immersion: 60 min (19ºC)	↓17.6%	—	Hct/[Hb] calc.
Young *et al.* 1987	Immersion: 90 min (18ºC)	↓17%	—	Hct/[Hb] calc.
Deuster *et al.* 1989	Immersion: up to 6 hours (5ºC – thermal diving suit)	↓17%	—	Hct/[Hb] calc.

Abbreviations: ΔPV = plasma volume change; ΔRCV = red cell volume change; ^{125}I albumin = radioiodinated serum albumin 125; ^{51}Cr = chromium 51; Hct = haematocrit; [Hb] = haemoglobin concentration.

where: V_U = unknown volume;
V_T = volume of tracer before administration;
C_T = concentration of tracer before administration;
C_U = concentration of unknown volume after tracer administration.

However, direct tracers rarely stay within the confines of the unknown compartment, primarily due to the hydrostatic and oncotic forces between the intravascular and interstitial spaces, and correction factors are needed for extravasation (Chien and Gregersen, 1962; Baker, 1963). Furthermore, the use of radioisotopes exposes the researcher and subject to a small but undesirable radiation dose. Hence, the development of indirect measures of blood volume (BV), RCV and PV utilising intravascular components, namely Hct and [Hb], were developed and utilised in many settings. The general principle of all indirect techniques is to provide a means for quantification of some variable which is somewhat difficult to measure, by measuring changes in a more-readily quantified variable, which has been shown to have a fixed relationship with the variable of interest.

The calculation of PV change can be achieved using independent and concomitant changes in Hct and [Hb]. These measures have the advantage of being simple to analyse, and do not require the subject to receive an injection of either dye or radioisotope. However, these indirect methods do not quantify the absolute body-fluid volume, but rather detect changes over short periods of time, thus the actual volume cannot be determined. Despite this limitation, this indirect technique, has become the most prevalent method for determining changes in PV (Table 2.1).

2.3 Blood volume measurement

Keith *et al.* (1915) outlined the principles of the general dilution method first employed by Valentin (1847), whereby a known amount of water was injected into the circulation and sampled after recirculation. However, the water was not wholly contained with the vasculature and the BV was underestimated. Thereafter, Haller (1854) attempted to determine BV by bleeding the entire blood supply from two criminals, measuring the collected volume of blood. Unfortunately, this technique did not collect blood in the microcirculation, and thus underestimated BV. In addition, this procedure does not lend itself to laboratory experiments in which repeated measurements are taken. Subsequently, Bischoff (1856) employed the same technique but washed the vasculature and tissues with water, thus deducing that blood could still be removed after exsanguination (see Keith *et al.,* 1915).

Clearly, these methods were impractical for clinical purposes, and different methods were developed.

Haldane and Smith (1899) initiated an indirect method to determine BV by using carbon monoxide gas as a label, which occurs naturally at very low partial pressures in room air. Haemoglobin has a high affinity with carbon monoxide and forms carboxyhaemoglobin. Hence, the concentration of carboxyhaemoglobin in the blood can be determined before and after carbon monoxide rebreathing, and the total BV estimated. However, a lack of reliability with the carboxyhaemoglobin test, and the loss of inhaled gas from the circulation, tended to cause an overestimation of BV. This technique was not examined again until 1948, when an accurate method for determination of carboxyhaemoglobin concentration and alveolar equilibration of carbon monoxide was devised for clinical practice (Sjöstrand, 1953; Thomsen *et al.,* 1991).

The emergence of dyes, such as vital red (Keith *et al.,* 1915) and Evans blue (Gregersen *et al.,* 1935), enabled researchers to refine the extraction of dye from the circulation and improve the accuracy of intravascular volume determination. Thereafter, the use of radioisotopes gained acceptance, after the ability to instil the solution into the circulation, and the subsequent determination using radiation scintillation counters, was achieved. To date, refinement of these techniques has occurred such that researchers can accurately and directly determine PV, RCV and hence BV.

2.4 Plasma volume determination methods

Many direct and indirect tests have been developed to measure PV. However, the most commonly used test involves Hct and [Hb], Evans blue dye, and radioiodinated serum albumin. During the present study, the indirect Hct/[Hb] calculation will be compared with an Evans blue dye column elution technique (Campbell *et al.,* 1958) and an Evans blue dye computer programme (Foldager and Blomqvist, 1991).

2.4.1 Evans blue dye

Evans blue dye[1] is a harmless azo-dye that binds to plasma albumin to directly

[1] Evans blue dye measures concentration using the principles of the general dilution model. This equates to volume and therefore the method can be viewed as a direct method.

measure PV (Campbell *et al.,* 1958; Farjanel *et al.,* 1997). Gregersen *et al.* (1935) were the first to describe a method for PV determination using Evans blue dye. Allen (1951) and Campbell *et al.* (1958) refined the process, with the inclusion of a detergent and cellulose matrix to extract the dye from the albumin. This process improved the accuracy of PV determinations by eliminating errors caused by lipaemia and haemolysis that interfere with absorbance at 615 nm, the wavelength for maximal absorbance of Evans blue dye (Campbell *et al.,* 1958; Greenleaf and Hinghofer-Szalkay, 1985). Subsequent authors have modified the process with the use of different extraction agents (Zweens and Frankena, 1981), mathematical correction calculations (Datsenko *et al.,* 1990) and different wavelength spectrophotometry, to exclude potential contaminants at a wavelength of 615 nm (Foldager and Blomqvist, 1991; Farjanel *et al.,* 1997).

Evans blue dye dilution methods have a high degree of accuracy when compared to radioisotopes (Chien and Gregersen, 1962). In a direct comparison with I^{131}- labelled human serum albumin, Evans blue dye has been shown to accurately determine PV at rest, and after PV expansion with an intravenous colloid infusion (Freinkel *et al.,* 1953; Schultz *et al.,* 1953; Parving *et al.,* 1973). However, radioisotopes attached to reinfused human blood, such as I^{131} albumin and I^{125} albumin, increase the risk of cross-contamination and blood-borne organisms infecting the recipient (Besa, 1975). Furthermore, the use of Evans blue dye for the measurement of PV is preferential due to the radiation dose imparted with the use of radioisotopes (Parving *et al.,* 1973). In addition to the PV determinations using an Evans blue dye column elution technique and computer programme, PV change will be measured using concomitant changes in Hct and [Hb]. Hence, these methods will be compared during control, thermoneutral and cold-water immersions.

2.4.2 Haematocrit and haemoglobin concentration

The indirect calculations of Hct and [Hb] are based on changes in the [Hb] and the relative constancy of the number of erythrocytes (Greenleaf and Hinghofer-Szalkay, 1985). The determination of Hct and [Hb] at rest, and during or after a period of stress, measures the relative change in intravascular components, and thus reflects changes in the intravascular volume and PV. Elkington *et al.* (1946) were the first to describe an equation utilising concomitant changes in both the Hct and [Hb]:

$$\frac{PV_2}{PV_1} = \frac{(1 - Hct_2)}{(1 - Hct_1)} \times \frac{[Hb_1]}{[Hb_2]}$$

where: PV = plasma volume
Hct = haematocrit
[Hb] = haemoglobin concentration
1 = initial measurement
2 = final measurement.

Thereafter, Strauss *et al.* (1951) expressed the equation in the form of percentage change:

$$\%\Delta PV = \left[\frac{[Hb_1]}{[Hb_2]} \text{ x } \frac{(1 - Hct_2)}{(1 - Hct_1)} - 1 \right] \text{x } 100$$

where: %ΔPV = percentage change in plasma volume
Hct = haematocrit
[Hb] = haemoglobin concentration
1 = initial measurement
2 = final measurement.

This often used equation of percentage change permits the researcher to equate an increase or decrease in PV, relative to a baseline. To increase the accuracy of the equation, correction factors are frequently incorporated into the calculation (Harrison *et al.,* 1982):

$$\%\Delta PV = \left[\frac{[Hb_1]}{[Hb_2]} \text{ x } \frac{(1 - Hct_2 \text{ x } \mathbf{CF})}{(1 - Hct_1 \text{ x } \mathbf{CF})} - 1 \right] \text{x } 100$$

where: %ΔPV = percentage change in plasma volume
Hct = haematocrit
[Hb] = haemoglobin concentration
1 = initial measurement
2 = final measurement
CF = correction for trapped plasma.

Haematocrit values obtained by microcentrifugation are often corrected, usually 0.96, for trapped plasma (Chaplin and Mollison, 1952) remaining in the packed cells of the microcapillary tube (see section 3.5.2). However, this is not uniformly adopted, as correction factors of 0.96 (Bœning *et al.,* 1972; Miki *et al.,* 1986; Ertl *et al.,* 1991), and 0.98 (Åstrand and Saltin, 1964) have been used. Furthermore, the application of Hct without any correction factor for trapped plasma has been used (Harrison *et al.,* 1986; Young *et al.,* 1987). Accordingly, Harrison *et al.* (1982) determined the percentage change in PV using the Hct/[Hb] calculation with different trapped plasma correction factors. The authors chose 16

consecutive paired values of Hct and [Hb] to provide a linear change in PV. From this analysis the error of trapped plasma on the change in PV was less than 5% (Figure 2.2). This project will not correct for trapped plasma in the Hct due to the lack of significant error. However, the effect of the ratio of the Hct_w to peripheral, or venous haematocrit (Hct_v) will be quantified to assess any error associated with the Hct/[Hb] calculation of PV during thermoneutral- and cold-water immersion.

2.5 Environmental influences

2.5.1 Thermoneutral-water immersion

Thermoneutral-water immersion (34.5ºC) elicits an increase in central blood volume and central venous pressure (Arborelius *et al.,* 1972; Greenleaf *et al.,* 1984; Bonde-Petersen *et al.,* 1992; Gabrielsen *et al.,* 1993). The mechanism involves a redistribution of blood into the thoracic region, due to the hydrostatic pressure on the immersed body, and reflected by the rise in cardiac output, and central venous and pulmonary pressures (Echt *et al.,* 1974; Lange *et al.,* 1974; Risch *et al.,* 1978a; Risch *et al.,* 1978b; Figure 2.3). Arborelius *et al.* (1972) demonstrated an acute increase in cardiac output of 32%, with a parallel increase in intrathoracic blood volume of 700 ml during upright thermoneutral immersion to the level of the neck. Furthermore, Johansen *et al.* (1997a) found the abdomen to be the main reservoir of blood causing central blood volume expansion, whereas the legs contributed to fluid shifts resulting in haemodilution. Tajima *et al.* (1999) confirmed this phenomenon by negating the effect of translocation of blood from the legs by studying legless men immersed in water.

Water immersion provokes an increase in interstitial hydrostatic pressure of the submerged vasculature, resulting in a reversal of the normal capillary membrane gradient, as postulated by von Diringshofen (1948), thus altering intravascular flux (Miki *et al.,* 1986; Hinghofer-Szalkay *et al.,* 1987; Miki *et al.,* 1989). The change in flux moves fluid from the extravascular space to the intravascular compartment where the increased tissue pressure opposes the fluid shift into the peripheral body cells, thus causing hypervolaemia (Greenleaf, 1984). This movement of fluid principally induces an increase in the PV during short-term (less than two hours) exposure (Khosla and Dubois, 1979; Greenleaf *et al.,* 1983). However, despite broad agreement on PV elevation during short-term thermoneutral immersion, discrepancies remain over the magnitude of the increase, which may be related to the method of PV determination (Johansen *et al.,* 1992).

Hct	0.35	0.355	0.36	0.365	0.37	0.375	0.38	0.385
[Hb]	12.25	12.43	12.60	12.78	12.95	13.13	13.3	13.48

Hct	0.39	0.395	0.4	0.405	0.41	0.415	0.42	0.425
[Hb]	13.65	13.83	14	14.18	14.35	14.53	14.7	14.88

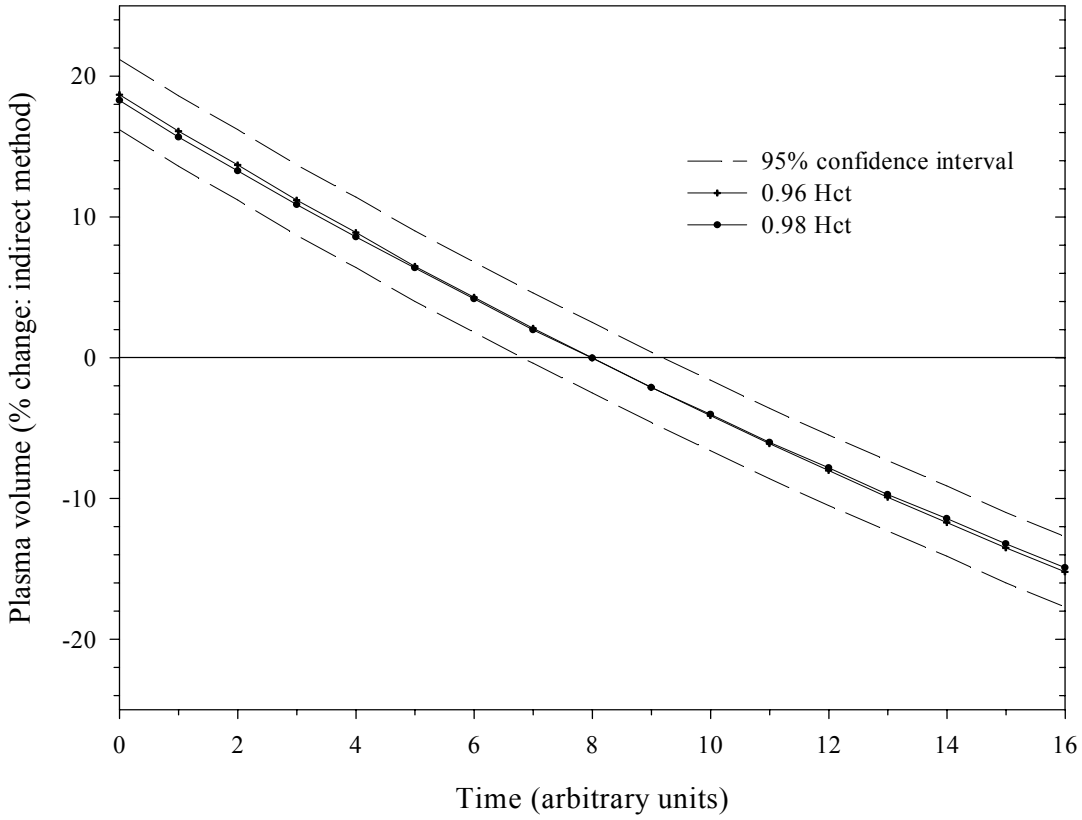


Figure 2.2: Error analysis of haematocrit correction factors (0.98, 0.96) on the change in plasma volume (PV) using the Hct/[Hb] calculation. Data represents PV change against arbitrary time units and 95% confidence intervals. (Modified from Harrison *et al.,* 1982). Values of the haematocrit and haemoglobin concentration listed above were used to calculate percentage change in plasma volume (baseline Hct = 0.39; baseline [Hb] = 13.65).

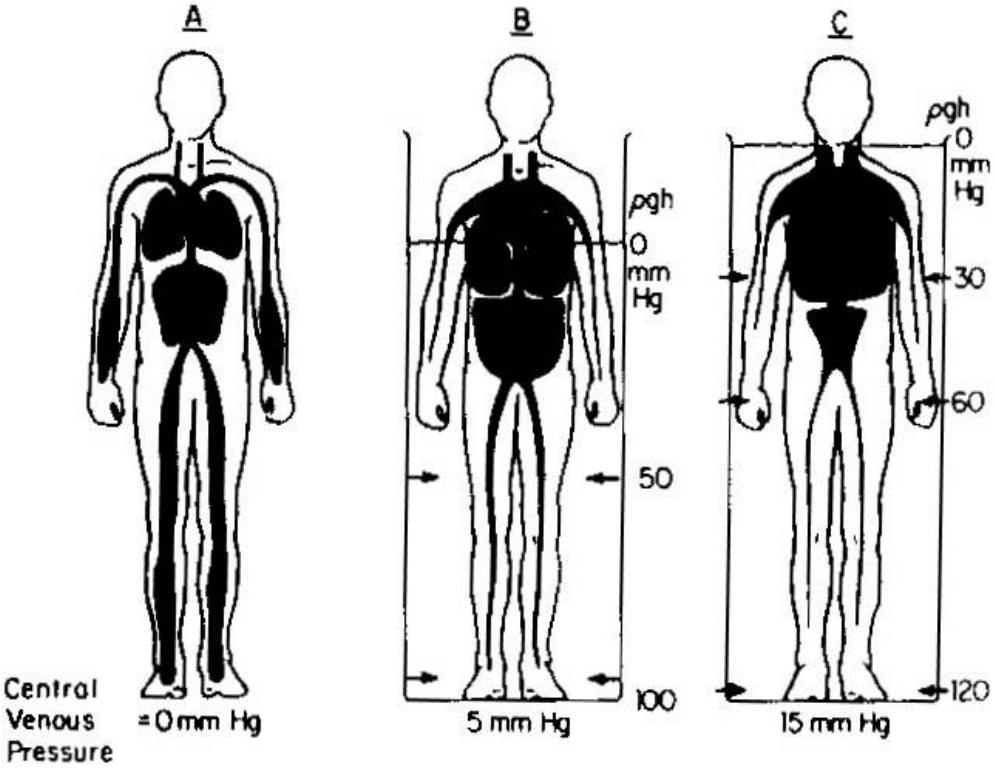


Figure 2.3: Central venous pressure and blood volume changes during standing in air (a), immersed to the level of the diaphragm (b), and immersed to the neck (c). Increasing water immersion depth causes redistribution of blood from the legs and visceral organs into the intrathoracic compartment with a concomitant increase in central venous pressure. (From Rowell, 1993).

The majority of these studies (McCally, 1964; Khosla and DuBois, 1979; Harrison *et al.,* 1986) utilised measurement of Hct and [Hb] changes for computation of the PV (Strauss *et al.,* 1951). Fewer studies have used a direct tracer, such as Evans blue, to determine PV during thermoneutral immersion (Greenleaf *et al.* 1980, 1981, 1983; Johansen *et al.* 1992, 1995). However, when direct and indirect methods of PV determination were compared, large discrepancies were demonstrated during short-term thermoneutral water immersion. Greenleaf *et al.* (1983) reported increases in PV of 8.8% and 4.3%, when using the Evans blue dye and Hct/[Hb] methods, respectively, in subjects immersed to the level of the neck for 20 min. In agreement, Johansen *et al.* (1992, 1995) immersed subjects for 12 hours in thermoneutral water and found after 30 min of immersion that PV had increased by 16% and 6.9%, using the Evans blue dye and Hct/[Hb] methods, respectively. Therefore, the Hct/[Hb] calculation underestimated the PV shift and the difference may possibly be explained by an alteration of the Hct_w to Hct_v ratio (Harrison *et al.,* 1986; Hinghofer-Szalkay *et al.,* 1987). The proposed mechanism suggests that the augmentation of hydrostatic forces, induced by water immersion on the peripheral vasculature, may cause a greater reduction in the Hct_v compared to the Hct_w (Knight *et al.,* 1986; Miki *et al.,* 1986). The decrease in Hct_v may increase the magnitude of the ratio and underestimate PV when determined using the Hct/[Hb] calculation (Johansen *et al.,* 1992). However, this hypothesis has yet to be demonstrated during water immersion and warrants investigation.

While the majority of studies report an increase in PV during short-term thermoneutral immersion, the contribution of the RCV is unknown. To our knowledge, direct tracer measurement of RCV in humans has not been performed during water immersion. Miki *et al.* (1986) found no change in RCV determined using ^{51}Cr-labelled erythrocytes in dogs immersed in thermoneutral water for 100 min. In humans, the majority of direct-tracer RCV studies have been conducted on resting subjects (Wennesland *et al.,* 1959; Baker, 1963; Sawka *et al.,* 1992), with a limited number of studies during environmental stress (Åstrand and Saltin, 1964; Costill and Saltin, 1974). Thus, the effect of water immersion on the RCV needs to be examined. In contrast, there is no evidence of direct tracer usage for the determination of PV during cold-water immersion. Hence, the effect of cold-water immersion on PV change, determined with a direct tracer-dilution method remains unclear. The current study will simultaneously determine PV and RCV, and hence, the F-cell ratio during cold-water immersion and determine the influence it has on the indirect PV method.

2.5.2 Cold-water immersion

In contrast to thermoneutral-water investigations, there are limited data regarding PV changes during cold-water immersion. In these studies, the general agreement was for a reduction in PV of 15 to 20%, despite differences in experimental conditions (Rochelle and Horvath, 1978; Young *et al.,* 1987; Deuster *et al.,* 1989). For instance, Rochelle and Horvath (1978) immersed unclothed subjects in 19^{o}C water for 60 min, and observed a 17.6% decline in PV. Similarly, Young *et al.* (1987) observed a 17% reduction in PV during 90 min of semi-reclined seating in 18^{o}C water in unclothed subjects. Furthermore, Deuster *et al.* (1989) observed a 17% reduction in PV in subjects wearing a thermally-protected diving suit, fully immersed for up to 6 hr in 5^{o}C water. Nevertheless, significant reductions in core temperature and increased diuresis were evident in all studies, indicating significant thermal and hydrostatic influences of cold-water immersion.

Despite similarities in thermal and urinary responses to cold-water immersion, the mechanism responsible for the observed PV reduction remains unclear (Young *et al.,* 1987). Rochelle and Horvath (1978) found an association between diuresis and PV reduction in subjects immersed for 60 min in 19ºC water, since urine production approximated that of PV loss. In addition, PV losses were negatively correlated with the subjects' body fat content, which also correlated with a decline in rectal temperature (T_{re}: Rochelle and Horvath, 1978). The strong relationship between body fat and thermoregulation in acute cold stress has been demonstrated repeatedly (McArdle *et al.,* 1984; Glickman-Weiss *et al.,* 1991; Tikuisis *et al.,* 2000). However, Young *et al.* (1987) found no relationship between the magnitude of PV reduction with shivering, body fat, or diuresis in subjects immersed for 90 min in 18ºC water.

Presumably, thermal stress, coupled with the hydrostatic pressure of cold-water immersion, produces a reduction in PV. However, the exact mechanism responsible for the reduction in PV has yet to be elucidated. Young *et al.* (1987) and Regan (1998) failed to show any significant relationship between body core cooling with a reduction in PV during cold-water immersion, regardless of exposure time. Previously, Vogelaere *et al.* (1992) hypothesised that the haemoconcentration observed during acute cold-air exposure may occur by intense peripheral vasoconstriction. Briefly, the mechanism suggests that vasoconstriction increases peripheral resistance and blood pressure, which modifies the Starling equilibrium at the capillary wall (Starling, 1896). The capillary fluid shift, accompanied by ions, moves fluid from the intravascular space to the interstitium. Certainly, the increased thermal conductivity

of cold water, compared to still air (Craig and Dvorak, 1966), produces intensive vasoconstriction, with a resultant increase in blood pressure (Strong *et al.,* 1985; Janský *et al.,* 1996). However, this mechanism of haemoconcentration during cold-air exposure has not been demonstrated during cold-water immersion, and the exact mechanism of PV reduction is yet to be elucidated.

Similarly, with thermoneutral immersion, the effect of the RCV on the intravascular compartment has not been determined directly during cold-water immersion in humans. In the few animal studies, RCV remained constant in dogs immersed in a 4ºC water bath (Fedor and Fisher, 1959). In support, Chen and Chien (1977) induced pronounced hypothermia (core temperature 26ºC) in dogs with the application of ice packs, and observed stability in the RCV over 110 min, measured using ^{51}Cr-labelled erythrocytes. Data are needed in humans to ascertain the role of the RCV during cold-water immersion. Therefore, the current study sought to quantify the ratio of Hct_w to Hct_v during thermoneutral and cold-water immersions by direct tracer-dilution measurement of PV and RCV.

2.6 F-cell ratio

The F-cell ratio, termed by Reeve *et al.* (1953a), is the proportion of erythrocytes of the total blood volume to the Hct_v. The F-cell ratio is characterised by the propensity of erythrocytes in the microcirculation, to be less than in the larger vessels, due to the reduced viscosity of blood in the smaller vessels (Fähræus, 1929). The smaller vessels cause erythrocytes to align in a fast moving central stream surrounded by a slower moving peripheral layer of plasma adjacent to the vessel wall (Fähræus and Lindqvist, 1931; Hahn *et al.,* 1942). This phenomenon is termed axial streaming, and was demonstrated by Ebert *et al.* (1941) using blood sampled from the antecubital vein, before and after arterial occlusion. The quantification of the F-cell ratio, usually reported as 0.91, was derived from data of Gibson *et al.* (1946), and supported by Chaplin *et al*. (1953) who measured the PV and RCV with simultaneous direct tracer-dilutions. It has previously been assumed that the F-cell ratio was constant, based primarily on the work of Costill and Dill (1974), who found no change in the ratio during dehydration induced by exercising in the heat. However, if the F-cell ratio changes during experimental conditions, the assumptions of the Hct/[Hb] calculation are invalidated, resulting in an incorrect PV determination (Greenleaf *et al.,* 1979; Harrison *et al.,* 1982). Furthermore, Harrison *et al.* (1982) have shown that mathematical manipulation of the F-cell ratio can produce large discrepancies in the PV change. Thus, a decrement of 0.02 in

the F-cell ratio results in a 3% PV change. More recently, Lundvall and Lindgren (1998) have shown an F-cell ratio shift can cause the Hct/[Hb] computation to underestimate the PV change by up to 30%, although the F-cell ratio shift was not quantified. In fact, data regarding the response of the F-cell ratio in humans exposed to acute stress is meagre. Therefore, our understanding of intravascular dynamics during immersion will be enhanced by the current study, in which the simultaneous direct measurement of PV and RCV, and the quantification of the F-cell ratio will be performed.

It is apparent that PV change during immersion studies, determined using the Hct/[Hb] calculation, underestimates the actual PV change, when compared to an Evans blue dye dilution method (Greenleaf *et al.,* 1983; Johansen *et al.,* 1992, 1995). Therefore, PV change determined using the Hct/[Hb] calculation during water immersion maybe erroneous. While the exact mechanism responsible for the underestimation of PV change has yet to be elucidated, it has been proposed that the F-cell ratio maybe responsible for the underestimation observed during thermoneutral immersion. This may be induced by a redistribution of erythrocytes from the peripheral vasculature to the larger, central circulation thus increasing the F-cell ratio (Johansen *et al.,* 1992). The hydrostatic pressure of water compresses the immersed vasculature and redirects blood into the central circulation (Arborelius *et al.,* 1972), thus possibly causing a simultaneous redistribution of erythrocytes. Accordingly, Young *et al.* (1987) have speculated that intense vasoconstriction, induced during cold-water immersion, may reduce the vascular volume of small vessels and lower the Hct_v, thus increasing the F-cell ratio. Presumably, the combined effects of thermal stress and the hydrostatic pressure of cold-water immersion would result in a larger shift of erythrocytes into the central circulation, and increase the magnitude of the F-cell ratio more than that during thermoneutral immersion. This remains speculative, as previous cold-water studies have determined the PV reduction only using the Hct/[Hb] calculation, and a comparison between direct and indirect methods of PV determination has not occurred. To our knowledge, the F-cell ratio has not been quantified during immersion at any water temperature. Therefore, there is a lack of information regarding intravascular volume changes, in particular, the influence of the F-cell ratio has on PV changes during thermoneutral and cold-water immersions. The current study will seek to provide such information and, in doing so, help to clarify intravascular volume changes during immersion.

2.7 Summary

Body-fluid distribution is influenced by several mechanisms that interact to maintain homeostasis. However, when the body is exposed to thermal stress, body-fluid distribution is altered, primarily through fluctuations in the hydrostatic, oncotic, and osmotic forces. These changes in body-fluid distribution, in particular intravascular volume, can be measured using different techniques, which either quantify the volume directly or measure components of the blood that are sensitive to fluid shifts. Water immersion is known to change the intravascular volume, primarily through hydrostatic pressure changes. Generally, short-term thermoneutral immersion, results in a temporary increase in PV, due to the hydrostatic pressure of the immersed vasculature exceeding that of the capillary hydrostatic pressure, thereby forcing fluid into the intravascular space. In contrast, cold-water immersion reduces PV, principally from the combined effects of hydrostatic water pressure, cold-induced diuresis and the added cold stress of the water temperature. The majority of these PV determinations were measured using the transformation equation of Hct/[Hb]. However, studies have demonstrated that this method underestimates PV change when compared to a direct tracer-dilution technique. As a result, alterations in F-cell ratio have been proposed as the mechanism responsible for the underestimation. This may be due to a redistribution of erythrocytes from the peripheral vasculature into the central circulation, induced by hydrostatic water pressure, thereby increasing the magnitude of the ratio and resulting in underestimations of PV when determined using the Hct/[Hb] method. Furthermore, cold-water immersion increases thermal strain and produces more pronounced vasoconstriction of the peripheral vasculature, which may induce greater F-cell ratio aberrations. To the best of our knowledge, there have been no comparative studies between direct and indirect measures of PV and RCV during acute thermoneutral and cold-water immersion. Furthermore, the indirect PV method has been shown to underestimate PV change during short-term thermoneutral immersion. Therefore, simultaneous measurement of PV and RCV in different water immersion temperatures using direct tracer-dilution techniques, will enable quantification of the F-cell ratio and allow the determination of its influence on the indirect calculation of PV. This study investigates the effect of hydrostatic and thermal influences on the F-cell ratio.

CHAPTER THREE: METHODS

3. METHODS

3.1 Subjects

Seven healthy, active males, prescreened for metabolic and cardiovascular disease, participated as subjects in this study. Females were not included due to hormonal fluctuations during the menstrual cycle and the possible effect this may have upon thermal responses, and subsequent body-fluid distribution (Graham *et al.,* 1989; Gonzalez and Blanchard, 1998). The subjects' characteristics are listed in Table 3.1. Each subject received a Subject Information Package, provided informed consent and completed a screening and physical activity questionnaire before participating. Subjects attended the laboratory on three occasions for a control, thermoneutral, and cold-water immersion protocol, each separated by 14 days in a counterbalanced design. Plasma volume (PV) and red cell volume (RCV) were measured simultaneously, both prior to, and at 60 min of the control and water immersion protocols. Three PV determination methods: Evans blue dye column elution (Campbell *et al.,* 1958), Evans blue dye computer programme (Foldager and Blomqvist, 1991), and the Hct/[Hb] calculation (Strauss *et al.,* 1951) were analysed. Total RCV was measured using radionuclide dilution: sodium radiochromate ($Na^{51}Cr$).

3.2 Procedures

3.2.1 Water immersion tests

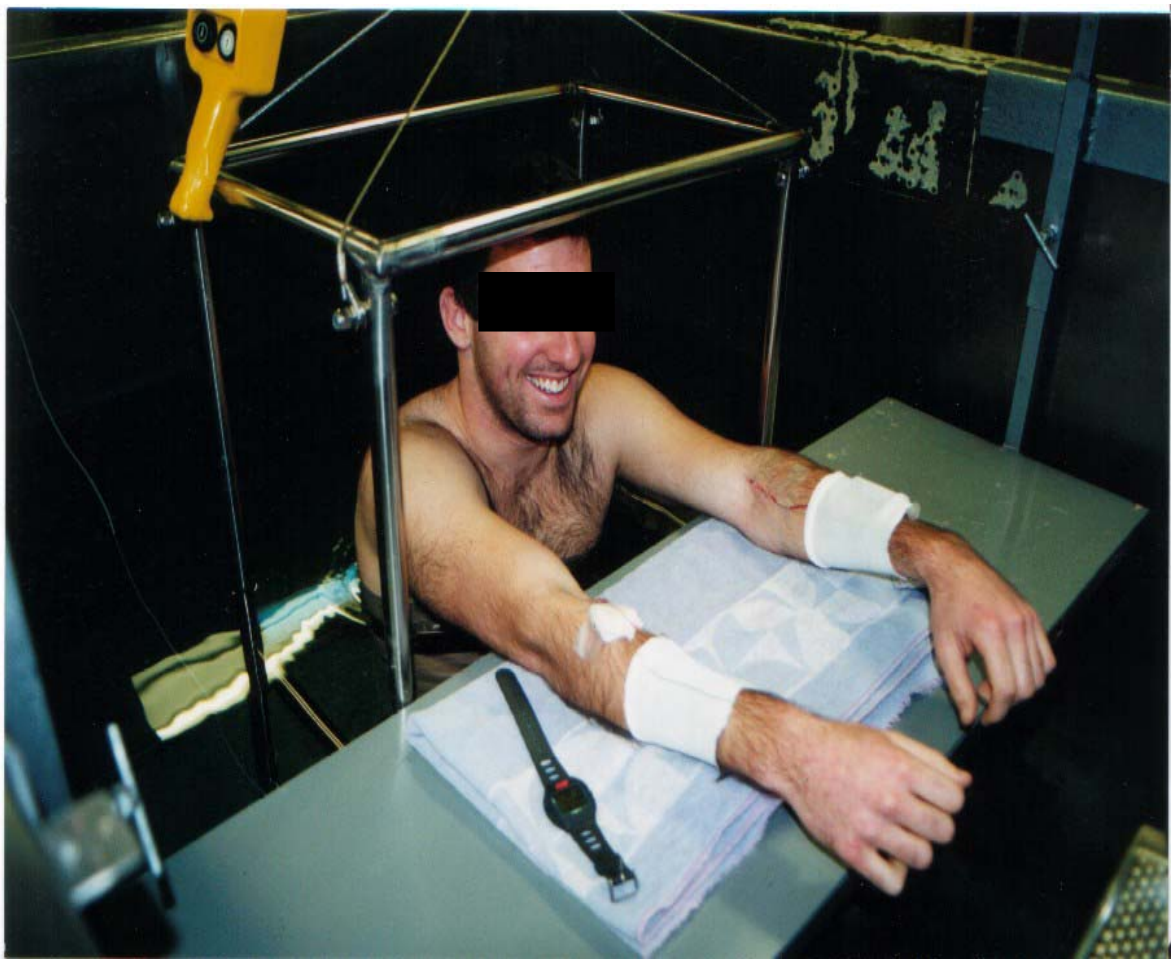
Subjects were exposed to two water-immersion tests: (i) thermoneutral (34.5°C ± S.D. 0.2) and (ii) cold water (18.6°C ± S.D. 0.2) for 60 min each. The mean air temperature was 21.2°C ± S.D. 1.1). All tests were separated by two weeks to allow for adequate Evans blue dye clearance. Subjects were immersed to the level of the third intercostal space with both arms above water level. This was achieved by placing the subject's arms on a tray above the water line (Figure 3.1). The subject maintained this upright seated posture and refrained from movement for the duration of the immersion period. Measurements during the immersion phase included: body-fluid volumes (PV and RCV), haematological variables (haematocrit (Hct) and haemoglobin concentration ([Hb])), air and water temperature, rectal temperature (T_{re}), and cardiac frequency (f_c). Blood was sampled after 45 min of immersion for background PV reference. Evans blue dye was injected after 50 min of immersion and blood

Table 3.1 Physical characteristics of subjects

Subject	Age (yr)	Height (cm)	Mass (kg)	Σ9SKF (mm)	B.S.A. (m^2)
S1	22	176.1	74.88	101.4	1.91
S2	19	189.5	80.03	46.4	2.07
S3	27	180.2	79.52	112.7	1.99
S4	19	185.8	75.56	57.8	1.99
S5	45	179.8	101.40	119.2	2.21
S6	28	186.1	84.39	76.7	2.09
S7	33	184.5	79.12	70.4	2.02
Mean	27.6	183.14	82.13	83.51	2.04
SD	9.2	4.61	9.06	27.99	0.09

Abbreviations: Σ9SKF = sum of nine skinfold sites (triceps, subscapular, biceps, iliac crest, supraspinale, abdominal, front thigh, medial calf and mid axilla skinfold thickness); B.S.A. = body surface area ($Mass^{0.425}$ x $Height^{0.725}$ x 0.007184; after: DuBois and DuBois, 1916); SD = standard deviation.

Figure 3.1 Position of a subject during an immersion test with both arms positioned on the tray above water level



samples were taken at 55, 57 and 60 min for PV determination. Haematocrit, [Hb] and RCV were measured in the 60 min sample. A total of 115 ml of blood was drawn during each test. Urine volume and change in mass were measured prior to, and immediately following, immersion.

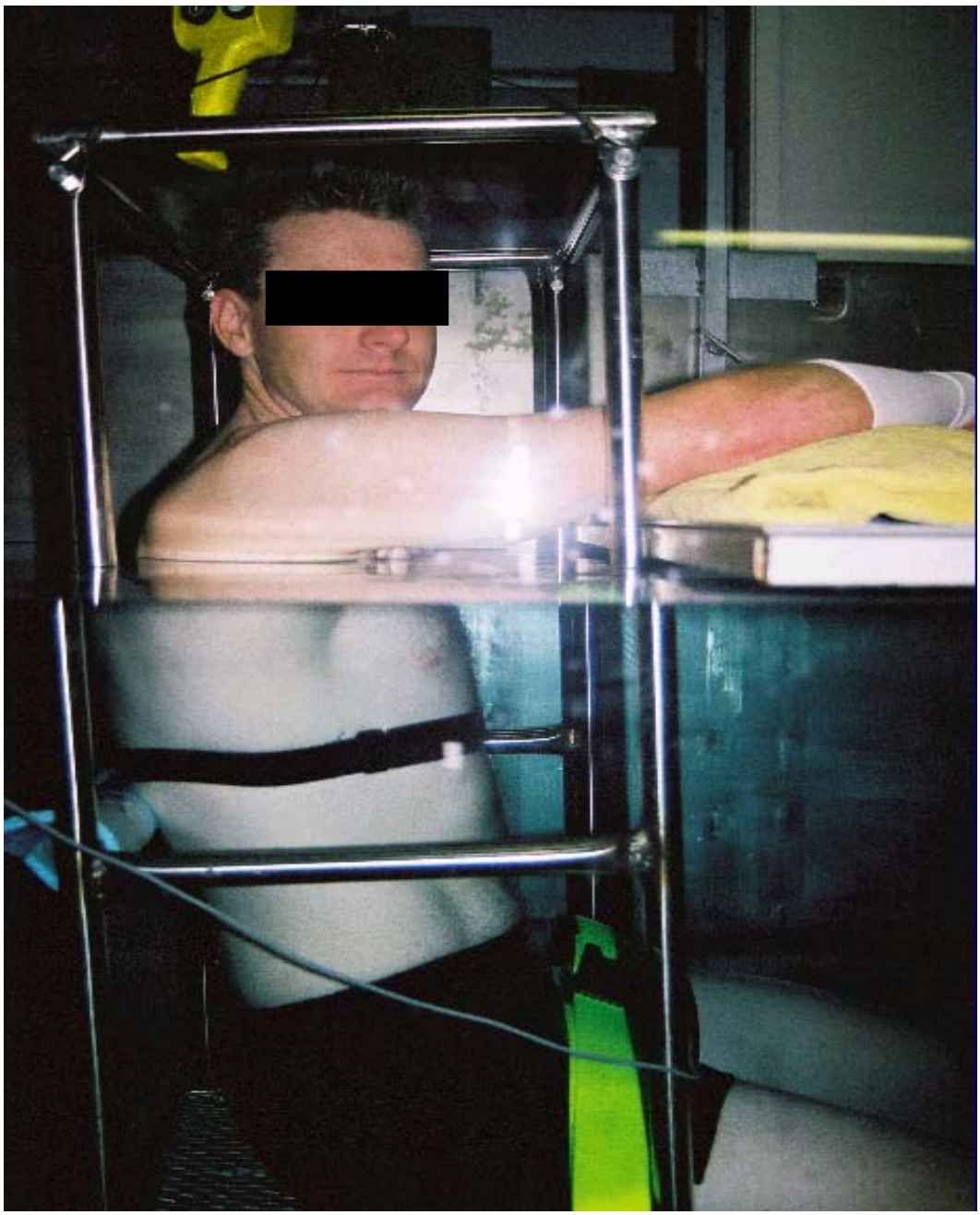
The water-immersion tank (height 1.7 m, width 1.3 m, and length 1.7 m) was constructed of stainless steel with a viewing window on one side. The subject was immersed until the feet were on the floor of the tank and the arms at shoulder height on the tray. The water was stirred continually at 1.5 $l \cdot sec^{-1}$ with a pump (Model 413, Onga, Melbourne, Australia). The water was maintained at a constant temperature with a water-cooling condensing unit (AH-0350-MHZ, Lovelock Luke, Melbourne, Australia) and a heat-exchanger (CN-2, Aqua Systems Inc., Melbourne, Australia) linked to a heating unit (415V Immersion Heater, Ice-Tech Air, Pty. Ltd., Wollongong, Australia).

The immersion chair was constructed from marine grade stainless steel (height 1.05 m, width 0.6 m, and depth 0.4 m) with a stainless steel mesh seat (mesh aperture 11.1 mm). This enabled the maximum surface area of water contact with the subject's body. Four wire ropes attached the chair overhead to a hoist (T202-8 1/1, Stahl Fördertechnik, Germany) enabling the operator to move the subject into the tank whilst maintaining an upright seated posture.

3.2.2 Control test

A control test was performed in which each subject was exposed to air (21.31ºC ± S.D. 1.1) for 60 min. The control tests were separated from the thermoneutral and cold-water immersion tests by at least 14 days in a counterbalanced design. The subjects were seated on the same chair used during the immersion phases, placed next to the water immersion tank to replicate the room ambient conditions of the thermoneutral and cold-water immersion tests. Special consideration was given to the subject's posture. The subject was requested to maintain a posture identical to that adopted during the thermoneutral and cold-water immersion tests, with feet placed on the floor and both arms placed on a box arranged on a table in front of the subject, ensuring the subject's arms were at shoulder level (Figure 3.2).

Figure 3.2: Position maintained during an immersion test with the subject immersed to the level of the third intercostal space.



3.2.3 Experimental standardisation

Subjects were requested to refrain from strenuous exercise and the consumption of caffeine and alcohol for a 12 hr period before the testing. The subject's dietary intake was unrestricted before the tests apart from a request for a low fat and low-protein meal the night before and day of testing. During testing, the subject did not consume food or liquid. Tests were performed at the same time of day to limit the effect of circadian variation in the measured variables. The study was conducted during August, September and early October 2000, which corresponded with Southern Hemisphere spring.

3.2.4 Subject preparation

Subjects arrived at the laboratory voided to obtain a background urine reference sample for radiation counting. After 30 min of upright seated posture, a 20-gauge catheter (Insyte, B.D., UT, U.S.A.) was inserted into a left antecubital vein and 30 ml of blood were collected without stasis. From this volume, 15 ml were treated with lithium-heparin (Li-Hep 15 I.U heparin·ml^{-1} of blood) and stored for background PV analysis, and 5 ml were treated with ethylenediamine tetra-acetic acid (EDTA: 1.8 mg·ml^{-1} of blood) and stored as a radiation background reference. The remaining 10 ml of blood were transferred to a sterile McCartney bottle pre-treated with 2 ml of 10% citrate phosphate dextrose with adenine (CPD-A, Sigma, MO, U.S.A.) for labelling of erythrocytes with sodium-radiochromate (see Section 3.3.2). The catheter was attached to a 30 cm length of teflon minimum volume extension tubing (Braun, Malaysia) and was affixed to the skin with an adhesive plaster (Opsite., Hull, England). The catheter and extension tubing were kept patent with a 1-ml injection of heparin-saline (50 I.U.·5 ml) after the initial blood samples. The subject maintained the same upright, seated posture throughout this period.

Following the sodium-radiochromate labelled erythrocyte preparation (approximately 90 min), a second 20-gauge catheter (Insyte, B.D., UT, U.S.A.) was inserted into the right antecubital fossa as per the left arm cannulation. This catheter was utilised for prepared injections of Evans blue dye (2.5 ml; Evans Blue Injection 25 mg·5 ml^{-1}, New World Trading Corporation, U.S.A.) and Na^{51}Cr-labelled erythrocytes. No sampling occurred from this catheter to reduce the effects of sampling error.

The injection of Evans blue dye immediately followed the Na^{51}Cr-labelled

erythrocytes injection. The Evans blue dye was injected over a 1-min period. The time at the end of injection was considered as the origin of PV determination. The Evans blue dye was injected into the catheter through a three-way tap. The syringe, three-way tap and catheter were flushed with 20 ml of sterile 0.9% sodium chloride several times to remove residual dye. This procedure maximised the volume of Evans blue dye injected. Following administrations, 1 ml of heparin-saline (50 I.U.·5 ml^{-1}) was injected to maintain catheter patency.

At 5, 7, 10 and 15 min, 9 ml of blood (lithium-heparin 15 I. U. heparin·ml^{-1} of blood) were sampled for PV analysis. Blood for Hct/[Hb] (2.7 ml K-EDTA 1.6 mg·ml^{-1}) and RCV (5.0 ml K-EDTA 1.6 mg·ml^{-1}) determination were drawn at 10 and 30 min, respectively. The catheter and extension tubing were flushed between samples with 2-ml of 0.9% sodium chloride. Following all blood sampling, the catheter was heparin-locked with 1 ml of heparin-saline (50 I.U.·5 ml^{-1}) to maintain catheter patency.

The subject was then required to void, self-insert a rectal thermistor 12 cm past the anal sphincter and change into swimming shorts. The rectal thermistor was connected to a data logger (1206 Series Squirrel, Grant Instruments Pty. Ltd., U.K.) and a cardiac frequency sensor chest strap (SportsTester PE4000, Polar®, Finland) was fitted. Baseline measurements were obtained and the body mass of the subject was determined. The subject was immediately transferred to the immersion chair for the commencement of the control, thermoneutral or cold-water immersion test. Water and ambient air temperatures were measured using thermistors (Type EU, Yellow Springs Instruments Co. Ltd., Yellow Springs, OH, USA.). Blood samples were drawn from the left antecubital fossa catheter at 45 min for PV background, followed by an injection of Evans blue dye at 50 min into the catheter in the right arm. At 55, 57 and 60 min, 9 ml of blood (lithium-heparin 15 I. U. heparin·ml^{-1} of blood) were drawn from the left arm catheter for PV determination. Blood for Hct/[Hb] and RCV (10.0 ml K-EDTA 1.6 mg·ml^{-1}) determination were drawn at 60 min. The test was terminated after 60 min and the subject's body mass was determined, and equipment and catheters were removed. The subject was requested to void and a commercial sports drink and shower were provided.

3.3 Erythrocyte volume determination

3.3.1 Sodium-radiochromate preparation

The preparation of $Na^{51}Cr$ was conducted under sterile conditions in accordance with the New South Wales Department of Health's Code of Safe Practice (1987). A modified technique (*after* Spears *et al.,* 1974), with previous validation at the University of Wollongong (Maw, 1994; Regan, 1998), was utilised (see Appendix B for details). Sodium-radiochromate (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) with an activity of 37.0 $MBq \cdot 1ml^{-1}$, was reconstituted in a 100 ml sterile 0.9% sodium chloride glass bottle (Astra Pharmaceuticals Pty. Ltd., North Ryde, Australia) and stored until use. The reconstituted concentration (37 $kBq \cdot ml^{-1}$) permitted accurate administration of $Na^{51}Cr$.

3.3.2 Erythrocyte labelling

A McCartney bottle, containing 2 ml of 10% CPD-A and 10 ml of whole blood, was centrifuged for 10 min at 1500 *g*. The buffy coat and supernatant were carefully removed and 18.5 kBq of $Na^{51}Cr$ was combined with the remaining erythrocytes. This dose was utilised during tests one and two. An adjusted dose, 29.6 kBq of $Na^{51}Cr$, was utilised during the third test. The increased dosage was necessary to obtain a sample to background ratio of no less than 2 to 1. This method has been repeated safely in the University of Wollongong, Department of Biomedical Sciences, Thermal Physiology Laboratory (Regan, 1998). This preparation was incubated at room temperature in a sterile fume cabinet for 30 min, thus optimising erythrocyte labelling. Following this period, 10 ml of sterile 0.9% sodium chloride were added to the erythrocytes and centrifuged at 1500 *g* for 10 min. This washing process was repeated three times with the resultant supernatant carefully removed after each centrifugation. The final preparation of $Na^{51}Cr$-labelled erythrocytes was reconstituted with sterile 0.9% sodium chloride to a total volume of 10 ml. The syringe was immediately weighed, to a precision of 0.0001g (ER-182A Electronic Balance, A&D Co. Ltd., Tokyo, Japan) and administered to the subject. Following administration the syringe was reweighed to determine the amount injected.

3.3.3 Sodium radiochromate standard

A standard was prepared during each test. Approximately, 0.5 ml of $Na^{51}Cr$-labelled erythrocytes were diluted in distilled water to a total volume of 250 ml. This dilution approximated that expected for the remaining $Na^{51}Cr$-labelled erythrocytes after their equilibration in the body (Maw, 1994). A 1-ml aliquot, determined volumetrically, was

removed and stored in a 5-ml plastic vial as the $Na^{51}Cr$-dose standard. The standard was refrigerated at 4°C until gamma-radiation (γ) counting later the same day. The standards were used to determine the exact injected dose of $Na^{51}Cr$.

3.4 Blood and urine radiation counting

3.4.1 Blood analysis

Three 5-ml blood samples (EDTA: 1.8 $mg \cdot ml^{-1}$ of blood) were collected after 30 min of rest, 30 min after the first Evans blue dye injection, and at 60 min of immersion or control for RCV determination. The blood was centrifuged at 1200 *g* for 10 min, and the plasma removed. One-millilitre of erythrocytes were haemolysed by adding 3 drops of 1 $mg \cdot ml^{-1}$ saponin (BDH, Merck Pty. Ltd., Victoria, Australia) and distilled water in a 5-ml plastic vial. This preparation was refrigerated at 4°C prior to γ counting later the same day.

3.4.2 Urine analysis

The subject was required to void on entry to the laboratory, after 150 min of rest, and at the completion of the test. Urine samples were measured for total volume and a 1 ml sample was dispensed into 5-ml plastic vials with 3 drops of 1 $mg \cdot ml^{-1}$ saponin (BDH, Merck Pty. Ltd., Victoria, Australia) and distilled water. This preparation was refrigerated at 4°C prior to RCV determination. The γ counting occurred on the same day of testing (see Section 3.4.1).

3.4.2.1 Radiation counting

A well-type γ scintillation counter (Wallac 1480, Wizard™ 3" Automatic Gamma Counter, Turku, Finland) provided absolute gamma counts of the erythrocyte, urine and standard specimens. Each sample was counted for 20 min.

3.5 Plasma volume determination

3.5.1 Evans blue dye

Background 5, 7, 10 and 15-min blood samples (lithium-heparin 15 I. U. $heparin \cdot ml^{-1}$ of blood) from the resting and immersion periods were centrifuged at 1200 *g* for 10 min. The plasma was removed and stored in 5-ml plastic vials at 4°C until analyses later the same day.

An Evans blue dye column elution technique (Campbell *et al.,* 1958) was used for the

measurement of PV. Briefly, this technique requires chemical cleaning of the plasma to eliminate potential plasma contaminants which may alter the determination of PV. In addition, the Evans blue dye extraction technique was compared with a PV back-extrapolation computational determination (Foldager and Blomqvist, 1991) during the pre-immersion, control, thermoneutral and cold-water immersion tests. The Evans blue dye computer programme required untreated plasma samples to be measured at two different wavelengths and a back extrapolation measurement determined PV. The computer programme technique has not been compared to the Evans blue dye column elution method and thus the reliability was determined. Furthermore, these PV determination methods were compared to the original method described by Gregersen *et al.* (1935) when sufficient plasma was available after centrifugation. This method used untreated plasma and usually requires fasting before the experiment that is not always feasible.

3.5.1.1 Column elution technique

The extraction technique used a detergent to displace dye from albumin, thus reducing the influence of lipaemic turbidity and haemolysis (Greenleaf and Hinghofer-Szalsky, 1985). Briefly, 2 ml of background plasma was volumetrically separated into two flasks. To one sample (the standard), 0.2 ml of an Evans blue solution (1 ml Evans blue dye from the same batch as the injection dissolved in 50 ml distilled water) was added (see Appendix A for details). One millilitre of the 10-min post Evans blue dye injection plasma sample was volumetrically added to a separate flask. Fifteen millilitres of detergent (30 ml Teepol 610S, Fluka Chemika, Steinham, Germany; 20 g Na_2PO_4, BDH Merck Pty. Ltd., Victoria, Australia and 1000 ml of distilled water) was combined with each sample and added to separate desalting columns (Sephadex® G-25 M, Amersham Pharmacia Biotech, Buckinghamshire, U. K.). This solution was allowed to pass through the columns and was followed by a further 5-ml detergent wash. A 10-ml salt solution (20 g Na_2PO_4, BDH Merck Pty. Ltd., Victoria, Australia; dissolved in 1000 ml distilled water) was added and allowed to pass through the columns. An elution agent (1:1 acetone; BDH Merck Pty. Ltd., Victoria, Australia, distilled water mixture) was passed through at a rate of one drop per second until the Evans blue dye had moved to the bottom of the column. Ten millilitres of sample was collected in a flask, treated with 15 ml of buffer (8 g KH_2PO_4, BDH Merck Pty. Ltd., Victoria, Australia; dissolved in 100 ml distilled water), and measured in a spectrophotometer (Shimadzu UV-1601, UV-visible spectrophotometer, Tokyo, Japan) at an absorbance of 615 nm.

3.5.1.2 Computer programme analysis

A computer programme (Foldager and Blomqvist, 1991), based on dye disappearance curve fitting, was utilised for resting, control and thermoneutral water immersion PV determinations (see Appendix C for details). Additionally, three subjects had computational PV determinations during the cold-water immersion test.

The dye disappearance curve was found by sampling a background and at least two plasma samples after an Evans blue dye injection. This study sampled background, 5, 7, 10, and 15 min plasma samples (lithium-heparin 15 I. U. heparin·ml^{-1} of blood) due to reported complete dye mixing after 5 min (Dr. Niels Foldager, Danish Aerospace Medical Centre, Copenhagen, Denmark, 2000; personal communication). The plasma samples were transferred to 5-ml plastic vials and stored at 4°C until analysis later the same day of collection. The samples were measured in a spectrophotometer (Shimadzu UV-1601, UV-visible spectrophotometer, Tokyo, Japan) at wavelengths of 620 and 740 nm, respectively. The absorbances were measured in triplicate and the mean values used in the programme.

3.5.1.3 Evans blue dye determination

Plasma samples taken for the column elution technique were also used to measure PV based on the original work of Gregersen *et al.* (1935). Background, and 10-min post injection blood samples (lithium-heparin 15 I. U. heparin· ml^{-1} of blood) were centrifuged for 10 min at 1200 *g* and the plasma removed and placed in 5-ml plastic vials and refrigerated at 4°C for analysis later the same day. A standard had previously been prepared with 1 ml of Evans blue dye (Evans Blue Injection 25 mg·5 ml^{-1}, New World Trading Corporation, U.S.A.) mixed with 50 ml of distilled water. One millilitre of background plasma was added to 0.2 ml of Evans blue dye solution to form a standard solution. The three samples (background, standard and 10-min sample) were measured in a spectrophotometer (Shimadzu UV-1601, UV-visible spectrophotometer, Tokyo, Japan) at an absorbance of 615 nm.

3.5.1.4 Evans blue dye clearance

Prior to the commencement of tests, an analysis of the amount of residual dye contained in the catheter and extension tubing after Evans blue dye administration and

subsequent flushing was undertaken. A 20-gauge catheter (Insyte, B.D., UT, U.S.A) attached to a 30 cm length of teflon minimum volume extension tubing (Braun, Malaysia) was connected to a three-way tap, assembled and primed with saline. Approximately 2.5 ml of blue food dye, used to simulate Evans blue dye, was drawn into a 5-ml syringe and injected through the three-way tap into the catheter and thereafter a collecting container. After this procedure, a 20-ml syringe filled with saline was attached to the other port of the three-way tap and the system was flushed several times. The catheter, tubing, and three-way tap were inspected after each flushing for residual dye. There was no visual evidence of blue food dye in the system and hence it was considered that a 20-ml saline flush was sufficient for flushing between subsequent injections of Evans blue dye.

3.5.2 Haematocrit and haemoglobin concentration

Blood was drawn at 10 min after Evans blue dye injections (rest and immersion), to correspond with the samples drawn for direct PV analysis. A 2.7-ml tube of blood (potassium-EDTA) was used for Hct and [Hb] determination. One millilitre of whole blood was transferred into a plastic storage tube and stored at –20°C for later analysis of [Hb]. The remaining blood was utilised for haematocrit determination immediately after collection.

Haemoglobin concentration was measured in duplicate samples using the cyanomethaemoglobin method (Total Hemoglobin, Sigma Diagnostics®, MO, U.S.A.). The method utilises the oxidation of haemoglobin to methaemoglobin in the presence of alkaline potassium ferricyanide. Methaemoglobin reacts with potassium cyanide to form cyanomethaemoglobin with the maximal spectrophotometric absorption at 540 nm. This colour intensity is proportional to the total [Hb]. Drabkin's reagent (sodium bicarbonate, 100 parts, potassium ferricyanide, 20 parts, and potassium cyanide, 5 parts) was reconstituted with 1000 ml of deionised water and 0.5 ml 30% Brij-35 solution to form Drabkin's solution. Cyanomethaemoglobin standard solution (Haemoglobin solution dissolved in 50 ml Drabkin's solution) was mixed with Drabkin's solution to form four standards (0, 6, 12 and 18 $g \cdot dl^{-1}$) and read in a spectrophotometer (Shimadzu UV-1601, UV-visible spectrophotometer, Tokyo, Japan) at 540 nm. The spectrophotometer automatically determined a four-point calibration curve ($r = 0.999$) from the concentration versus the absorbance of the standards. Twenty microlitres of the subject's whole blood was added to 5 ml of Drabkin's solution, vortexed, and allowed to stand at room temperature for 20 min. The preparations were read in the

spectrophotometer at 540 nm and converted to a concentration ($g \cdot dl^{-1}$).

The microhaematocrit technique (Hct: IEC MB CENTRIFUGE, International Equipment Co., MA, U.S.A.) was used to determine the packed cell volume. Haematocrit, measured in capillary tubes (Hirshmannn, Germany), 75 mm in length, with a blood capacity of 75 µl, was measured in triplicate. Blood entered the tube by capillary attraction until approximately 15 mm remained unfilled. The tubes were sealed at one end with plasticine (Silligum, Copenhagen, Denmark) and centrifuged at 3500 *g* for 10 min. A microhaematocrit reader (Digimatic, Mitutoyo, Japan) with an accuracy of 0.01 mm, measured the packed cell volume in millimetres as a portion of the PV. The Hct was calculated as a percentage of the packed cell volume to the total blood volume (BV). Trapped plasma, usually reported as 4%, was not corrected for during this study due to negligible error compared to measurement errors (Harrison *et al.,* 1985).

3.5.3 Determination of intravascular fluid volume compartments

The introduction of a known volume and concentration of indicator into an unknown space to be measured is referred to as the dilution technique. The general principle of the dilutional technique maintains that the indicator must be uniformly distributed and restricted to the unknown compartment. In this study, the measurement of PV and RCV with Evans blue dye and $Na^{51}Cr$, respectively, comply with this principle.

3.5.3.1 Erythrocyte volume

Erythrocyte volume was determined from the $Na^{51}Cr$-labelled erythrocytes with measurement of $Na^{51}Cr$ concentration in the urine to correct for $Na^{51}Cr$ losses. The erythrocyte volume was calculated as (after Chien and Gregersen, 1962):

$$RCV = \frac{(S_c \times S_d \times S_v) - (U_c \times U_v)}{E_c} \qquad (1)$$

where: RCV = erythrocyte volume;

S_c = [Na ^{51}Cr] of the Na^{51} Cr;

S_d = dilution of the Na^{51} Cr standard (see section 3.3.3);

S_v = volume of the [Na ^{51}Cr] injection;

U_c = [Na ^{51}Cr] in the urine collected;

U_v = volume of the urine collected;

E_c = [Na ^{51}Cr] in the erythrocyte aliquot.

3.5.3.2 Plasma volume

Plasma volumes were determined at rest and at 60 min of immersion. The simple technique and column elution technique utilised the same calculation. Plasma volume was thus calculated as (after Campbell *et al.,* 1958):

$$PV = \frac{(V \times D \times v \times \varepsilon_{std})}{((\varepsilon_t - \varepsilon_b) \times 1.03)} \quad (2)$$

where: PV = plasma volume;

V = volume of Evans blue dye injected;

D = dilution of the standard sample (1:250);

v = volume of sample extracted (1 ml);

ε_{std} = absorbance of the standard sample;

ε_t = absorbance of the test blood sample;

ε_b = absorbance of the blank blood sample;

1.03 = correction factor for the uptake of dye by the tissues (3%: Campbell *et al.,* 1958).

The direct dilutional technique of PV determination was compared with the indirect calculation of percent change in PV. The indirect calculation was determined by the concomitant relative changes in Hct and [Hb] and was thus calculated as (after Strauss *et al.,* 1951):

$$\%\Delta PV = \left(\left(\frac{[Hb_b] \times (1 - Hct_a)}{[Hb_a] \times (1 - Hct_b)} \right) - 1 \right) \times 100\% \quad (3)$$

where: %ΔPV = percent change in plasma volume;

$[Hb_a]$ = haemoglobin concentration at the time of immersion;

$[Hb_b]$ = initial haemoglobin concentration;

Hct_a = venous haematocrit at time during immersion;

Hct_b = initial venous haematocrit.

The F-cell ratio was calculated directly as a function of the RCV over the total intravascular volume and the venous haematocrit. The F-cell ratio was thus calculated as: (after Balga *et al.,* 2000):

$$f = \frac{\text{RCV}}{(\text{PV} \times \text{RCV}) \times \text{Hct}_\text{v}} \qquad (4)$$

where: f = F-cell ratio;

RCV = erythrocyte volume;

PV = plasma volume;

Hct_v = venous Hct.

3.6 Apparatus

3.6.1 Body-core temperature

Rectal temperature was measured continuously at 15-sec intervals during all tests. Rectal thermistors (Type 401, Yellow Springs Instruments Co. Ltd., Yellow Springs, OH, U.S.A.) self-inserted by the subject 12 cm past the anal sphincter were secured to the skin at the lumbar region with adhesive tape. A data logger (1206 Series Squirrel, Grant Instruments Pty. Ltd., U.K.) recorded T_{re} and was subsequently downloaded to computer. Oesophageal thermistors (Type FF, Yellow Springs Instruments Co. Ltd., Yellow Springs, OH, USA.) were used in three subjects for one trial each, but were discontinued due to unsuitability of oesophageal intubation in the remaining subjects.

3.6.2 Environmental temperature

Water and air temperatures were continuously monitored during the immersion and control tests with thermistors (Type EU, Yellow Springs Instruments Co. Ltd., Yellow Springs, OH, USA.). Air temperature was measured approximately 40 cm above the subject during all trials. Water temperature was recorded at the bottom of the tank filled with 90 cm of water.

3.6.2.1 Calibration

Rectal and skin thermistors were calibrated against a calibrated referenced mercury thermometer (Dobbie total immersion mercury thermometer, ± 0.05°C: Dobbie Instruments, Melbourne, Australia). The thermistors were coupled together around the thermometer bulb

and placed in an open water bath. The initial water temperature was set at 5°C and was increased by 5°C every 15 min until 45°C was attained. A data logger (1206 Series Squirrel, Grant Instruments Pty. Ltd., U.K.) recorded each 15-min interval with corresponding readings from the immersed thermometer. A regression equation was formulated from these data and used to correct the raw data from the thermistors.

3.6.3 Cardiac frequency

Cardiac frequency was continuously recorded from ventricular depolarisation (SportsTester PE4000, Polar®, Finland). Data logged were downloaded to a computer for analysis.

3.6.4 Mass, height and skinfolds

Body mass was determined using a high-resolution platform scale (A&D, Model No. fw-150k, CA, U.S.A.). Height was determined using a stadiometer (Holtain Ltd., U.K.) and skinfold measurements were taken at nine sites (see Table 3.1) with Harpenden skinfold calipers (British Indicators, West Sussex, U.K.).

3.7 Analyses

Statistical analysis consisted of repeated-measures analysis of variance (ANOVA) and Students *t*-test between trials and measurement methods. When statistical significance was detected, Students *t*-test and Student-Newman-Keuls test were used to isolate differences. All data are presented as means with standard errors of the means, unless otherwise stated. *Alpha* was set at the 0.05 level for all analyses.

CHAPTER FOUR: RESULTS

4. RESULTS

4.1 Methodological evaluation

Prior to testing, an error analysis of the calculation of plasma volume (PV), using the Evans blue dye column elution method and the indirect calculation using changes in haematocrit (Hct) and haemoglobin concentration ([Hb]), was undertaken. In addition, constants required for the calculation of PV using the back extrapolation technique of the Evans blue dye computer programme (Foldager and Blomqvist, 1991) were determined. These factors were vital to this investigation to identify the greatest sources of error before commencing the investigation, and thus facilitate error reduction.

4.1.1 Plasma volume techniques

4.1.1.1 Evans blue dye column elution method

An error analysis, using values obtained from a previous study (Regan, 1998) for the Evans blue dye column elution calculation (see Section 3.5.3.2), is presented in Table 4.1(a). The indicated values calculated a PV of 3070 ml, approximately the average value for adult males. Subsequently, for each expected value, the variable was increased by 5% and the resultant error calculated. For each variable, the percentage change in PV was 5% or less. Therefore, the impact of each variable does not affect the calculation of PV more than the internal error of the variable.

4.1.1.2 Haematocrit and haemoglobin concentration calculation

Similar to the error analysis of the Evans blue dye column elution method, Hct and [Hb] values for a decrease in PV of 9.7% were increased by 5% and the difference calculated. The difference was less than 5% for each variable (Table 4.1(b)). Thus, the individual variables will not skew the determination of PV.

4.1.1.2 Evans blue dye computer programme

The Evans blue dye computer programme (Foldager and Blomqvist, 1991) required the determination of constants specific for the composition of the standard solution and the back-extrapolation calculations (see Appendix C for details). Briefly, 15 plasma samples were measured in a spectrophotometer at wavelengths of 620 and 740 nm, respectively, the absorbance data were plotted with 740 nm on the X-axis and 620 nm on the Y-axis, and the slope of the regression line and y-intercept were determined (Figure 4.1). Thereafter, the

Table 4.1(a): Error analysis of the Evans blue dye technique for determination of plasma volume. The expected values for each variable are listed in the first row and subsequent rows indicate variables increased by 5% (bold).

	V	D	St	v	T	PV	% change
Expected	2.5	250	0.172	1	0.034	3070	-
V	**2.63**	250	0.172	1	0.034	**3223**	-5.0
D	2.5	**262.5**	0.172	1	0.034	**3223**	-5.0
St	2.5	250	**0.181**	1	0.034	**3223**	-5.0
v	2.5	250	0.172	**1.05**	0.034	**3223**	-5.0
T	2.5	250	0.172	1	**0.036**	**2923**	4.76

Abbreviations: PV = (V x D x St x *v*) / (T x 1.03); where PV = plasma volume; V = volume of Evans blue dye injected (ml); D = dilution of standard sample (1:250); St = absorbance of standard; *v* = volume of dye extracted (1 ml); T = absorbance of TEST sample minus the absorbance of BLANK; 1.03 = correction factor for dye uptake by the tissues.

Table 4.1(b): Error analysis of plasma volume percentage change using the indirect calculation. The expected values for each variable are listed in the first row and subsequent rows indicate variables increased by 5% (bold).

	$[Hb_b]$	$[Hb_a]$	Hct_a	Hct_b	%PV change	Difference
Expected	15	16	0.47	0.45	-9.7	-
$[Hb_b]$	**15.75**	16	0.47	0.45	**-5.1**	-4.6
$[Hb_a]$	15	**16.8**	0.47	0.45	**-14.0**	4.3
Hct_a	15	16	**0.49**	0.45	**-13.7**	4.0
Hct_b	15	16	0.47	**0.47**	**-5.8**	-3.9

Abbreviations: %PV change = ((([Hb_b]/[Hb_a])*((1-Hct_a)/(1-Hct_b))-1)* 100; where %PV change = plasma volume percentage change; [Hb_a] = haemoglobin concentration after treatment; [Hb_b] = haemoglobin concentration at rest; Hct_a = haematocrit after treatment; Hct_b = haematocrit at rest.

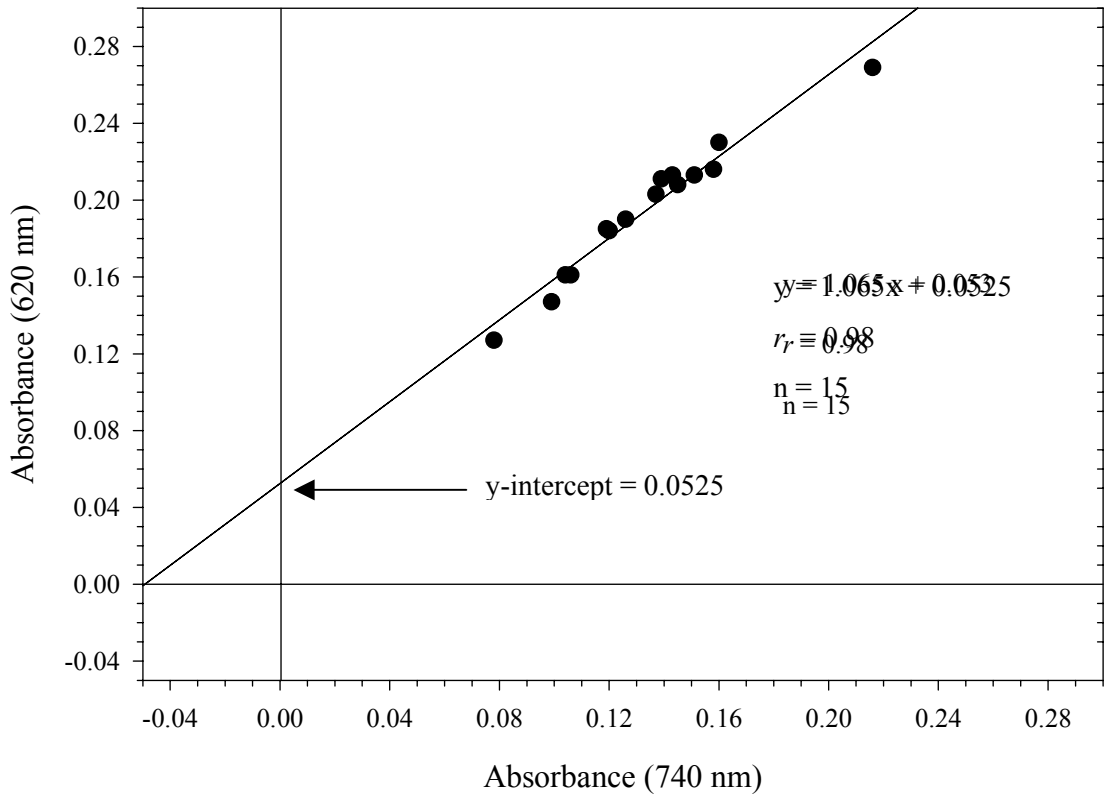


Figure 4.1: Regression of plasma samples measured at absorbance 620 (y-axis) and 740nm (x-axis). Calculated slope and y-intercept of the regression equation is used in the calculation of the constants for the Evans blue dye computer programme (Foldager and Blomqvist, 1991).

absorbance of the standard solution was determined at the wavelengths of 620 and 740 nm, respectively, and the corrected optical density at 620 nm (optical density which is due to Evans blue dye) was calculated from:

$$\text{Corrected 620 nm} = \text{620 nm} - (\text{slope of regression line x 740 nm} + \text{y-intercept}) \quad (1)$$

Substituting the values:

$$\text{Corrected 620 nm} = 0.429 - (1.065 \times 0.075 + 0.0525) = 0.2966 \text{ nm}$$

From this calculation, the specific extinction constant was calculated from:

$$\text{Specific extinction} = \frac{\text{corrected 620 nm}}{\text{standard solution concentrat ion}}$$

Substituting the values:

$$\text{Specific extinction} = \frac{0.2966}{0.00405} = 73.24 \text{ units} \cdot \text{mg}^{-1} \cdot \text{ml}^{-1} \quad (2)$$

Thus, when plasma was sampled from a subject, the 620 nm reading was corrected by substituting the 620 and 740 nm values using equation (1). This value was converted to a dye concentration value by multiplying with the specific extinction constant (2). This procedure enabled the computer programme to calculate PV at the time of injection by comparing the measured 620 nm reading with the corrected 620 nm value at intervals post injection, and determining the best fitting curve using linear regression (Foldager and Blomqvist, 1991).

4.1.2 Baseline intravascular volumes

4.1.2.1 Plasma volume

The pre-immersion baseline PV, calculated using the Evans blue dye column elution method, averaged 3492 (± 158), 3420 (± 126), and 3543 (± 128) ml for the control, thermoneutral and cold-water immersions, respectively. There were no significant differences between these volumes (P>0.05).

4.1.2.2 Erythrocyte volume

There was no significant difference in the pre-immersion baseline red cell volumes

(RCV) among the control, thermoneutral and cold-water immersions (P>0.05). Red cell volume averaged 2861 (± 60), 2617 (± 166), and 2724 (± 138) ml for the baseline of the control, thermoneutral and cold-water immersions, respectively.

4.1.2.3 Blood volume

Blood volume (BV) was calculated from PV (Evans blue dye column elution method) and RCV. The pre-immersion baseline BV, averaged 6352 (± 178), 6037 (± 250), and 6267 (± 211) ml for the control, thermoneutral and cold-water immersions, respectively. Despite a 316 ml difference between the control and thermoneutral baseline BV values, there was no significant difference between the three pre-immersion volumes (P>0.05).

The pre-immersion baseline volumes of PV, RCV, and BV, when corrected for body mass, were larger than those reported by the International Committee for Standardization in Haematology (1980) as normal. However, the volumes were comparable with body-fluid volumes reported in previous investigations (Dyrbye and Kragelund, 1970; Maw, 1994; Regan, 1998) using analogous dilution techniques. Furthermore, the subjects' body composition, based on their sum of nine skinfold measurements, were considered lean (Gore and Edward, 1992), and their intravascular volumes are typical of the population (Sjöstrand, 1953).

4.1.3 Correlation between plasma volume techniques

4.1.3.1 Evans blue dye column elution versus Evans blue dye computer programme

Plasma volume calculated using the Evans blue dye computer programme (Foldager and Blomqvist, 1991), at baseline (control, thermoneutral and cold) and at 60 min of control, correlated significantly (r=0.83) with PV, determined using the Evans blue dye column elution method at baseline (P<0.05; Figure 4.2). Plasma volume, averaged for the combined baseline (control, thermoneutral and cold) and at 60 min of control, was 3437 ml (± 70) and 3473 ml (± 84), using the Evans blue dye computer programme and the Evans blue dye column elution technique, respectively. However, PV comparisons between the Evans blue dye computer programme and Evans blue dye column elution technique during thermoneutral immersion, revealed limitations with the computer programme. There was a low non-significant correlation with PV determined at 60 min of thermoneutral immersion (r=0.30; P>0.05; n=4). Three of the four plasma samples were haemolysed, primarily from hydrostatically-induced peripheral vasoconstriction, resulting in exaggerated spectrophotometric absorbance measurements. Gross haemolysis in the post-injection plasma samples will increase the absorbance at

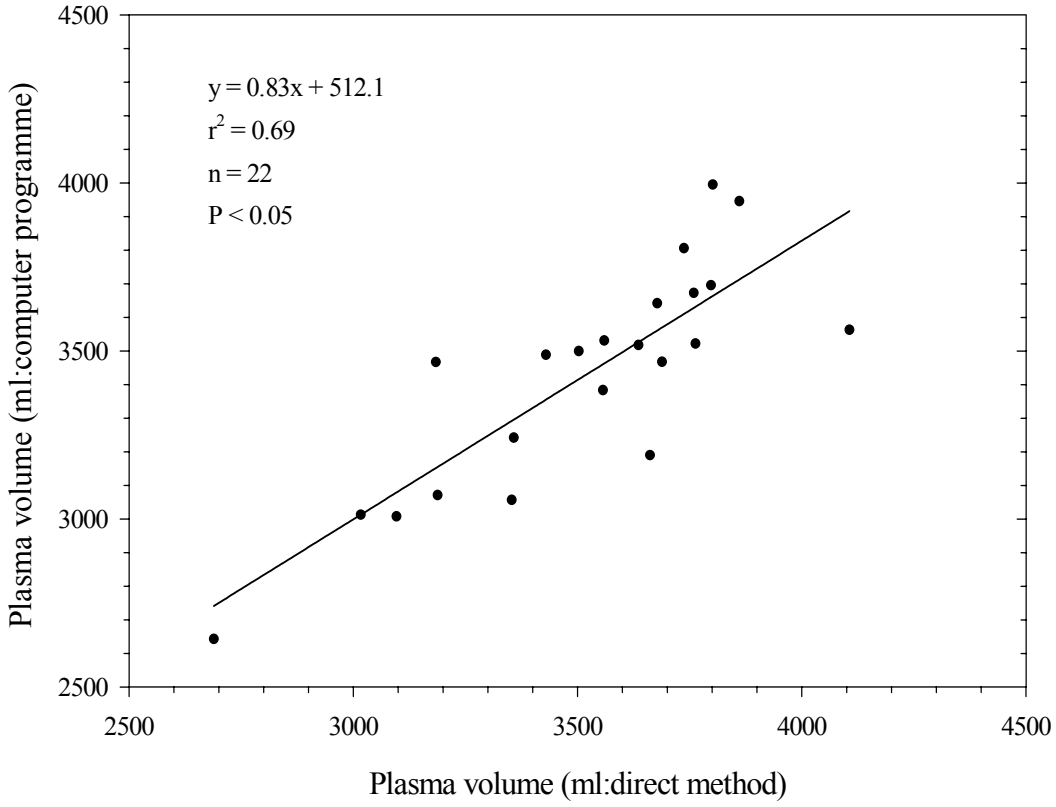


Figure 4.2: Regression of plasma volume measured using the Evans blue dye column elution (direct) technique and an Evans blue dye computer programme (Foldager and Blomqvist, 1991) method. Data are plasma volume at baseline (control: air (21.2°C), thermoneutral (34.5°C) and cold-water (18.6°C) immersions) and after 60 min of control (air 21.2°C).

wavelengths between 615 and 740 nm, resulting in an underestimation of PV (Dr. Niels Foldager, Danish Aerospace Medical Centre, Copenhagen, Denmark, 2000; personal communication). However, in non-haemolysed plasma, PV determined using the Evans blue dye computer programme after 60 min of cold-water immersion was highly correlated with the Evans blue dye column elution method (r = 0.99; P<0.05; n = 3). Previously, the Evans blue dye computer programme had been utilised during thermoneutral immersion and postural manipulation studies (Johansen *et al.,* 1992, 1998) without comparison against another direct tracer-dilution technique. Therefore, the Evans blue computer programme has been evaluated during 60 min of control, thermoneutral and cold-water immersions and found to accurately reflect actual PV, in non-haemolysed samples, using an Evans blue dye column elution technique.

4.1.3.2 Evans blue dye column elution versus Evans blue dye method

There was a low correlation between PV determined by the Evans blue dye column elution (Campbell *et al.,* 1958) and the untreated Evans blue dye methods (Gregersen *et al.* 1935; r = 0.13; P>0.05). The PV determined at the pre-immersion baselines using the untreated Evans blue dye method was greater in magnitude, when compared to that determined using the Evans blue dye column elution method. Moreover, the untreated dye technique revealed a decline in PV during the control, thermoneutral and cold-water immersions, which was larger in magnitude than the cold-water trial. This is clearly erroneous, in both the direction and magnitude of PV change. Factors such as lipaemia and haemolysis increase absorbance at 615 nm, (the wavelength for Evans blue dye maximal absorbance), thereby altering PV (Zweens and Frankena 1981; Greenleaf and Hinghofer-Szalkay 1985; Farjanel *et al.,* 1997). While subjects were not required to fast overnight during this study, they were requested to lower fat and protein intake 24 hr before testing. Several plasma samples were haemolysed and others exhibited turbidity indicative of lipaemia, thus affecting PV determinations. Recent data from our laboratory (Fogarty, 2001) have confirmed this aspect, and contrasts with the Evans blue dye column elution technique which eliminates problems with plasma contamination (Greenleaf and Hinghofer-Szalkay, 1985; Farjanel *et al.,* 1997).

Therefore, the present study compared three Evans blue dye dilution methods and demonstrated the need for overnight fasting (Johansen *et al.,* 1992) with haemolysis-free plasma samples. To ensure accuracy and reliability a dye-extraction technique should be utilised when haemolysis or lipid turbidity is present to remove these potential contaminants which interfere with the absorbance and hence PV determination.

4.2 Characteristisation of experimental conditions

4.2.1 Environment

Air temperature averaged 21.2 ± 0.4°C over the control, thermoneutral and cold-water immersions, and was stable across the three tests (Figure 4.3: $P>0.05$), ranging from 19.7 to 22.5°C throughout the three tests. Prior to testing, it was determined that either heating or cooling the water tank produced large fluctuations in air temperature due to the venting of air from the heater-cooler unit. Therefore, water temperature was stabilised one hour prior to immersion and the heater-cooler unit was only used intermittently for short periods (<3 min) during the immersion phase to regulate water temperature. However, a slight decrease in air temperature of approximately 0.4°C was observed between commencement of immersion and 10 min. This is likely to be due to the marginal increase in water temperature during the thermoneutral immersion period that produced decreases in air temperature.

The water temperature averaged 34.5 ± 0.09°C and 18.6 ± 0.07°C for the thermoneutral and cold-water immersions, respectively (Figure 4.3). At the commencement of immersion, the thermoneutral water temperature was below 34.5°C, however the water temperature increased rapidly to the desired temperature and was constant throughout testing. This constancy is reflected in the narrow range of water temperature during the seven trials (33.4 to 34.9°C). The water temperature during the cold-water immersion was remarkably constant and ranged from 18.2 to 19.1°C. There was no significant increase in water temperature during the 60 min of immersion.

4.2.2 Thermoregulatory responses during thermoneutral and cold-water immersions

Averaged rectal temperature (T_{re}) was 37.0 ±0.07°C, 37.2 ± 0.07°C and 36.8 ± 0.27°C over 60 min of the control, thermoneutral and cold-water immersions, respectively ($P>0.05$). The cold-water stimulus evoked a strong hypothermic response (<35ºC; Giesbrecht, 2000) accounting for a 1.40°C drop in T_{re} after 60 min of immersion with a significant decline from 40 min of immersion when compared to time zero ($P<0.05$; Figure 4.4). Furthermore, T_{re} decreased significantly during thermoneutral immersion from 45 min to 60 min ($P<0.05$). In addition, two subjects (S2 and S5) with the lowest and highest recorded sum of 9 skinfold sites, 46.4 and 119.2 mm respectively, highlighted the different T_{re} responses to cold-water immersion. Subject 2 demonstrated a marked drop in T_{re} after 25 min of immersion, while subject 5 produced an attenuated decrease in T_{re} during the 60 min of immersion. The relationship between a greater decline in T_{re} and low body fat has been previously

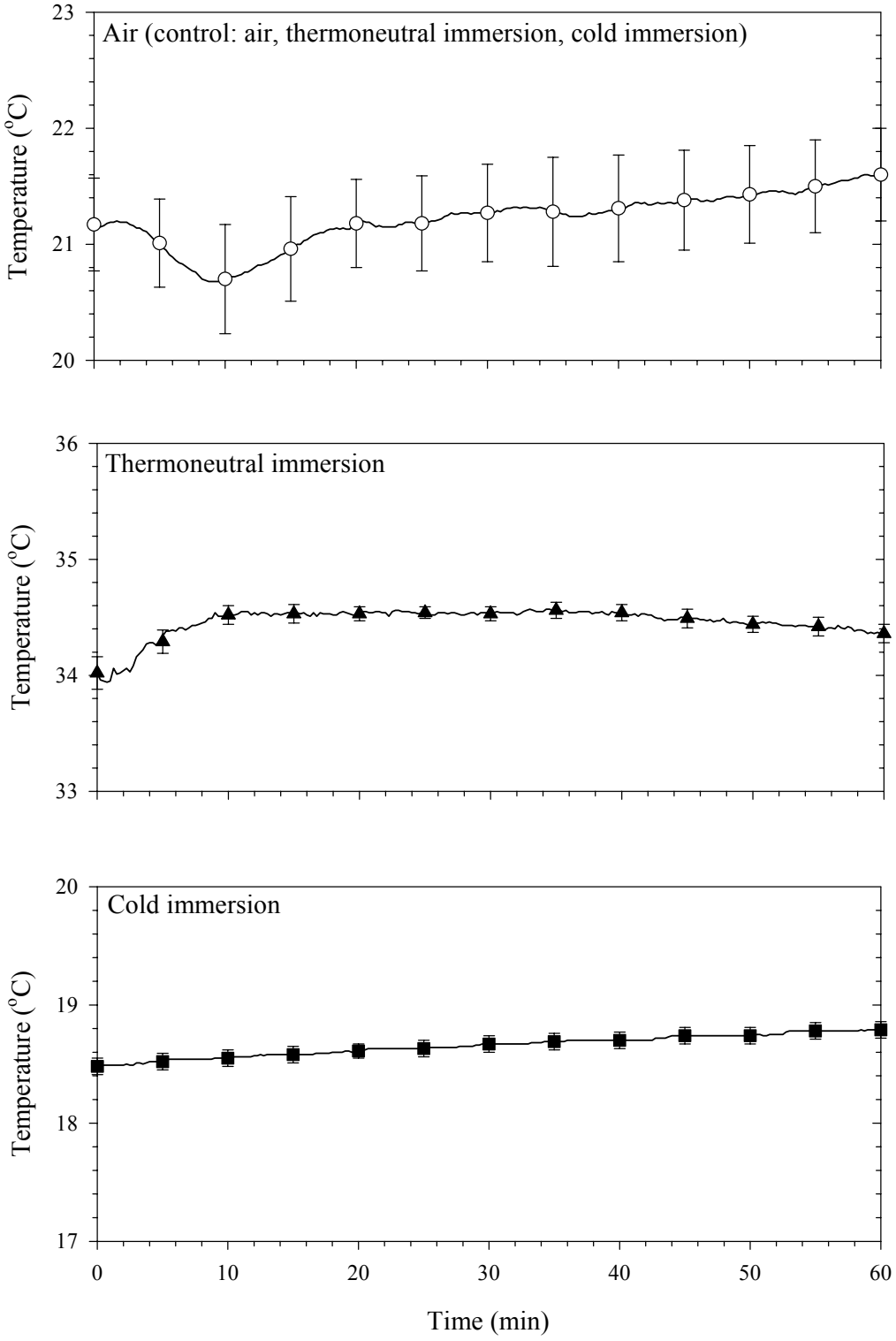


Figure 4.3: Averaged air temperature of control, thermoneutral and cold-water immersions and water temperature of thermoneutral (34.5ºC) and cold-water immersion (18.6ºC) over 60 min of testing. Data are means with standard errors of the mean.

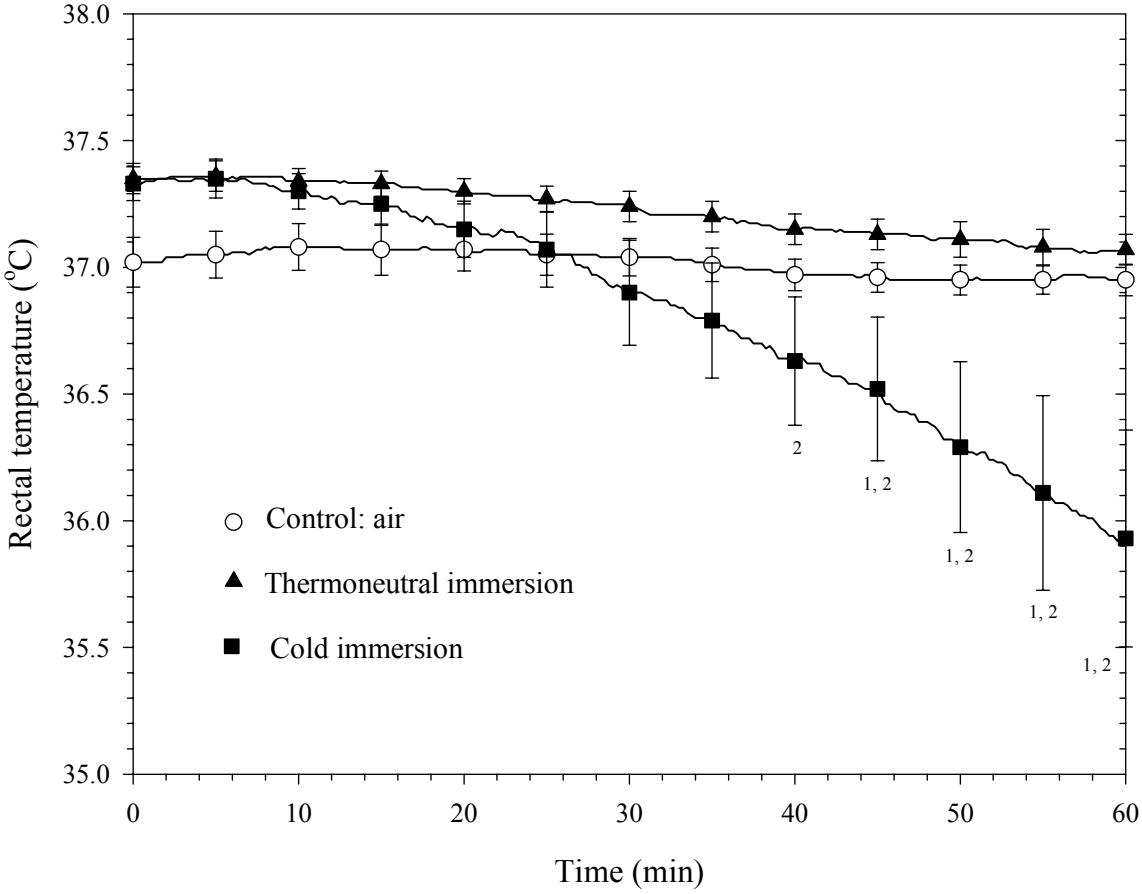


Figure 4.4: Rectal temperature during 60 min of control (air 21.2ºC), thermoneutral (34.5ºC) and cold-water (18.6ºC) immersions. Data are means with standard errors of the means. (1 = significantly different from thermoneutral: 2 = significantly different from time zero).

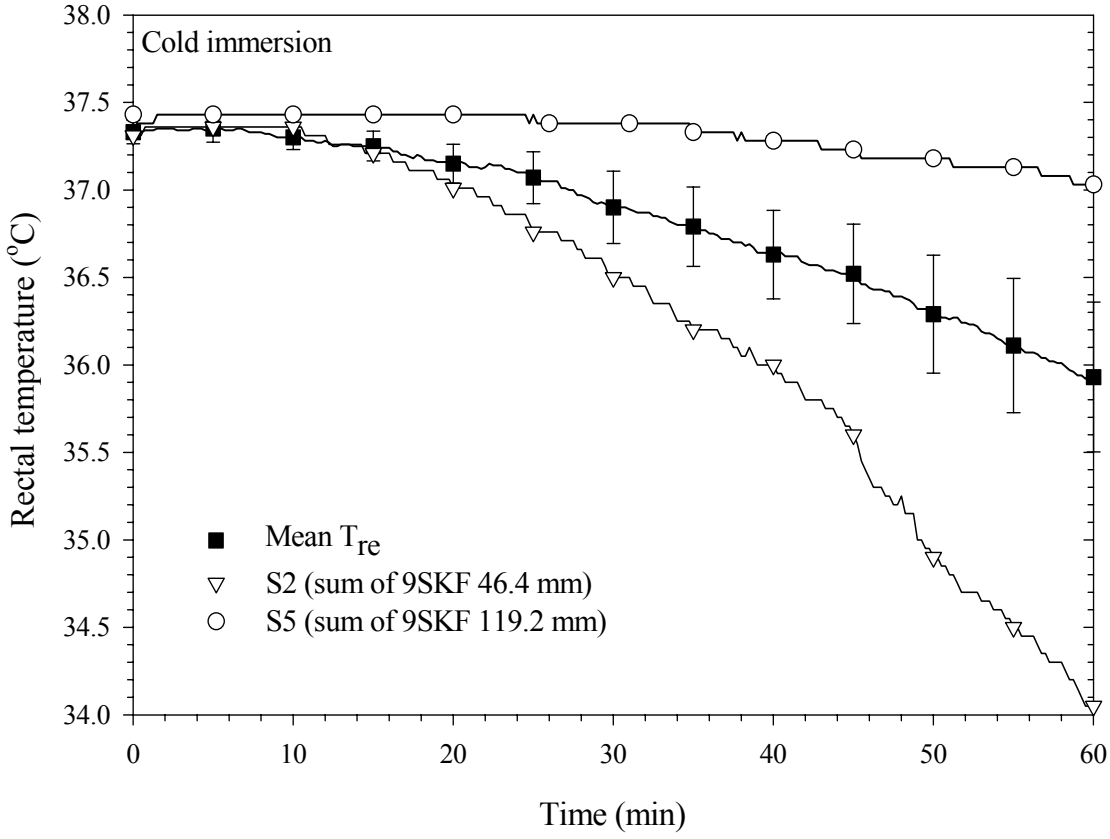


Figure 4.5: Response differences in rectal temperature (T_{re}) of two subjects (S2 and S5) with the highest and lowest sum of nine skinfold sites (Σ9SKF) and the mean T_{re} response during 60 min of cold-water immersion (18.6ºC). Data are means with standard errors of the mean.

demonstrated during cold-water studies immersion studies (McArdle *et al.,* 1984; Tikuisis *et al.,* 2000). However, linear regression analysis failed to demonstrate any relationship between T_{re} decline and the sum of 9 skinfold sites during the cold-water immersions in this study.

The majority of cold-water studies measure T_{re} only, therefore, for the purpose of this study, thermal strain was quantified using T_{re}. However, oesophageal temperature (T_{es}) commonly increases upon cold-water immersion, primarily due to cold-induced vasoconstriction (Craig and Dvorak, 1966) and then progressively declines after approximately 15 min of continuous immersion (Lee *et al.,* 1997; Regan, 1998; Zeyl *et al.,* 2001). In addition, mean skin and mean body temperatures usually show rapid decreases upon cold-water immersion and then plateau at the lower temperature for the remainder of the trial (Bittel, 1987). This is demonstrated in Figure 4.6 for oesophageal, mean skin and body temperatures during 60 min of cold-water immersion, with T_{re} responses similar to those in the current study (from Regan, 1998). Oesophageal and skin temperatures were not measured in this study, primarily because the effect is well documented and this study sought differences in thermal strain from thermoneutral immersion which are evident from the pronounced decline in T_{re} after 60 min of cold immersion.

In contrast to the cold-water immersion test, T_{re} declined by 0.07°C and 0.30°C during the control and thermoneutral tests, respectively (P>0.05). Craig and Dvorak (1966) found a small decline in T_{re} without any change in mean body temperature in 34.6°C water that conferred thermoneutrality. Therefore, in the current study, thermoneutrality was established during the thermoneutral immersion test with only a small decrease in T_{re}. These data substantiate that intravascular fluid changes during the thermoneutral test would occur by the hydrostatic pressure of the water, whereas the combined effect of hydrostatic pressure and the thermal stress of cold water may affect intravascular changes during cold-water immersion.

There was a significant increase in cardiac frequency (f_c) at the commencement of the cold-water immersion with respect to control (P<0.05; Figure 4.7). Thereafter the cold-water immersion provoked some transient bradycardia, after an initial immersion tachycardia, which is consistent with a cold-shock response (Tipton *et al.,* 1998). There was minimal variation in f_c during the control and thermoneutral immersions, oscillating between a narrow range of 10 beats·min^{-1} over the 60 min period. The mean f_c calculated over the 60-min period was 65 (± 0.2), 60 (± 0.3) and 62 (± 0.3) beats·min^{-1} for the control, thermoneutral and cold-water

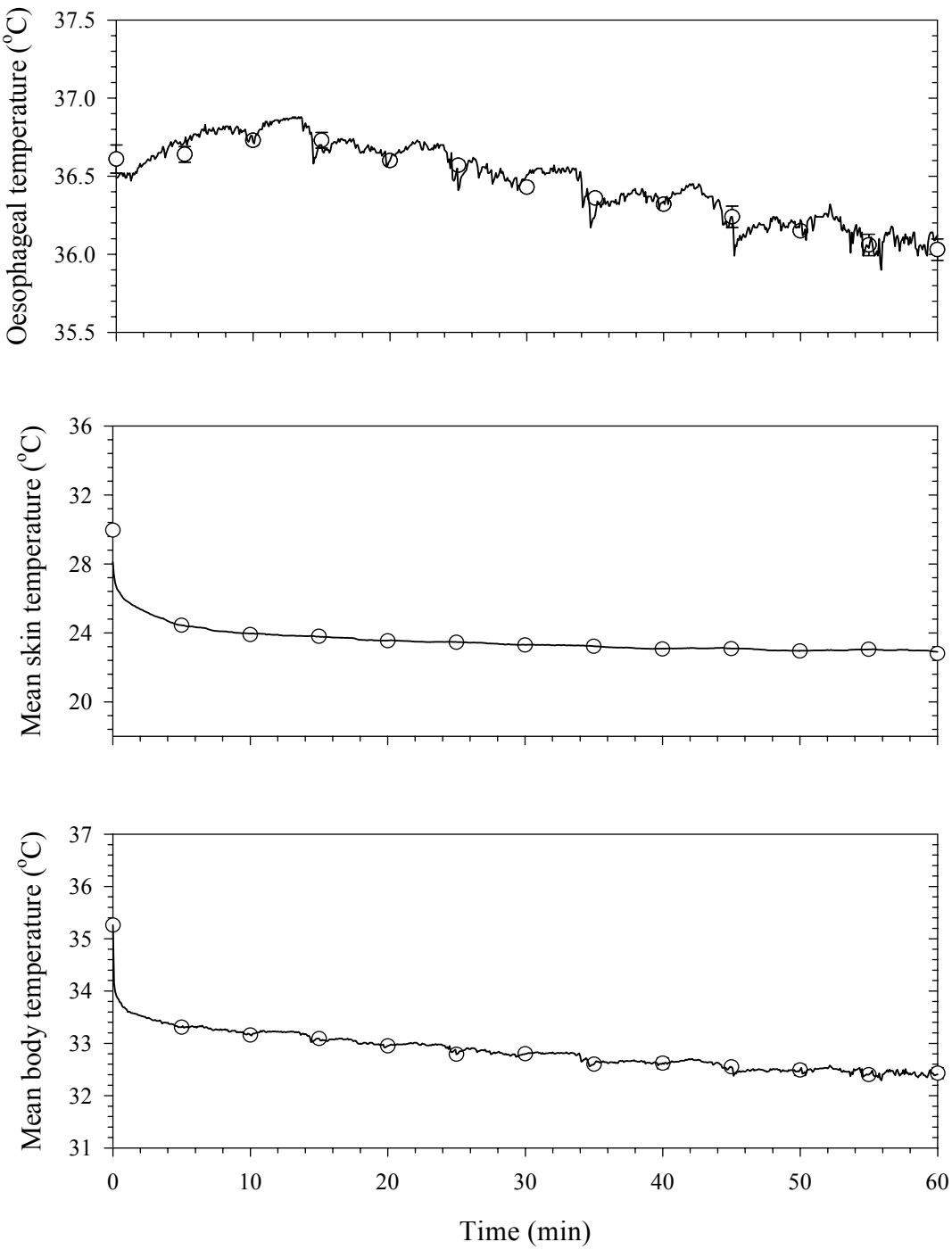


Figure 4.6: Oesophageal, mean skin and mean body temperature ($\overline{T}_b = (0.67*T_{es}) + (0.33*T_{sk})$ after Bittel, 1987) during 60 min of cold-water immersion (18.12ºC). Data are means with standard errors of the means. Averaged 5-min data represents 2 min prior to each 5-min interval. From Regan (1998).

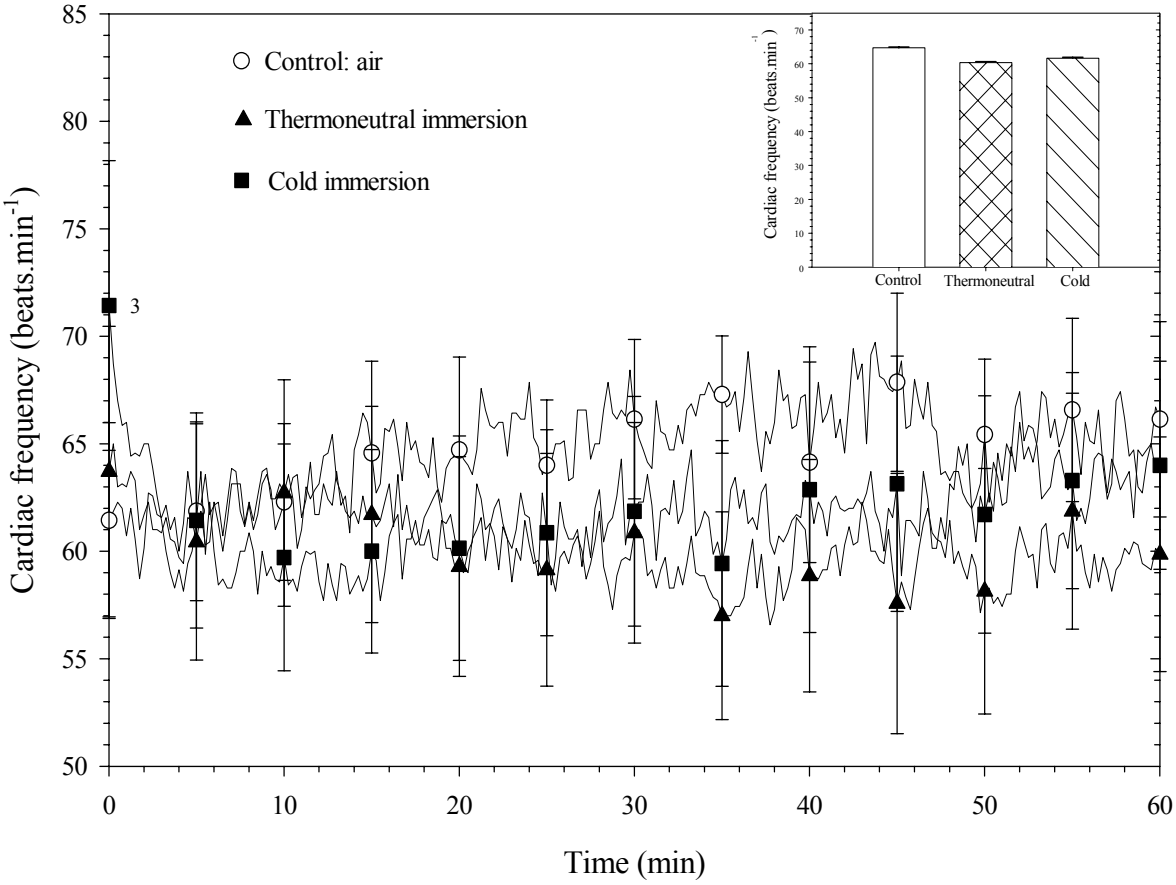


Figure 4.7: Cardiac frequency during 60 min of control (air 21.2°C), thermoneutral (34.5°C) and cold-water (18.6°C) immersions. Insert graph represents the mean cardiac frequency across the control, thermoneutral and cold-water immersion tests. Data are means with standard error of the means. (3 = significantly different from control).

immersions, respectively (P>0.05). However, thermoneutral immersion elicited intermittent bradycardia for 25.5 min of the total immersion. This response has been demonstrated in numerous studies, although the exact mechanism instigating the thermoneutral immersion bradycardia remains to be elucidated (Greenleaf *et al.,* 1981; Gabrielsen *et al.,* 1993).

4.3 Body-fluid distribution during thermoneutral and cold-water immersions

4.3.1 Body mass and urinary changes during thermoneutral and cold-water immersions

There was no significant change in body mass during 60 min of control, thermoneutral and cold-water immersions (P>0.05). Urine flow was calculated as the total urine production during the control and immersion phases of each test. The subject voided immediately prior to and upon completion of immersion. Both immersion conditions produced significant increases in urine production when compared to control, with cold-water immersion producing the greatest diuresis (both P<0.05; Figure 4.8). Furthermore, the diuresis observed during the cold immersion was significantly greater than that during thermoneutral immersion (P<0.05; Figure 4.8). Calculated urine flow ($ml \cdot min^{-1}$) was 2.0, 3.1 and 5.1 $ml \cdot min^{-1}$ for the control, thermoneutral and cold immersions, respectively.

4.3.2 Plasma volume changes during thermoneutral and cold-water immersions

Plasma volume determined by the Evans blue dye column elution method, increased significantly from 3420 (± 126) to 4083 (± 144) ml after 60 min of thermoneutral immersion. This increase represented a 16.2 (± 1.4)% change in PV (P<0.05). During the cold-water immersion, PV decreased significantly by 17.9 (± 3.0)%, from 3543 (± 128) to 3018 (± 136.7) ml (P<0.05; Figure 4.9). Furthermore, PV during thermoneutral immersion differed significantly when compared to PV during cold-water immersion after 60 min (P<0.05). Despite the difference between immersion conditions, the changes in PV during the thermoneutral and cold-water immersions could not be accounted for by changes in T_{re}, urine output, or body mass when analysed using linear regression (P>0.05).

4.3.3 Erythrocyte volume during thermoneutral and cold-water immersions

The thermoneutral and cold-water immersions produced no significant differences in RCV relative to pre-immersion volumes (both P>0.05; Figure 4.10). There was a slight decrease in RCV from 2617 (± 166) to 2549 (± 198) ml during the thermoneutral immersion and a larger decrease of 2724 (± 138) to 2472 (± 92) ml during the cold-water immersion (P>0.05). In contrast, RCV decreased significantly from 2861 (± 60) to 2510 (± 63) ml during

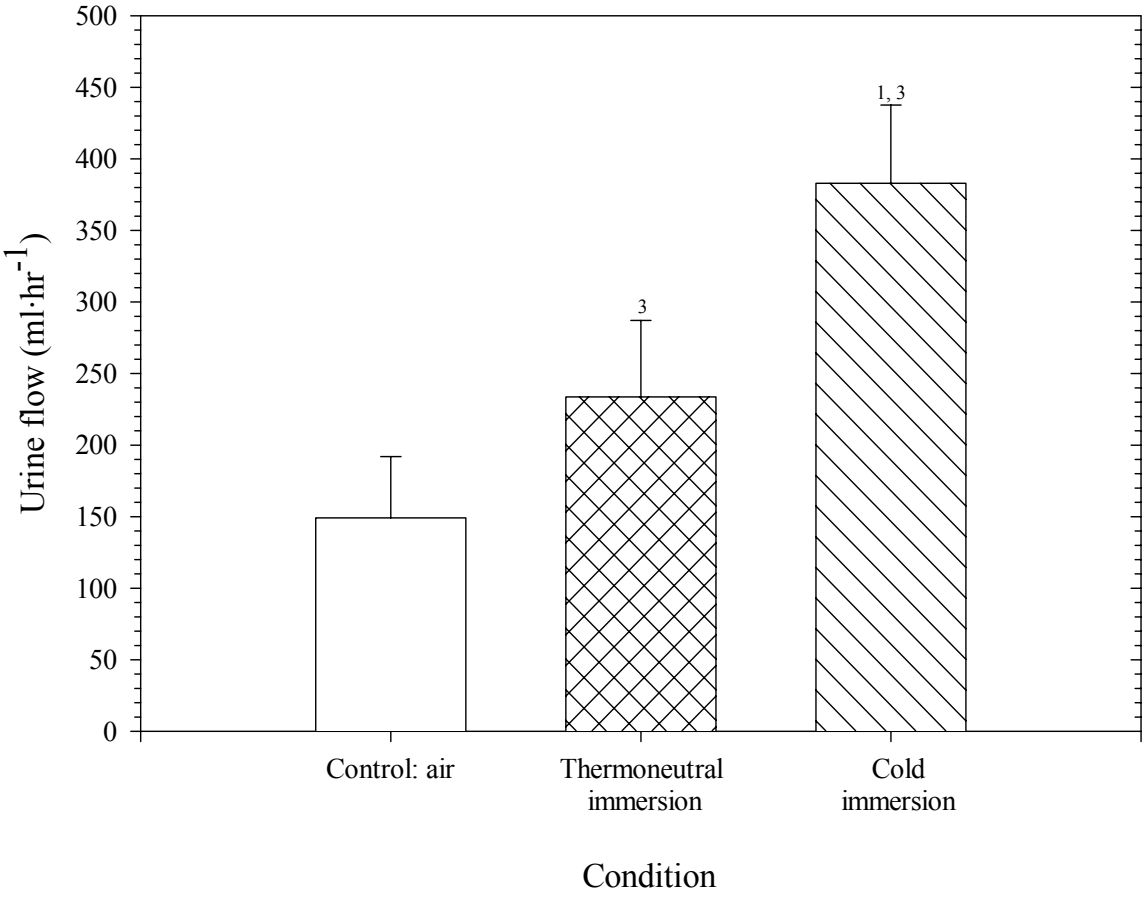


Figure 4.8: Urine flow over 60 min of control (air 21.2°C), thermoneutral (34.5°C) and cold-water (18.6°C) immersions. Data are means with standard errors of the means. (1 = significantly different from thermoneutral; 3 = significantly different from control).

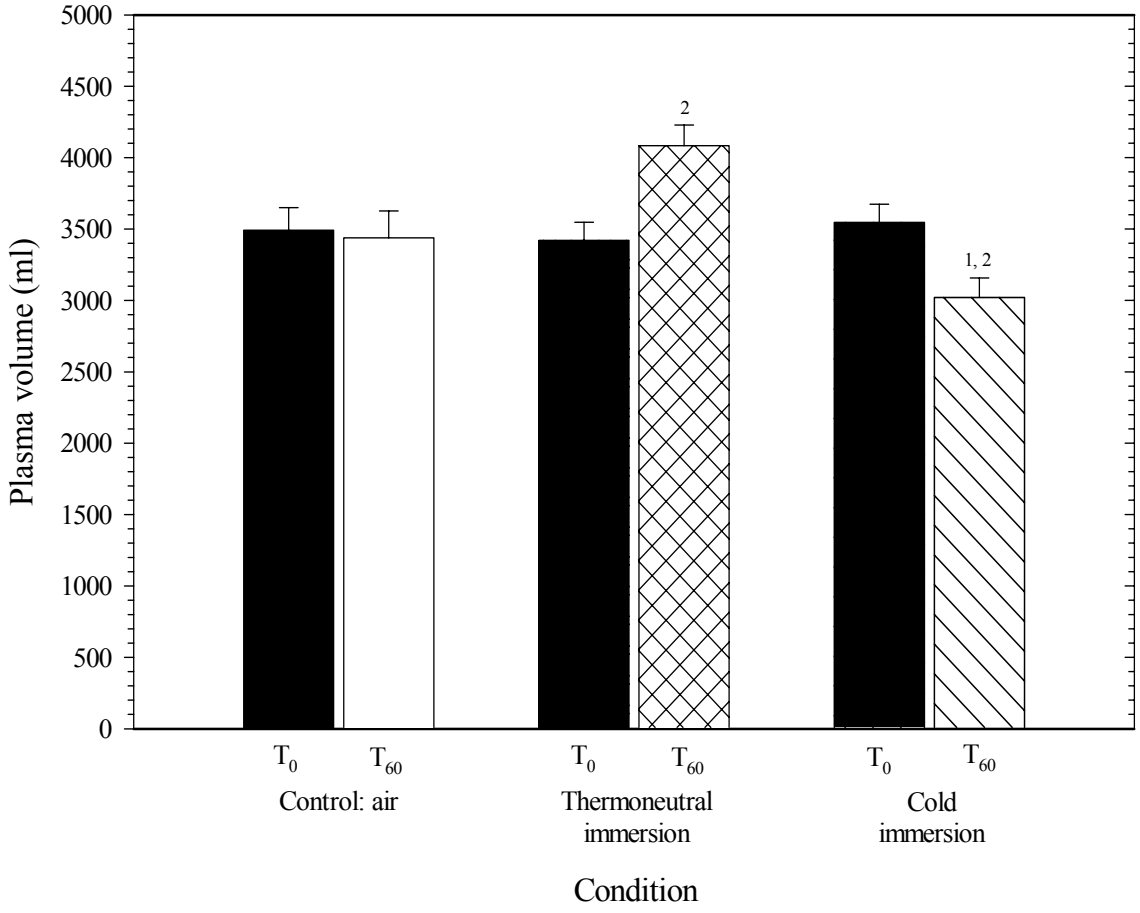


Figure 4.9: Plasma volume, determined using the Evans blue dye column elution method (Campbell *et al.,* 1958), at T_0 (time zero) and after 60 min (T_{60}) of control: air (21.2°C), thermoneutral (34.5°C) and cold-water (18.6°C) immersions. Data are means with standard errors of the means. (1 = significantly different from thermoneutral; 2 = significantly different from time zero).

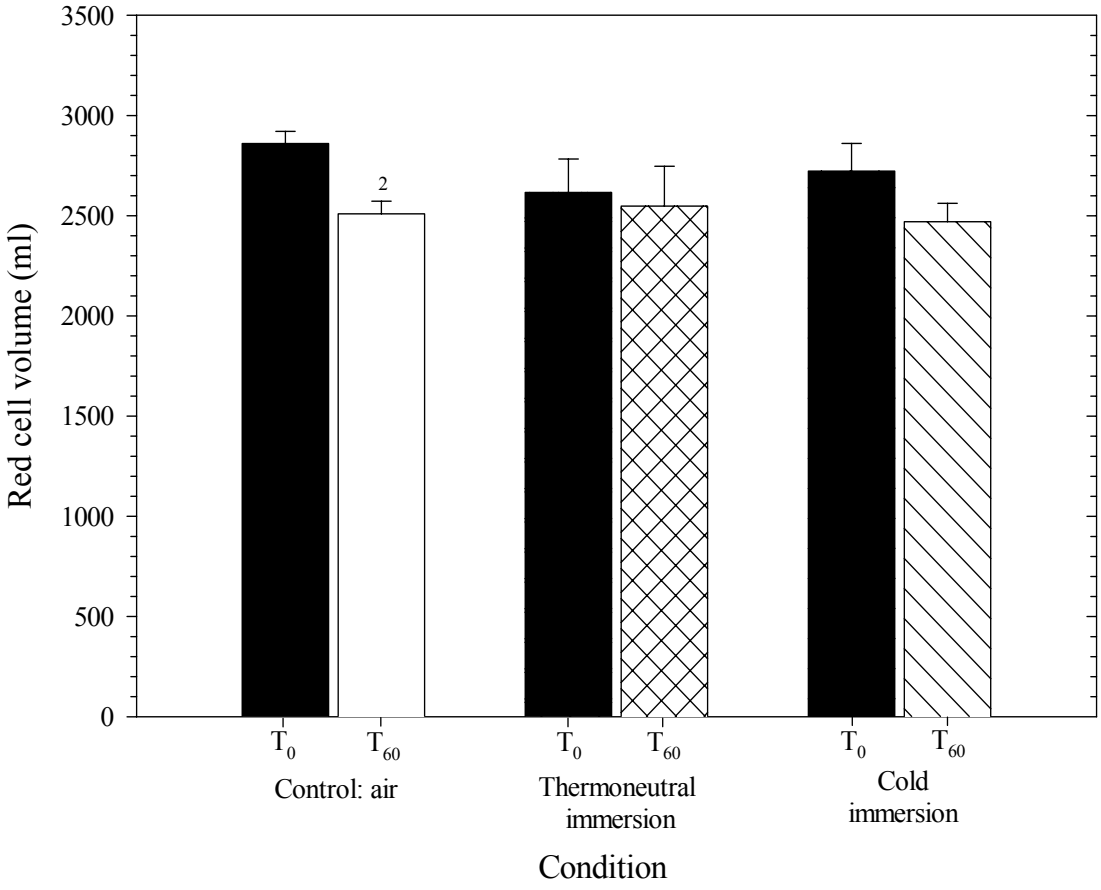


Figure 4.10: Red cell volume before (T_0) and after 60 min (T_{60}) of control: air (21.2°C), thermoneutral (34.5°C) and cold-water (18.6°C) immersions. Data are means with standard errors of the means. (2 = significantly different from time zero).

the control test (P<0.05). Furthermore, all subjects demonstrated a decline in RCV during the control, however two subjects increased RCV during the thermoneutral immersion and one subject during the cold immersion. These data demonstrate, for the first time, that RCV decreases during 60 min of upright seated thermoneutral and cold-water immersions when measured with a direct-tracer dilution method.

4.3.4 Blood volume during thermoneutral and cold-water immersions

Blood volume increased significantly after 60 min of thermoneutral immersion, from pre-immersion, by 8.6 (± 0.6)%, whereas BV decreased significantly by 14.4 (± 3.1)% during the cold-water immersion (both P<0.05; Figure 4.11). The changes in BV could be partially explained by the shifts in PV during the thermoneutral and cold immersions. A decrease in RCV during the cold-water immersion increased the magnitude of when compared to the thermoneutral immersion. Likewise, BV decreased significantly from the pre-immersion volume during the control test, despite there being no significant change in PV during the same period (P>0.05). The decrease in BV during the control may be due to the decrease in RCV observed during the control (P<0.05).

4.3.5 Indirect plasma volume change during thermoneutral and cold-water immersions

The indirect PV change, determined using the Hct/[Hb] calculation (see Section 3.5.3.2), increased significantly from pre-immersion, by 8.5 (± 0.83)% during 60 min of thermoneutral immersion (P<0.05). In contrast, PV decreased significantly by 8.0 (± 1.2)% during cold-water immersion (P<0.05; Figure 4.12). However, the indirect PV changes, during both immersion tests, were significantly different from the PV changes determined using the Evans blue dye column elution method (P<0.05). The Hct/[Hb] calculation underestimated relative PV change by 43 (± 9.1)% and 52 (± 6.8)% during thermoneutral and cold-water immersions, respectively (both P< 0.05; Figure 4.13). Moreover, the change in PV, determined using the indirect Hct/[Hb] calculation was not significantly different from that derived using the Evans blue dye column elution method during the control (P>0.05).

Despite significant discrepancies between the direct and indirect methods, during both thermoneutral and cold-water immersions, the change in PV, for both the direct and indirect methods, were highly correlated (r=0.86; P<0.05; Figure 4.14). This was across the three tests and demonstrates the homogeneity of PV difference between the two different methods. It is apparent that the indirect method underestimates PV change during water immersion and that the magnitude of underestimation is approximately 50%.

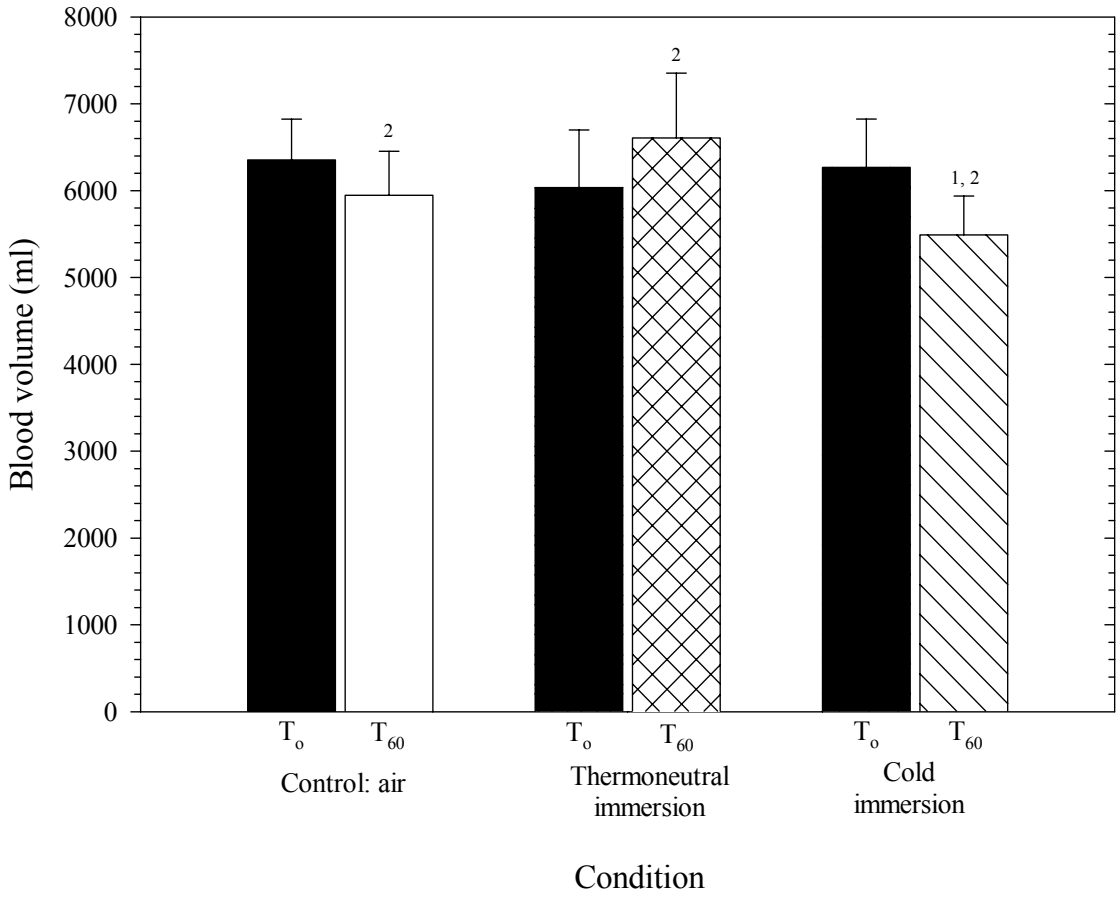


Figure 4.11: Blood volume before (T_0) and after 60 min (T_{60}) of control: air (21.2°C), thermoneutral (34.5°C) and cold-water (18.6°C) immersions. Data are means with standard errors of the means. (1 = significantly different from thermoneutral; 2 = significantly different from time zero).

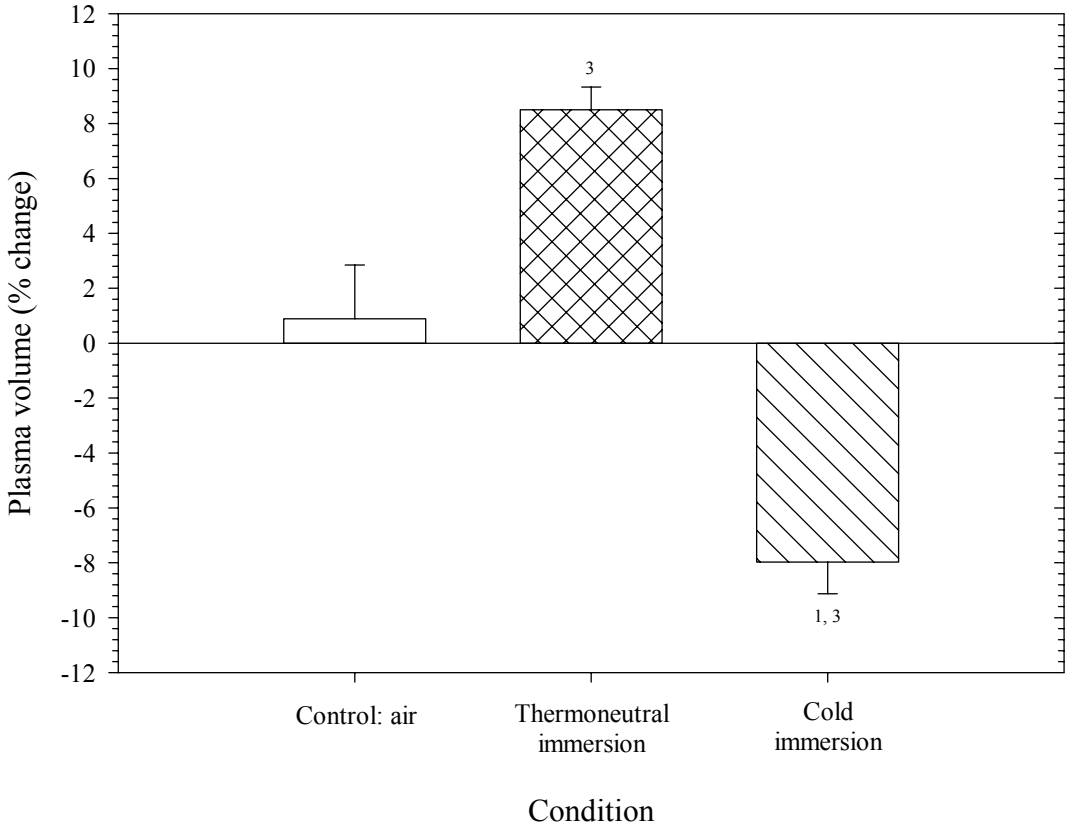


Figure 4.12: Plasma volume percentage change determined using the Hct/[Hb] method (Strauss *et al.* 1951), after 60 min of control: air (21.2ºC), thermoneutral (34.5ºC) and cold-water (18.6ºC) immersions. Data are means with standard errors of the means. (1 = significantly different from thermoneutral; 3 = significantly different from control).

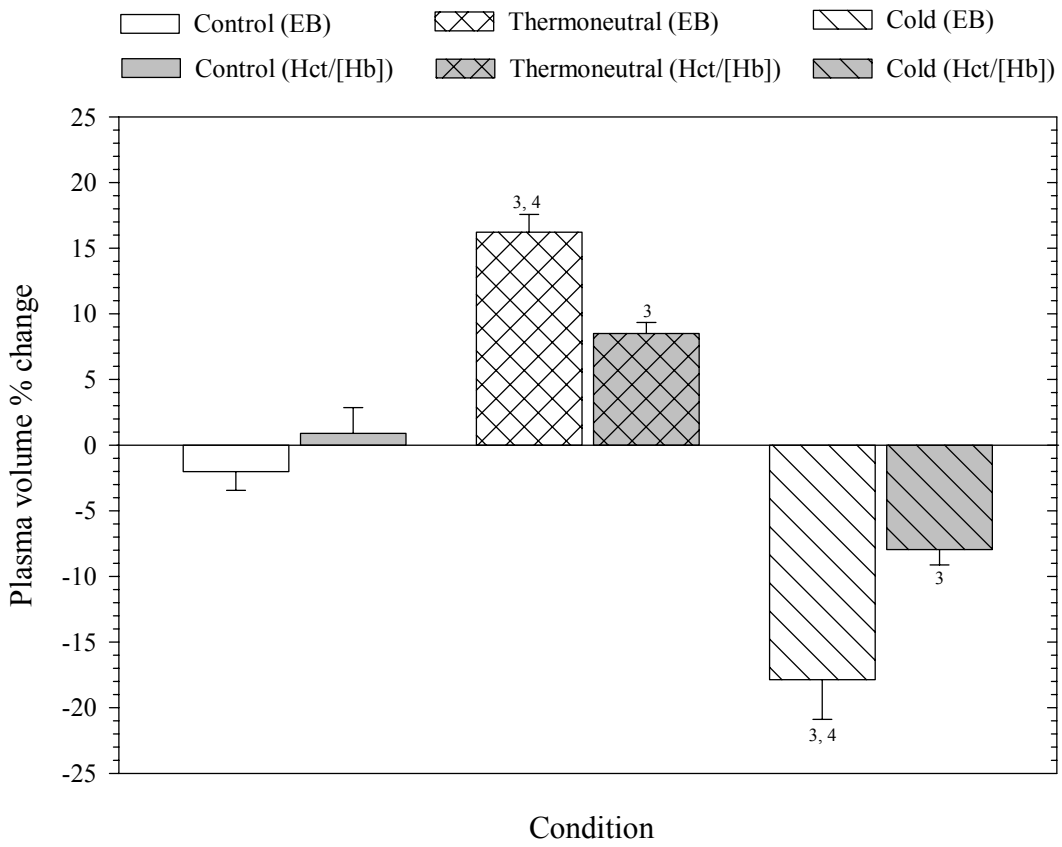


Figure 4.13: Plasma volume percentage change determined with Evans blue dye column elution (EB) and haematocrit to haemoglobin concentration ratio (Hct/[Hb]) methods. Data are PV percentage changes after 60 min of control: air (21.2°C), thermoneutral (34.5°C) and cold-water (18.6°C) immersions. Data are expressed as means with standard errors of the means.

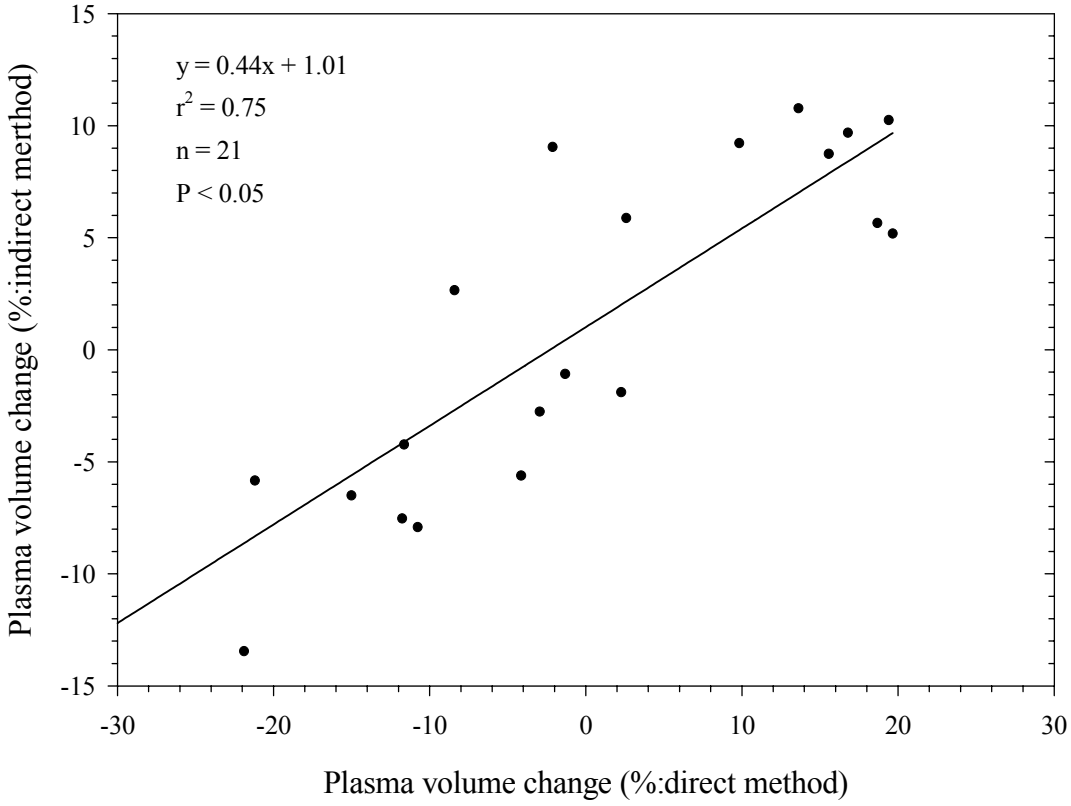


Figure 4.14: Regression of plasma volume (PV) changes measured using the Evans blue dye column elution technique (direct) and the haematocrit to haemoglobin concentration ratio (Hct/[Hb]: indirect) methods. Data are plasma volume percentage changes at baseline and after 60 min of control: air (21.2°C), thermoneutral (34.5°C) and cold-water (18.6°C) immersions.

4.3.6 F-cell ratio during thermoneutral and cold-water immersions

The pre-immersion F-cell ratio remained stable and equivalent across the three trials ($P>0.05$; Figure 4.15). Mean pre-immersion F-cell ratio was 1.08, averaged from all baseline measurements. The F-cell ratio was not significantly different between the control, thermoneutral and cold-water immersions after 60 min and averaged 1.01, 0.96 and 1.06, respectively ($P>0.05$). However, the F-cell ratio decreased significantly with respect to baseline in both the control and thermoneutral tests (both $P<0.05$; Figure 4.15). The decrease in the F-cell ratio during the control occurred despite no significant difference in PV change, determined using three different methods (Evans blue dye column elution method; Evans blue dye computer programme; Hct/[Hb] calculation).

There was considerable intra- and inter-individual variation of the F-cell ratio during the three trials (Table 4.1). For instance, the F-cell ratios calculated for Subject 1 were 1.03, 0.95, 1.01 and 0.92 for the three baselines (T_0) of control, thermoneutral and cold-water immersions and after 60 min of control, respectively. Furthermore, the F-cell ratio at rest ranged from 0.92 to 1.20 demonstrating the variability between subjects. Moreover, the F-cell ratio variability was more pronounced during both water immersions compared with the control. The F-cell ratio ranged from 0.81 to 1.14 and 0.96 to 1.17 during the thermoneutral and cold-water immersions, respectively.

The whole-body haematocrit (Hct_w), calculated from the PV and RCV, averaged 43.9 (± 1.3), 42.4 (± 1.7), 38.3 (± 1.6), and 45.1 (± 1.4)%, for the combined baseline tests (T_0), and at 60 min of control, thermoneutral and cold-water immersions, respectively (Table 4.1). In comparison, venous haematocrit (Hct_v) of the combined baselines (T_0), and at 60 min of control, thermoneutral and cold- water immersions averaged 40.9 (± 1.0), 42.0 (± 1.0), 40.3 (± 1.6) and 42.6 (± 1.4)%, respectively. Trapped plasma during the microcentrifugation process of Hct determination (see Section 3.5.2), usually reported as 4% (Chaplin and Mollison, 1952), was not corrected for during this study. Harrison *et al.* (1985) determined that the effect of measurement error is greater than the error associated with random errors.

The larger F-cell ratios were due to elevated RCV and lower Hct_v. Plasma volume measurements during both thermoneutral and cold-water immersions were consistent with literature data in both the magnitude and the percentage change (Greenleaf *et al.,* 1981; Young *et al.,* 1987; Johansen *et al.,* 1992). Despite larger RCV, when compared with prior data (Costill and Saltin, 1974; Sawka *et al.,* 1992), these volumes remain within the upper

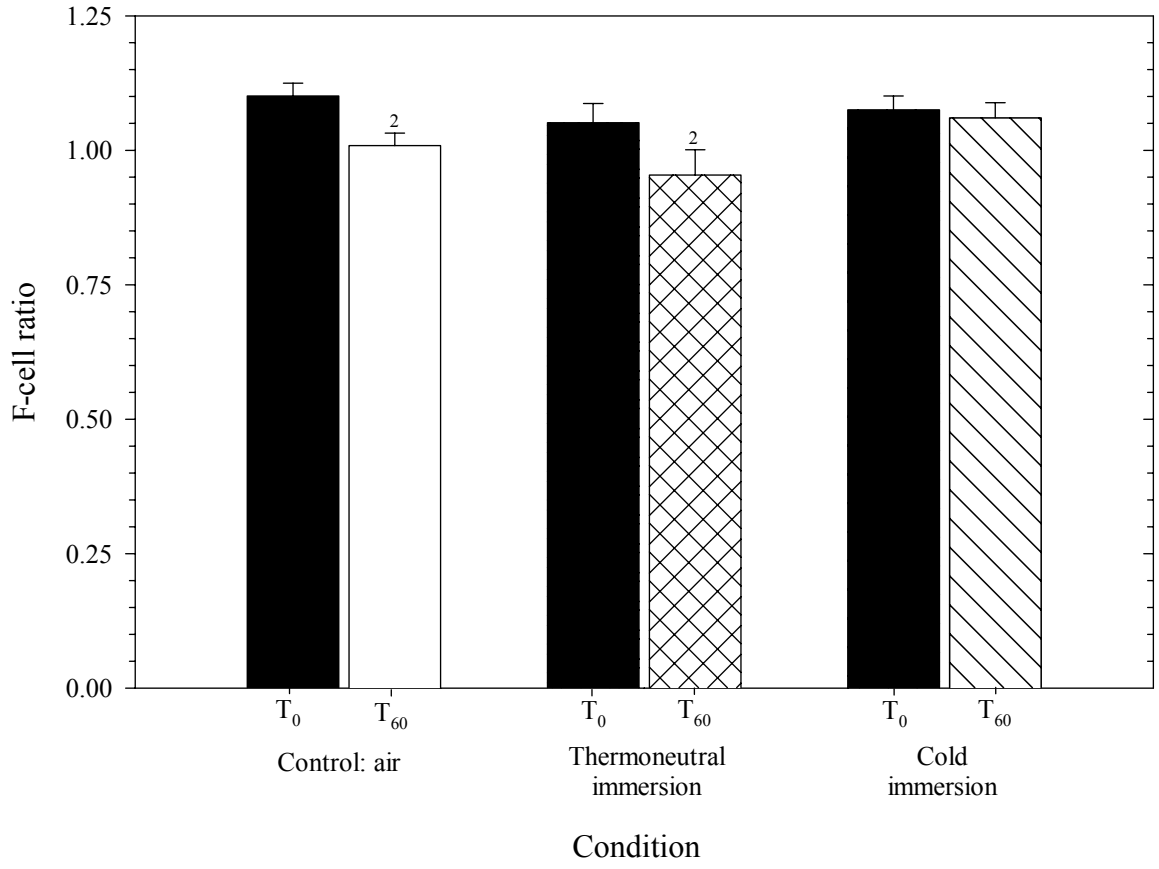


Figure 4.15: F-cell ratio, (ratio between the whole-body haematocrit and large-vessel haematocrit, where the whole-body haematocrit is derived from PV and RCV) before (T_0) and after 60 min (T_{60}) of control: air (21.2°C), thermoneutral (34.5°C) and cold-water (18.6°C) immersions. Data are means with standard errors of the means (2 = significantly different from rest).

Table 4.1 Comparison of whole-body and venous haematocrit

Condition	Subject	Hct_w	Hct_v	F-cell ratio
Baseline (T_0) (Control: air)	S1	52.1	44.7	1.17
	S2	43.5	40.1	1.09
	S3	42.5	41.1	1.03
	S4	43.4	42.5	1.02
	S5	44.5	38.4	1.16
	S6	46.6	39.9	1.17
	S7	43.6	40.7	1.07
Baseline (T_0) (Thermoneutral immersion)	S1	39.7	45.2	0.95
	S2	37.0	36.7	1.04
	S3	41.9	44.0	0.95
	S4	47.5	39.6	1.20
	S5	45.5	40.8	1.11
	S6	39.5	39.6	1.00
	S7	47.0	42.3	1.11
Baseline (T_0) (Cold immersion)	S1	45.1	44.6	1.00
	S2	38.1	33.7	1.13
	S3	41.0	40.7	1.01
	S4	48.0	40.2	1.19
	S5	43.2	39.9	1.08
	S6	42.5	40.3	1.05
	S7	47.7	44.0	1.05
Control: air (T_{60})	S1	52.6	46.7	1.12
	S2	41.0	41.1	1.00
	S3	41.0	44.6	0.92
	S4	39.3	38.8	1.01
	S5	42.2	41.3	1.02
	S6	40.4	40.3	1.00
	S7	40.7	41.4	0.98
Thermoneutral immersion (T_{60})	S1	37.0	44.7	0.83
	S2	37.1	33.3	1.11
	S3	35.7	43.8	0.81
	S4	46.6	40.8	1.14
	S5	38.8	40.0	0.97
	S6	33.5	36.4	0.92
	S7	39.4	43.1	0.91
Cold immersion (T_{60})	S1	39.4	45.3	0.97
	S2	39.8	36.3	1.10
	S3	50.8	44.2	1.06
	S4	48.6	45.4	1.07
	S5	45.9	39.1	1.17
	S6	41.2	42.9	0.96
	S7	49.2	45.2	1.09
Mean	---	42.9	41.3	1.04
±SEM	---	1.7	1.2	0.03

Abbreviations: Hct_w = whole-body haematocrit, Hct_v = venous haematocrit, SEM = standard errors of the mean, T_0 = time zero, and T_{60} = time 60 min of immersion.

recommended ranges of the International Committee for Standardization for Haematology, (1980) and are within the normal limits from other reports of similar populations (Dyrbye and Kragelund, 1970; Maw, 1994; Regan, 1998). Therefore, the red cell compartment measurements have increased the magnitude of the F-cell ratio.

CHAPTER FIVE: DISCUSSION

5. DISCUSSION

This project investigated changes in intravascular volume during thermoneutral and cold-water immersions. The purpose was twofold. First, to ascertain the influence of thermal stress and hydrostatic pressure during thermoneutral and cold-water immersion on the plasma volume (PV) and red cell volume (RCV), and hence the F-cell ratio. There was a hydrostatically-induced increase in PV during thermoneutral immersion. In contrast, PV decreased during cold immersion, presumably due to the combined effect of vasoconstriction and hydrostatic pressure. However, there was no significant change in RCV during thermoneutral and cold-water immersions, yet the F-cell ratio decreased significantly from rest during the control: air and thermoneutral immersion. To date, this is the first time that the F-cell ratio has been quantified during water immersion in humans. The second purpose was to compare three different PV methods, namely a direct, indirect and an Evans blue dye computer programme. The comparison of PV methods revealed that the Evans blue dye computer programme correlated significantly (r=0.83) with the pre-immersion baseline PV data of the Evans blue dye column elution method. However, the untreated Evans blue dye method of Gregersen *et al.* (1935) showed a low, non-significant correlation (r=0.13) with the Evans blue dye column elution method. In contrast, the indirect PV calculation method underestimated actual PV changes by approximately 50%, during both thermoneutral and cold-water immersions when compared to an Evans blue dye column elution method. Therefore, the data of the current study does not support the hypothesis that water immersion increases the F-cell ratio, despite finding differences between PV methods in thermoneutral and cold immersions. The lack of a clear response during the immersion tests does not refute these hypotheses, rather it reinforces the variability found in intravascular measurements during previous water immersion studies.

5.1 Plasma volume comparisons

5.1.1 Evans blue dye column elution and untreated Evans blue dye

The untreated Evans blue dye method (Gregersen *et al.,* 1935) demonstrated a low correlation when compared with PV determined using the Evans blue dye column elution. The most likely reason is that lipid turbidity and haemolysis interfered with the plasma samples and altered the absorbance at a wavelength of 615 nm, the wavelength of maximal Evans blue dye absorption (Greenleaf and Hinghofer-Szalkay, 1985; Farjanel *et al.,* 1997).

The subjects were not required to fast or stay in the laboratory overnight (Greenleaf *et al.,* 1980; Johansen *et al.*, 1992) thus the dietary intake could not be regulated in the 12 hr before testing. Although the subjects were required to decrease fat and protein intake in the 24-hr before testing, several plasma samples were turbid, indicative of lipaemia and fat consumption prior to testing. Furthermore, haemolysis was present in many plasma samples that compounded the measurement of Evans blue dye at a wavelength of 615 nm (Farjanel *et al.,* 1997). Thus, the untreated Evans blue dye method cannot determine PV accurately in individuals who do not either fast or lower fat intake prior to the testing. Therefore, a technique, such as the Evans blue dye column elution method, will more accurately determine PV as the method eliminates potential contaminants by extracting the dye from the albumin (Campbell *et al.,* 1958; Greenleaf and Hinghofer-Szalkay, 1985).

5.1.2 Evans blue dye column elution and computer programme

While the Evans blue dye computer method has been used extensively during water immersion and postural manipulation investigations (Johansen *et al.* 1992; Johansen *et al.* 1992; Johansen *et al.* 1998), it has not been compared directly with a dye extraction technique. In the present study, the PV determined by an Evans blue dye column elution method, agreed with data from the Evans blue dye computer programme (Foldager and Blomqvist, 1991) during pre-immersion baseline volumes and control (see Figure 4.2). To date, this is the first time that such a comparison has been made, irrespective of experimental state.

In contrast to the baseline measurements, PV determined at 60 min of thermoneutral immersion revealed a low correlation between the methods (r=0.30). Three of four blood samples were haemolysed during the thermoneutral immersion phase. The haemolysis may be due to venous stasis provoked by hydrostatically-induced peripheral vasoconstriction. Three subjects were sufficiently vasoconstricted to permit blood sampling only for the Evans blue dye column elution, Hct, [Hb] and RCV analysis. Haemolysis contaminates plasma samples (Farjanel *et al.,* 1997) and dramatically increase the absorbance of Evans blue dye at the maximal wavelength (615 nm), thus altering PV determination. Despite the Evans blue dye computer programme technique having a high tolerance for haemolysis and lipaemia, by measuring at an absorbance of 740 nm, haemolysed samples will change the PV with this method (Dr. Niels Foldager, Danish Aerospace Medical Centre, Copenhagen, Denmark, 2000; personal communication). Furthermore, the original tests of the Evans blue dye computer

programme (Johansen *et al.,* 1992; Johansen *et al.,* 1995; Johansen *et al.,* 1998), utilised a central venous catheter positioned in a large intrathoracic vein, thereby negating the effects of hydrostatically-induced peripheral vasoconstriction and venous stasis, when compared with data from a peripheral venous catheter (Ladegaard-Pedersen and Engell, 1969). In addition, the high correlation (r = 0.99: P<0.05) between the PV methods during the cold-water immersion emphasises the importance of obtaining haemolysis-free plasma samples. Blood sampling was free from haemolysis and the Evans blue dye computer programme equated well with the PV determined by the Evans blue dye column elution method after 60 min of cold immersion. Therefore, the Evans blue dye computer programme accurately reflects PV during rest and cold-water immersion, provided the plasma samples are procured without stasis, when compared with the Evans blue dye column elution method.

5.2 Thermoneutral immersion

5.2.1 Cardiovascular and thermoregulatory responses

Whilst cardiac frequency (f_c) and rectal temperature (T_{re}) were measured in this study, the primary emphasis was on the F-cell ratio and the different methods of PV determination and not the thermal effects of different temperatures during immersion. However, the cardiovascular and thermal responses to thermoneutral and cold-water immersions are well known, and the measurement of f_c and T_{re} during this study, was sufficient to determine these responses and thus elicit the thermal strain of water immersion.

Intermittent bradycardia was observed during the entire thermoneutral immersion period. Similar decreases in f_c have been reported during immersion periods as brief as five min (Miwa *et al.,* 1997) and up to eight hours (Begin *et al.,* 1976; Greenleaf *et al.,* 1980; Larsen *et al.,* 1994). Thermoneutral immersion causes an increase in intrathoracic blood volume due to central translocation of peripheral blood by hydrostatic compression of the water (Christie *et al.,* 1990; Epstein, 1996). This augments cardiac output and preload, primarily from increases in stroke volume (Arborelius *et al.,* 1972; Risch *et al.,* 1978). The increase in central venous pressure recorded in several studies, distends the intrathoracic vessels and the atria of the heart, stimulating the baroreceptors resulting in reduction of f_c (Norsk *et al.,* 1986; Gabrielsen *et al.,* 1993).

The differing response shown in rectal temperature (T_{re}) during the three tests was due to the thermal stress. There was no change in T_{re} while seated upright in ambient air of

21.0ºC. Despite a decrease of 0.3ºC in T_{re} after 60 min of thermoneutral immersion, water temperature of 34.5ºC normally elicits thermoneutrality. Numerous studies have confirmed this phenomenon during immersion periods of one hour (Ertl *et al.,* 1991) and longer (Greenleaf *et al.,* 1981). Regan (1998) observed a drop of 0.4ºC in T_{re} in resting subjects immersed in 33.3ºC water, however, oesophageal temperature remained stable over the immersion period suggesting thermoneutrality. Craig and Dvorak (1966) found that a water temperature of 34.6ºC conferred neutrality, however water temperatures below 35ºC decreased T_{re} during immersion.

5.2.2 Intravascular changes

In the present study, there was a significant increase in PV of 16.2 ± 1.4% after 60 min of thermoneutral immersion. An increase in PV of the same magnitude has previously been reported utilising a direct tracer-dilution technique similar to the method of PV determination in this study (Johansen *et al.,* 1992; Johansen *et al.,* 1995). Data from previous investigations suggest the increase in PV during thermoneutral immersion may result from a net decrease in the hydrostatic pressure gradient across the capillary wall (Miki *et al.,* 1986). This has been demonstrated in dogs during water immersion, with the magnitude of increase in tissue pressure surpassing that of capillary pressure, thereby refuting the von Diringshofen (1948) postulate that a decrease in the arteriovenous pressure results in haemodilution (Miki *et al.,* 1986; Miki *et al.,* 1987a; Miki *et al.,* 1987b). Furthermore, the depth of immersion has been shown to influence the degree of PV expansion in the dog (Miki *et al.,* 1989) with increases in interstitial fluid pressure proportional to the column of external water pressure. This has not been demonstrated in humans, although gradual increases in central venous pressure have been associated with graded water immersion to the neck, indicating an increase in central blood volume (Arborelius *et al*., 1972; Gabrielsen *et al.,* 1993). This condition suggests that PV expansion in humans increases according to the immersion depth.

The magnitude and time course of PV expansion during immersion has recently been investigated with continuous measurements of blood density (Yamazaki *et al.,* 2000). The authors found that PV increased during thermoneutral immersion, to the level of the neck, immediately upon immersion and continued to increase to 20 to 25 min, reaching a plateau level when diuresis commenced. Miki *et al.* (1986) observed a peak PV increase in dogs after 35 min of immersion and a consequent diuresis from 20 to 40 min. Although the present study determined PV after one hour of immersion, it is likely that maximal PV expansion had

occurred earlier, since a significant diuresis was evident from post-immersion urine volumes (see Figure 4.8). These volumes were calculated over the entire immersion phase, and are similar to those reported in previous studies (Bœning *et al.,* 1972; Johansen *et al.,* 1995).

Although there is general consensus that short-term thermoneutral immersion elicits an increase in PV, the magnitude of change remains speculative (Greenleaf, 1984). Plasma volume, when calculated from measurements of haematocrit (Hct) and haemoglobin concentration ([Hb]), have varied between 7.2 and 13.5% (McCally, 1964; Harrison *et al.,* 1986; Miki *et al.,* 1986; Ertl *et al.,* 1991) during short-term thermoneutral immersion. Clearly, there is a lack of consistency among these studies with differences in PV changes previously attributed to variations in the experimental protocol and postural changes (Hagan *et al.,* 1978; Lundvall *et al.,* 1994). However, differences in PV determined by the indirect method, utilised in the majority of studies, have been shown to underestimate actual PV change during short-term thermoneutral immersion (Johansen *et al.*, 1992; Johansen *et al.*, 1995). Greenleaf *et al.* (1983) were the first to demonstrate disproportionate increases in PV during thermoneutral immersion. They found that PV increased by 8.8%, using an Evans blue dye dilution method, but increased by only 4.3% with the Hct/[Hb] calculation after 20 min of immersion, representing an approximate 50% underestimation of PV by the Hct/[Hb] calculation. Although the magnitude of PV expansion was greater in the current study, PV increased by 8.5% when determined using the Hct/[Hb] calculation which underestimated relative PV change by 43% ($P<0.05$), when compared to the Evans blue dye column elution method. In agreement with data from the current study, Johansen *et al.* (1992) have shown that the Hct/[Hb] calculation underestimated actual PV changes by approximately 50% during thermoneutral immersion, when compared to an Evans blue dye dilution technique (Foldager and Blomqvist, 1991).

One possible explanation for the underestimation of PV is the efficacy of the Hct/[Hb] calculation to correctly determine PV change. Several authors have commented on the inaccuracy of utilising concomitant changes in Hct/[Hb] to determine PV changes during different interventions, such as immersion and posture changes (Harrison, 1985; Hinghofer-Szalkay *et al.,* 1987; Johansen *et al.,* 1998; Lundvall and Lindgren, 1998). There are several assumptions that need to be satisfied for the validity of the Hct/[Hb] calculation to accurately determine PV change (Harrison *et al.,* 1986; Johansen *et al.,* 1998). For instance, the RCV and the F-cell ratio must remain stable throughout the experiment. Red cell volume has been

postulated to remain constant during immersion, yet this has only been shown from indirect calculations rather than direct determination (Greenleaf *et al.,* 1979). During the present study, there was no change in the RCV measured directly (see Figure 4.10), during the thermoneutral immersion. To our knowledge, this is the first time that the RCV has been determined directly in humans with a radioisotope during thermoneutral immersion. Constancy in the RCV has been demonstrated in dogs immersed to the neck, using ^{51}Cr-labelled erythrocytes measured continuously with an extracorporeal circuit that circulated blood through a gamma radiation counter (Miki *et al.,* 1986). Furthermore, Costill and Saltin (1974) reported a constant RCV after 4% body mass dehydration, thus confirming the stability of the RCV during different interventions. Therefore, changes in the F-cell ratio may account for the significant difference between the indirect and direct methods of PV determination during thermoneutral immersion.

5.2.2.1 F-cell ratio

While the effects of PV changes during environmental stress have been examined extensively, there are few human studies that have quantified the F-cell ratio during such stress. The scant available studies have explored these changes during heat and exercise (Costill and Saltin, 1974) and postural manipulations in different thermal environments (Maw, 1994). To our knowledge, no human studies have directly measured the F-cell ratio during water immersion and therefore, presumed F-cell ratio changes have only been theorised. Several authors have hypothesised an increase in the F-cell ratio may explain PV discrepancies, when determined indirectly with changes in Hct/[Hb], during thermoneutral and cold-water immersions (Harrison *et al.,* 1986; Young *et al.,* 1987; Johansen *et al.,* 1992). The concept is probably due to volume of erythrocytes in the peripheral vasculature, especially the capacitance vessels where there are relatively fewer cells compared to the plasma (Fähræus, 1929; Fähræus and Lindqvist, 1931). When the peripheral vasculature is exposed to the hydrostatic and thermally-mediated pressure of water immersion, the microcirculation will be compressed and the resultant Hct_v will be relatively lower than the Hct_w, thus increasing the F-cell ratio (Harrison *et al.,* 1986; Hinghofer-Szalkay *et al.,* 1987). However, despite large discrepancies in PV change between the Evans blue dye column elution method and the Hct/[Hb] calculation in thermoneutral immersion, there was no increase in the F-cell ratio in the present study. In fact, there was a significant decline in the F-cell ratio during the control and thermoneutral immersion. This is the first time that a reduction in the F-cell ratio has been found, and is difficult to explain when the overwhelming

body of evidence is in favour of a net gain in the F-cell ratio, induced by a decreased Hct_v, and mediated by the hydrostatic pressure of water. Furthermore, the F-cell ratios are close to unity, which is generally larger than those reported in the literature.

One possible reason for the increase of the resting and control F-cell ratios is the variability. Najean and Deschrywer (1984) determined F-cell ratios in a hospital population, to be slightly lower than the literature, whereas Maw (1994) found an increase in the mean F-cell ratios in a cohort similar to that of the present study. The variation in the F-cell ratio of 0.84 to 1.10 reported by Maw (1994), were similar to that measured in the current study (0.81-1.20). Furthermore, there was considerable intra-individual variation within subjects between tests. For example, one subject during baseline of the three conditions and the control, demonstrated F-cell ratios of 1.03, 1.01, 0.95 and 0.92, respectively. The most likely explanation is that blood flow dynamics change considerably between subjects, and are not consistent (Maw, 1994). In addition, there are clinical conditions, such as splenomegaly, that are capable of producing an F-cell ratio close to one (Chaplin *et al.,* 1953). However, it seems highly unlikely that the subjects in the present study exhibited this condition as they were prescreened for metabolic and cardiovascular disease.

The F-cell ratios determined in the present study may be attributable to the relatively large RCVs and comparative lower Hcts. Red cell volumes were higher than reported previously (Hicks *et al.,* 1956; Wennesland *et al.,* 1959; Retzlaff *et al.,* 1969; Sawka *et al.,* 1992), yet within the upper range of relative fluid volumes as determined by the International Committee for the Standardization of Haematology (1980). The three resting relative RCVs were 35.3, 32.2 and 33.5 $ml \cdot kg^{-1}$ compared with 17 to 38 $ml \cdot kg^{-1}$ reported by Dyrbye and Kragelund (1970) and 33 $ml \cdot kg^{-1}$ advocated by the International Committee for the Standardization of Haematology (1980). Furthermore, the magnitude of the RCVs are equivalent to those reported by Maw *et al.* (1995), obtained utilising a similar radioisotope method. Additionally, Hct_v were lower in the current study than previous resting studies (Gibson, 1946; Huff and Feller, 1955; Sawka *et al.,* 1992) and this is reflected in increased F-cell ratios close to unity. Furthermore, relative pre-immersion baseline PV averaged 42.9 $ml \cdot kg^{-1}$, in accordance with data in the literature (Bentley and Lewis, 1976; International Committee for the Standardization of Haematology, 1980; Pearson *et al.,* 1995; El-Hemaidi *et al.,* 1997) thus the magnitude of the F-cell ratio was increased due to the red cell compartment.

One possible explanation for the decrease in the F-cell ratio during the control may result from postural factors. Previously, the F-cell ratio has been found to be homogenous in hospital patient populations (Gibson *et al.,* 1946; Chaplin *et al.,* 1953; Fudenburg *et al.,* 1961) measured in a supine posture, which confers greater stability in Hct distribution (Lundvall and Bjerkhoel, 1994). Maw (1994) found inconsistent F-cell ratios between individuals in an upright posture, suggesting that blood flow dynamics were responsible. In addition, blood sampled from an arm catheter during upright sitting may reflect local dependent Hct changes rather than a total body circulatory shift (Lundvall and Bjerkhoel, 1995). These findings imply that upright seated subjects may exhibit variations in intravascular dynamics, such as the F-cell ratio, independent of actual change when compared to resting supine subjects. However, the position of the arm utilised for blood sampling in the resting phase of the present study was at heart level and slightly below the axillary crease during the control and immersion phases, thus negating the effects of hydrostatic gradients (Johansen *et al.,* 1998) affecting the F-cell ratio.

In seems logical to explore the role of the spleen in changing the F-cell ratio. The spleen contains a reservoir of erythrocytes that are ejected into the circulation at times of stress. There are no studies of splenic contraction during resting water immersion for comparison. However, Hurford *et al.* (1990) found Korean Ama divers exhibiting splenic contraction after sustained periods of breath-hold diving in 25ºC water to a depth of approximately 6 metres. Splenic contraction has been demonstrated during 5 min of maximal recumbent cycle ergometry (Sandler *et al.,* 1984) and splenic emptying during graded bicycle exercise (Laub *et al.,* 1993). Furthermore, Reeve *et al.* (1953a) induced splenic dilation and contraction in dogs after injections of nembutal and adrenaline, and observed a reduction in the F-cell ratio. The authors conclude that a discharge of pooled erythrocytes from the spleen, approximately 18–29% of the dog's total RCV into the large vessels, increased the Hct_v relative to the Hct_w, thus lowering the F-cell ratio. However, for the spleen to contract during immersion, a sufficient sympathetic stimulus would be required (Chen and Chien, 1977), contrary to the usual parasympathetic activity elicited during thermoneutral immersion (Connelly *et al.,* 1990; Miwa *et al.,* 1997). It is doubtful whether the volume of splenic blood needed to increase the Hct_w is sufficient to lower the F-cell ratio. Therefore, the release of a reserve of pooled erythrocytes induced by splenic contraction, thus lowering the F-cell ratio, is dubious during thermoneutral immersion.

5.3 Cold-water immersion

5.3.1 Cardiovascular and thermoregulatory responses

In the present study, f_c increased significantly upon cold-water immersion compared to control (see Figure 4.6). Similar increases in f_c during the first minute of cold-water immersion have been observed in equivalent conditions (Keatinge and Evans, 1961; Tipton *et al.,* 1998a). The initial increase in f_c during cold-water exposure has been attributed to noradrenaline release (Keatinge and Evans, 1961), or activation of thermoreceptors that increase the activity of the sympathetic nervous system (Šrámek *et al.,* 2000). However, Tipton *et al.* (1998b) found the initial response of cold-water immersion to be mediated by central rather than peripheral receptors. Following the initial increase in f_c, bradycardia was sustained between 10 and 15 min and oscillated between a narrow band of 7 beats·min^{-1} for the remainder of the immersion period. Vogelaere *et al.* (1995) found similar f_c responses during cold-water immersion and suggested two possible mechanisms, either vagal stimulation from general nociceptive activation, or peripheral vasoconstriction increasing blood pressure. The stimulation of baroreceptors during cold-water immersion increases blood pressure and is likely to cause a reflex bradycardia (Strong *et al*., 1985).

There was a continuous decrease in T_{re} during the cold-water immersion period with significantly lower T_{re} compared to thermoneutral immersion during the last 20 min that indicated body core cooling. Thermal strain is increased during cold-water immersion due to the greater conductivity of water by increasing the thermal gradient between the body and the environment (McArdle *et al.,* 1984; Young *et al.,* 1987). Rectal temperature declined at a rate of 1.40ºC·hr^{-1}, with 50% of the reduction occurring during the last 20 min of immersion. Lee *et al.* (1997) and Castellani *et al.* (1998) found similar decrements in T_{re} after 60 min immersions in 15ºC and 20ºC water. Therefore, cold-water immersion provides a strong thermal stress, that is evident in the decrease in T_{re} when compared to the thermoneutral and control conditions.

5.3.2 Intravascular changes

Plasma volume data during cold immersion are meagre, however the few studies available have demonstrated decreases in PV that are greater in magnitude than observed on exposure to cold air (Young *et al.,* 1987; Vogelaere *et al.,* 1992). Plasma volumes determined during cold-water immersion, measured indirectly with the Hct/[Hb] calculation, have varied between 7.1% and 17.6% (Rochelle and Horvath, 1978; Young *et al.,* 1987; Regan, 1998). In

the current study, there was a significant reduction in PV of 17.9 ± 3.0%, measured for the first time with a direct tracer-dilution method. The magnitude of this PV reduction is in agreement with other data from other studies using the indirect method (Rochelle and Horvath, 1978; Young *et al.,* 1987; Deuster *et al.,* 1989).

The mechanism responsible for a reduction in PV during cold exposure is unclear, yet responses that arise from the cold stress may be responsible. There is an increase in intrathoracic BV, due to hydrostatically-induced compression of the peripheral microvasculature and intense vasoconstriction, centralising blood flow and volume (Park *et al.,* 1999) thus provoking diuresis similar to that observed during thermoneutral immersion (Stadaeger *et al.,* 1992). Rochelle and Horvath (1978) speculated, in a group of surfers and non-surfers immersed for one hour in 19ºC water, that the PV reduction was due to diuresis, since PV loss approximated urine loss. In the present study, the significant diuresis of 5.1 $ml \cdot min^{-1}$ was greater than the thermoneutral immersion diuresis. However, Young *et al.* (1987) found no direct relationship between diuresis or shivering and PV change during acute cold-water exposure.

Another possible mechanism for the hypovolaemia is an haemodynamic change induced by the cold stress. Cold water produces intense vasoconstriction (Bonde-Petersen *et al.,* 1992), which increases peripheral resistance and consequently blood pressure (Janský *et al.,* 1996), altering the equilibrium of the capillary wall fluid flux and favouring a net shift of fluid from the intravascular to the interstitial spaces (Vogelaere *et al.,* 1992), thus inducing haemoconcentration. However, this hypothesis has yet to be substantiated.

In the present study, PV decreased by 8.0%, when measured by the Hct/[Hb] calculation which is less than previous studies (Rochelle and Horvath, 1978; Young *et al.,* 1987; Deuster *et al.,* 1989). However, the Hct/[Hb] calculation underestimated PV change by 52% ($P<0.05$) when compared to the Evans blue dye column elution method. This is the first time that PV changes during cold immersion have been compared between methods, and suggests the Hct/[Hb] calculation may not represent the actual PV change. As mentioned previously, the Hct/[Hb] calculation may be erroneous if the RCV shifts or the affinity of the Hct_v to Hct_w changes (Harrison *et al.,* 1982). A constant RCV occurs in normal and splenectomized dogs cooled to a body core temperature of 26ºC (Chen and Chien, 1977) determined with a direct tracer-dilution radioisotope measure. During the present study, it has

been shown for the first time in humans that RCV remains constant during cold immersion as measured by $Na^{51}Cr$, verifying the assumption inherent to the validity of the Hct/[Hb] calculation (Johansen *et al.,* 1998). However, Young *et al.* (1987) suggested the magnitude of PV change may be underestimated by changes to the peripheral vasculature induced by intense vasoconstriction during cold-water stress, reducing the vascular volume of the peripheral vessel and lowering the Hct_v, thus increasing the F-cell ratio (Harrison *et al.,* 1986). In accordance with a lower Hct_v, Knight *et al.* (1986) found regional differences in Hct_v by comparing immersed and non-immersed Hcts during upright immersion in 28ºC water and proposed an alteration in the F-cell ratio as the possible cause. Therefore, PV determined with the Hct/[Hb] calculation underestimates actual PV change, when compared to a direct tracer-dilution method and suggests evidence for an increased F-cell ratio.

5.3.2.1 F-cell ratio

While the present study established PV underestimation of the Hct/[Hb] calculation when compared with a direct tracer-dilution method and highlighted RCV constancy, the F-cell ratio did not increase as anticipated during cold-water immersion (Young *et al.,* 1987). In fact, the F-cell ratio remained constant and was in contrast to the decrease found during the control and thermoneutral tests. A stable F-cell ratio has been observed in cooled dogs (Chen and Chien, 1977). However, hypothermia in the dogs was achieved by covering the body surface with ice-filled plastic bags and shivering thwarted with drug administration, which is quite different from the hydrostatic and thermal stress applied during upright seated cold-water immersion in humans. For instance, the surface area covered by the water is greater and thermal conductivity is approximately 25 times that of air (Toner and McArdle, 1996). Furthermore, cold water exerts hydrostatic pressure (Rochelle and Horvath, 1978). Therefore, despite agreement of F-cell constancy between the studies, the differences in methodology and canine and human physiology precludes inferring unequivocal conclusions.

Another possible explanation is the release of pooled erythrocytes from the spleen negating the hydrostatic and thermally-mediated changes provoked during cold immersion. Splenic contraction occurs during maximal exercise (Laub *et al.,* 1993), adrenaline administration in dogs (Reeve *et al.,* 1953a, 1953b), and during extended breath-hold diving in Korean Ama female divers (Hurford *et al.,* 1990) which induce adrenergic responses to stress. Similarly, increases in sympathetic tone as measured by noradrenaline release have been observed in humans during cold immersion (Young *et al.,* 1987; Janský *et al.,* 1996).

Therefore, erythrocyte discharge from the spleen upon cold immersion may counteract the intense vasoconstriction of the capacitance vessels and cancel the effect of any F-cell ratio change (Chen and Chien, 1977).

A regional change in Hct_v is a more likely explanation for the lack of an increase in the F-cell ratio. The relatively larger RCV and comparatively lower Hct_v in the current study, when compared with previous studies (Wennesland *et al.,* 1959; Sawka *et al.,* 1992), may have contributed to the large F-cell ratios calculated during the resting phases. Thus, a local change in Hct_v during the immersion phase would alter the resultant F-cell ratio. Knight *et al.* (1986) found decreases in immersed limb Hct_v during head-out cold immersion (28ºC) when compared with non-immersed limb Hct_v and demonstrated regional differences that may account for the lack of an F-cell ratio change found in this study. The Hct_v determined in the present study was sampled from an antecubital vein in an arm resting above the water level on a specially designed tray, which may not represent Hct_v alteration in the body. If the Hct_v was lower in the immersed vasculature than that in the non-immersed limb, the F-cell ratio will increase and support the hypothesis previously expounded (Young *et al.,* 1987).

5.4 GENERAL CONCLUSIONS

The indirect measurement of PV change, utilising the Hct/[Hb] calculation, has repeatedly shown an underestimation in the magnitude of change when compared to a direct, tracer-dilution technique during thermoneutral-water immersion (Greenleaf *et al.*, 1983; Johansen *et al.,* 1992). Furthermore, hypovolaemia has been demonstrated with the utilisation of the Hct/[Hb] calculation during cold-water immersion, yet comparisons between alternative PV methods have not been performed. A probable explanation for these discrepancies is a change in the F-cell ratio (Harrison *et al.,* 1986; Johansen *et al.,* 1992) which may result from a disproportionate decrease in the Hct_v compared to the Hct_w when exposed to hydrostatic and thermal influences. Accordingly, the purpose of this study was to measure F-cell ratios during both thermoneutral and cold-water immersions to determine the effect on the indirect calculation of PV change.

The present study found significant increases in PV during thermoneutral immersion induced by the hydrostatic water pressure on the immersed vasculature. In contrast, there was a decrease in PV during cold-water immersion which is likely to result from the combined effects of hydrostatic pressure and a thermal influence due to the pronounced decline in body

core temperature. This is in accordance with the first and second hypotheses and agrees with previous findings.

Despite significant physiological differences, RCV remained constant during both immersion tests. However, the F-cell ratio declined during thermoneutral immersion and the control. There were significant discrepancies between the direct and indirect PV methods during both thermoneutral and cold-water immersion. The magnitude of the discrepancies between direct tracer-dilution and indirect methods during thermoneutral immersion was in accordance with previous reports (Johansen *et al.,* 1992; Johansen *et al.,* 1997b). Uniquely, this study is the first to show disparity between PV methods determined during cold-water immersion. While the third and fourth hypotheses can be accepted, the data did not establish that an increase in the F-cell ratio was responsible for the underestimation of the indirect PV method. This cannot be interpreted that the F-cell ratio does not influence the underestimation observed during both thermoneutral and cold-water immersions. Many authors have proposed an increase in the F-cell ratio as the most likely explanation due to compression of the peripheral microcirculation, induced by hydrostatic pressure, thereby translocating blood of low Hct into the large vessel vasculature, thus decreasing the Hct_v relative to the Hct_w (Harrison *et al.,* 1986).

Plasma volume measured using the Evans blue dye column elution method correlated highly with the Evans blue dye computer programme during pre-immersion and control conditions. This is the first study, to the best of our knowledge, that has compared the computer programme to a direct tracer-dilution method and found agreement in the PV determined. However, in the presence of haemolysis, the PV determined using the computer programme did not agree with that of the column elution method. This is a limitation of the programme method and reinforces the need for stasis free blood sampling.

5.5 RECOMMENDATIONS FOR FUTURE RESEARCH

Since the Hct/[Hb] calculation is the most prevalent measure to determine change in PV, comparison with direct tracer-dilution techniques are needed to determine the actual magnitude of change during different physiological states. Acute PV changes have been shown during exercise, heat stress, different hydration states, and postural manipulation, therefore, further investigation is needed to determine the magnitude of change with direct

and indirect measures of PV. Verification of differences between the measures should warrant investigation of possible F-cell ratio changes despite the findings of the current study.

The radioisotopic changes in splenic capacity, seen previously in exercising humans, could be used in conjunction with F-cell ratio determinations during different physiological states. This may permit quantification of the shift in erythrocytes from the spleen or other organs. Furthermore, radionuclide scanning may highlight areas of different erythrocyte density and demonstrate changes brought about by different stresses.

Further investigation is essential for a better understanding of thermal and body-fluid changes during cold-water immersion. The vast majority of studies have concentrated on thermoneutral immersion, with less emphasis on cold-water exposure and the discrete exploration of hydrostatically-induced variables. Studies are needed to determine the effect cold-water exposure has on PV and how this mechanism is mediated.

The use of a centrally placed venous catheter during thermoneutral and cold-water immersion may facilitate the administration of tracers and blood sampling. Severe vasoconstriction retards peripheral blood sampling, especially during the later stages of cold-water immersion, and the use of a central venous catheter would minimise venous stasis and facilitate uniform blood sampling. Arterial cannulation, commonly used in hospital settings, would permit blood sampling of an immersed limb. Arterial catheters inserted into either the anterior tibial, femoral or radial arteries and sealed during immersion of the limb would assist blood sampling without undue discomfort. Ultimately, catheters placed in central and peripheral veins or arteries, with dry and immersed limbs, may provide evidence of regional changes in haematological variables and determine the effect of the F-cell ratio during water immersion.

CHAPTER SIX: REFERENCES

Alexander, J. K., Hartley, L. H., Modelski, M., and Grover, R. F. (1967). Reduction of stroke volume during exercise in man following ascent to 3100 m altitiude. *Journal of Applied Physiology.* 23: 849-858.

Allen, T. H. (1951). Extraction of T-1824 in the presence of gross hemolysis and lipaemia. *Proceedings of the Society for Experimental Biology and Medicine.* 76: 145-147.

Arborelius, M., Jr., Balldin, U. I., Lilja, B., and Lundgren, C. E. G. (1972). Hemodynamic changes in man during immersion with the head above water. *Aerospace Medicine.* 43: 592-598.

Åstrand, P., and Saltin, B. (1964). Plasma and red cell volume after prolonged severe exercise. *Journal of Applied Physiology.* 19: 829-832.

Baker, C. H. (1963). Cr^{51}-labeled red cell, I^{131}-fibrinogen, and T-1824 dilution spaces. *American Journal of Physiology.* 204: 176-180.

Balga, I., Solenthaler, M., and Furlan, M. (2000). Should whole-body red cell mass be measured or calculated? *Blood Cells, Molecules, and Diseases.* 26: 25-31.

Bass, D. E., and Henschel, A. (1956). Responses of body fluid compartments to heat and cold. *Physiological Reviews.* 36: 128-144.

Begin, R., Epstein, M., Sackner, M. A., Levinson, R., Dougherty, R., and Duncan, D. (1976). Effects of water immersion to the neck on pulmonary circulation and tissue volume in man. *Journal of Applied Physiology.* 40: 293-299.

Bentley, S. A., and Lewis, S. M. (1976). The relationship between total red cell volume, plasma volume and venous haematocrit. *British Journal of Haematology.* 33: 301-307.

Berne, R. M., and Levy, M. N. (1990). *Prinicples of Physiology*. St. Louis: C. V. Mosby.

Besa, E. C. (1975). Physiological changes in blood volume. *CRC Critical Reviews in Clinical Laboratory Sciences.* 6: 67-79.

Bittel, J. H. M. (1987). Heat debt as an index for cold adaptation in men. *Journal of Applied Physiology.* 62: 1627-1634.

Bœning, D., Ulmer, H. V., Meier, U., Skipa, W., and Stegemann, J. (1972). Effects of a multi-hour immersion on trained and untrained subjects: 1. Renal function and plasma volume. *Aerospace Medicine.* 43: 300-305.

Bonde-Petersen, F., Schultz-Pedersen, L., and Dragsted, N. (1992). Peripheral and central blood flow in man during cold, thermoneutral, and hot water immersion. *Aviation, Space, and Environmental Medicine.* 63: 346-350.

Campbell, T. J., Frohman, B. A., and Reeve, B. M. (1958). A simple, rapid, and accurate method of extracting T-1824 from plasma, adapted to the routine measurement of blood volume. *Journal of Laboratory and Clinical Medicine.* 52: 768-777.

Castellani, J. W., Young, A. J., Sawka, M. N., and Pandolf, K. B. (1998). Human thermoregulatory responses during serial cold-water immersions. *Journal of Applied Physiology.* 85: 204-209.

Chaplin, H., Mollison, P. L., and Vetter, H. (1953). The body/venous hematocrit ratio: its constancy over a wide hematocrit range. *The Journal of Clinical Investigation.* 32: 1309-1316.

Chen, R. Y. Z., and Chien, S. (1977). Plasma volume, red cell volume, and thoracic duct lymph flow in hypothermia. *American Journal of Physiology.* 233: H605-H612.

Chien, S., and Gregersen, M. I. (1962). Determination of body-fluids. In: *Physical Techniques in Biological Research, IV, Special Methods,* Nastuk, W. L. (Eds). Academic Press**:** New York. pp1-105.

Christie, J. L., Sheldahl, L. M., Tristani, F. E., Wann, L. S., Sagar, K. B., Levandoski, S. G., Ptacin, M. J., Sobocinski, K. A., and Morris, R. D. (1990). Cardiovascular regulation during head-out water immersion exercise. *Journal of Applied Physiology.* 69: 657-664.

Connelly, T. P., Sheldahl, L. M., Tristani, F. E., Lavandoski, S. G., Kalkhoff, R. K., Hoffman, M. D., and Kalbfleisch, J. H. (1990). Effect of increased central blood volume with water immersion on plasma catecholamines during exercise. *Journal of Applied Physiology.* 69: 651-656.

Convertino, V. A., Greenleaf, J. E., and Bernauer, E. M. (1980). Role of thermal and exercise factors in the mechanism of hypervolemia. *Journal of Applied Physiology: Respiratory, Environmental & Exercise Physiology.* 48: 657-64.

Costill, D. L., and Saltin, B. (1974). Changes in the ratio of venous to body hematocrit following dehydration. *Journal of Applied Physiology.* 36: 608-610.

Craig, A. B., Jr., and Dvorak, M. (1966). Thermal regulation during water immersion. *Journal of Applied Physiology.* 21: 1577-1585.

Datsenko, B. M., Pilipenko, H. I., Gubsky, V. I., and Sherlanov, R. A. (1990). Measurement of circulating plasma volume by T-1824 indicator. *Laboratornoe - Deld.* 11: 32-34.

Deuster, P. A., Smith, D. J., Smoak, B. L., Montgomery, L. C., Singh, A., and Doubt, T. J. (1989). Prolonged whole-body cold water immersion: fluid and ion shifts. *Journal of Applied Physiology.* 66: 34-41.

Dill, D. B., and Costill, D. L. (1974). Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *Journal of Applied Physiology.* 37: 247-248.

Dill, D. B., Horvath, S. M., Dahms, T. E., Parker, R. E., and Lynch, J. R. (1969). Hemoconcentration at altitude. *Journal of Applied Physiology.* 27: 514-8.

DuBois, D., and DuBois, E. F. (1916). Clinical calorimetry: a formula to estimate the approximate surface area if height and weight be known. *Archives of Internal Medicine.* 17: 863.

Dyrbye, M. O., and Kragelund, E. (1970). Simultaneous determination of the apparent ^{3}HOH, ^{82}Br, ^{125}I human albumin and ^{51}Cr red cell volumes in human subjects. *Scandinavian Journal of Clinical and Laboratory Investigation.* 26: 61-66.

Echt, M., Lange, L., and Gauer, O. H. (1974). Changes of peripheral venous tone and central transmural venous pressure during immersion in a thermo-neutral bath. *Pflügers Archiv.* 352: 211-217.

Edelman, I. S., and Leibman, J. (1959). Anatomy of body water and electrolytes. *American Journal of Medicine.* 27: 256-277.

El-Hemaidi, I., Nunan, T. O., and Pearson, T. C. (1997). Red cell mass and plasma volume measurement. *Nuclear Medicine Communications.* 18: 189-190.

Epstein, M. (1996). Renal, endocrine and hemodynamic effects of water immersion in humans. In: *Handbook of Physiology, Environmental Physiology,* Fregly, M. J. and Blatteis, C. M. (Eds). Oxford University Press**:** New York. pp845-853.

Ertl, A. C., Bernauer, E. M., and Hom, C. A. (1991). Plasma volume shifts with immersion at rest and two exercise intensities. *Medicine and Science in Sports and Exercise.* 23: 450-457.

Fähræus, R. (1929). The suspension stability of the blood. *Physiological Reviews.* 9: 241-274.

Fähræus, R., and Lindqvist, T. (1931). The viscosity of the blood in narrow capillary tubes. *American Journal of Physiology.* 96: 562-568.

Farjanel, J., Denis, C., Chatard, J. C., and Geyssant, A. (1997). An accurate method of plasma volume measurement by direct analysis of Evans blue spectra in plasma without dye extraction: origins of albumin - space variations during maximal exercise. *European Journal of Applied Physiology and Occupational Physiology.* 75: 75-82.

Fedor, E. J., and Fisher, B. (1959). Simultaneous determination of blood volume with Cr^{51} and T-1824 during hypothermia and rewarming. *American Journal of Physiology.* 196: 703-705.

Fogarty, A. L., Armstrong, K. A., Gordon, C. J., Groeller, H., Woods, B. F., Stocks, J. M., and Taylor, N. A. S. (2001). The impact of thermal protective clothing on cardiovascular function during combined exercise and heat stress. *Tenth International Conference on Environmental Ergonomics*, Fukuoka, Japan (In press).

Foldager, N., and Blomqvist, C. G. (1991). Repeated plasma volume determination with the Evans blue dye dilution technique: The method and a computer program. *Computers in Biology and Medicine.* 21: 35-41.

Freinkel, N., Schreiner, G. E., and Athens, J. W. (1953). Simultaneous distribution of T-1824 and I^{131}- labelled human serum albumin in man. *The Journal of Clinical Investigation.* 32: 138-148.

Fudenburg, H., Baldini, M., Mahoney, J. P., and Damshek, W. (1961). The body hematocrit/venous hematocrit ratio and the "splenic reservoir". *Blood.* 16: 71-82.

Gabrielsen, A., Johansen, L. B., and Norsk, P. (1993). Central cardiovascular pressures during graded water immersion in humans. *Journal of Applied Physiology.* 75: 581-585.

Gaebelein, C. J., and Senay, L. C., Jr (1980). Influence of exercise type, hydration, and heat on plasma volume shifts in men. *Journal of Applied Physiology.* 49: 119-123.

Ganong, W. F. (1979). *Review of Medical Physiology*. Los Altos: Lange Medical.

Gauer, O. H., and Henry, J. P. (1963). Circulatory basis of fluid volume control. *Physiological Reviews.* 43: 423-481.

Gauer, O. H., Henry, J. P., and Behn, C. (1970). The regulation of extracellular fluid volume. *Annual Review of Physiology.* 39: 547-595.

Gibson, J. G., Peacock, W. C., Seligman, A. M., and Sack, T. (1946). Circulating red cell volume measured simultaneously by the radioactive iron and dye methods. *The Journal of Clinical Investigation.* 25: 838-847.

Giesbrecht, G. G. (2000). Cold stress, near drowning and accidental hypothermia: a review. *Aviation Space and Environmental Medicine* 71, 733-752.

Glickman-Weiss, E. L., Goss, F. L., Robertson, R. J., Metz, K. F., and Cassinelli, D. A. (1991). Physiological and thermal responses of males with varying body compositions during immersion in moderately cold water. *Aviation, Space, and Environmental Medicine.* 62: 1063-1067.

Gonzalez, R. R., and Blanchard, L. A. (1998). Thermoregulatory responses to cold transients: effects of menstrual cycle in resting women. *Journal of Applied Physiology.* 85: 543-553.

Gore, C. J., and Edwards, D. (1992). *Australian Fitness Norms: A Manual for Fitness Assessors*. Adelaide, Australia: Health Development Foundation.

Graham, T. E., Viswanathan, M., Van Dijk, J. P., Bonen, A., and George, J. C. (1989). Thermal and metabolic responses to cold by men and by eumenorrheic and amenorrheic women. *Journal of Applied Physiology.* 67: 282-290.

Green, H. J., Sutton, J. R., Coates, G., Ali, M., and Jones, S. (1991). Response of red cell and plasma volume to prolonged training in humans. *Journal of Applied Physiology.* 70: 1810-1815.

Greenleaf, J. E. (1984). Physiological responses to prolonged bed rest and fluid immersion in humans. *Journal of Applied Physiology.* 57: 619-633.

Greenleaf, J. E., Convertino, V. A., and Mangseth, G. R. (1979). Plasma volume during stress in man: osmolality and red cell volume. *Journal of Applied Physiology.* 47: 1031-1038.

Greenleaf, J. E., and Hinghofer-Szalkay, H. (1985). *Plasma volume methodology; Evans blue, hemoglobin-hematocrit, and mass density transformations.* NASA Technical Memorandum 86834; Ames Research Center, Moffett Field, CA.

Greenleaf, J. E., Morse, J. T., Barnes, P. R., Silver, J., and Kiel, L. C. (1983). Hypervolemia and plasma vasopressin response during water immersion in men. *Journal of Applied Physiology.* 55: 1688-1693.

Greenleaf, J. E., Shvartz, E., and Keil, L. C. (1981). Hemodilution, vasopressin suppression, and diuresis during water immersion in man. *Aviation, Space, and Environmental Medicine.* 52: 329-336.

Greenleaf, J. E., Shvartz, E., Kravik, S., and Keil, L. C. (1980). Fluid shifts and endocrine responses during chair rest and water immersion in man. *Journal of Applied Physiology.* 48: 79-88.

Gregersen, M. I., Gibson, J. J., and Stead, E. A. (1935). Plasma volume determination with dyes: errors in colorimetry; use of the blue dye T-1824. *American Journal of Physiology.* 113: 54-55.

Guyton, A. C. (1961). Physiologic regulation of arterial pressure. *American Journal of Cardiology.* 8: 401-407.

Guyton, A. C. (1991). *Textbook of Medical Physiology*. Philadelphia: W. B. Saunders.

Hagan, R. D., Diaz, F. J., and Horvath, S. M. (1978). Plasma volume changes with movement to supine and standing postions. *Journal of Applied Physiology.* 45: 414-418.

Hahn, P. F., Ross, J. F., Bale, W. F., Balfour, W. M., and Whipple, G. H. (1942). Red cell and plasma volumes (circulating and total) as determined by iron and by dye. *Journal of Experimental Medicine.* 75: 221-232.

Hainsworth, R., and Drinkhill, M. J. (1995). Regulation of blood volume. In: *Cardiovascular Regulation,* Jordan, D. and Marshall, J. (Eds). Portland Press**:** London. pp77-92.

Haldane, J., and Smith, J. L. (1899). The mass and oxygen carrying capacity of the blood in man. *Journal of Physiology (London).* 25: 331-340.

Harrison, M. H. (1985). Effects of thermal stress and exercise on blood volume in humans. *Physiological Reviews.* 65: 149-209.

Harrison, M. H., Graveney, M. J., and Cochrane, L. A. (1982). Some sources of error in the calculation of relative change in plasma volume. *European Journal of Applied Physiology and Occupational Physiology.* 50: 13-21.

Harrison, M. H., Keil , L. C., Wade, C. A., Silver, J. E., Geelen, G., and Greenleaf, J. E. (1986). Effect of hydration on plasma volume and endocrine responses to water immersion. *Journal of Applied Physiology.* 61: 1410-1417.

Hayes, P. M., Lucas, J. C., and Shi, X. (2000). Importance of post-exercise hypotension in plasma volume restoration. *Acta Physiologica Scandinavica.* 169: 115-124.

Hicks, D. A., Hope, A., Turnball, A. L., and Verel, D. (1956). The estimation and prediction of normal blood volume. *Clinical Science.* 15: 557-565.

Hinghofer-Szalkay, H., Harrison, M. H., and Greenleaf, J. E. (1987). Early fluid and protein shifts in men during water immersion. *European Journal of Applied Physiology and Occupational Physiology.* 56: 673-678.

Huff, R. L., and Feller, D. D. (1956). Relation of circulating red cell volume to body density and obesity. *The Journal of Clinical Investigation.* 35: 1-10.

Hurford, W. E., Hong, S. K., Park, K. S., Ahn, D. W., Shiraki, K., Mohri, M., and Zapol, W. M. (1990). Splenic contraction during breath-hold diving in the Korean Ama. *Journal of Applied Physiology.* 69: 932-936.

International Commission on Radiological Protection (1975). *Report of the Task Group on Reference Man (Publication No.23)*. Oxford, England: Pergamon Press.

International Committee for Standardization in Haematology (1980). Recommended methods for measurement of red-cell and plasma volume. *Journal of Nuclear Medicine.* 21: 793-800.

Jain, S. C., Bardhan, J., Swamy, Y. V., Krishna, B., and Nayar, H. S. (1980). Body fluid compartments in humans during acute high-altitude exposure. *Aviation, Space, and Environmental Medicine.* 51: 234-6.

Janský, L., Šrámek, P., Šavlíková, J., Uličný, B., Janáková, H., and Horký, K. (1996). Change in sympathetic activity, cardiovascular functions and plasma hormone concentrations due to cold water immersion in men. *European Journal of Applied Physiology and Occupational Physiology.* 74: 148-152.

Johansen, L. B., Bie, P., Warberg, C., Christensen, N. J., and Norsk, P. (1995). Role of hemodilution on renal responses to water immersion in humans. *American Journal of Physiology.* 269: R1068-R1076.

Johansen, L. B., Foldager, N., Stadeager, C., Seltz Kristensen, M., Bie, P., Warberg, C., Kamegal, M., and Norsk, P. (1992). Plasma volume, fluid shifts, and renal responses in humans during 12 h of head-out water immersion. *Journal of Applied Physiology.* 73: 539-544.

Johansen, L. B., Gharib, C., Allevard, A. M., Sigaudo, D., Christensen, N. J., Drummer, C., and Norsk, P. (1997b). Haematocrit, plasma volume and noradrenaline in humans during simulated weightlessness for 42 days. *Clinical Physiology.* 17: 203-210.

Johansen, L. B., Jensen, T. U. S., Pump, B., and Norsk, P. (1997a). Contribution of abdomen and legs to central blood volume expansion in humans during immersion. *Journal of Applied Physiology.* 83: 695-699.

Johansen, L. B., Videbaek, R., Hammerum, M., and Norsk, P. (1998). Underestimation of plasma volume changes in humans by hematocrit/hemoglobin method. *American Journal of Physiology.* 274: R126-R130.

Keatinge, W. R., and Evans, M. (1961). The respiratory and cardiovascular response to immersion in cold and warm water. *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences.* 46: 83-94.

Keith, N. M., Rowntree, L. G., and Geraghty, J. T. (1915). A method for determination of plasma and blood volume. *Archives of Internal Medicine.* 16: 547-576.

Khosla, S. S., and DuBois, A. B. (1979). Fluid shifts during initial phase of immersion diuresis in man. *Journal of Applied Physiology.* 46: 703-708.

Knight, D. R., Santoro, T., and Bondi, K. R. (1986). Distortion of calculated whole-body hematocrit during lower-body immersion in water. *Journal of Applied Physiology.* 61: 1885-1890.

Ladegaard-Pedersen, H. J., and Engell, H. C. (1969). Blood volume determination using one catheter located in a central vein. *Acta Chirurgica Scandinavica.* 135: 105-108.

Landis, E. M. (1927). Micro-injection studies of capillary permeability. *The American Journal of Physiology.* 82: 217-238.

Landis, E. M., and Pappenheimer, J. R. (1972). Exchange of substances through the capillary wall. In: *Circulation,* Hamilton, W. F. (Eds). American Physiological Society**:** Washington. pp961-1034.

Lange, L., Lange, S., Echt, M., Gauer, O. H., and Dannenberg, H. (1974). Heart volume in relation to body posture and immersion in a thermo-neutral bath. *Pflügers Archiv.* 352: 219-226.

Larsen, A. S., Johansen, L. B., Stadaeger, C., Warberg, J., Christensen, N. J., and Norsk, P. (1994). Volume-homeostatic mechanisms in humans during graded water immersion. *Journal of Applied Physiology.* 77: 2832-2839.

Laub, M., Hvid-Jacobsen, K., Hovind, P., Kanstrup, I.-L., Christensen, N. J., and Nielsen, S. L. (1993). Spleen emptying and venous hematocrit in humans during exercise. *Journal of Applied Physiology.* 74: 1024-1026.

Lee, D. T., Toner, M. M., McArdle, W. D., Vrabas, I. S., and Pandolf, K. B. (1997). Thermal and metabolic responses to cold-water immersion at knee, hip, and shoulder levels. *Journal of Applied Physiology.* 82: 1523-1530.

Levick, J. R. (1995). Changing perspectives on microvascular fluid exchange. In: *Cardiovascular Regulation,* Jordan, D. and Marshall, J. (Eds). Portland Press**:** London. pp127-152.

Lundvall, J., and Bjerkhoel, P. (1994). Failure of hemoconcentration during standing to reveal plasma volume decline induced in the erect posture. *Journal of Applied Physiology.* 77: 2155-2162.

Lundvall, J., and Bjerkhoel, P. (1995). Pronounced and rapid plasma volume reduction upon quiet standing as revealed by a novel approach to the determination of the intravascular volume change. *Acta Physiologica Scandinavica.* 154: 131-142.

Lundvall, J., and Lindgren, P. (1998). F-cell shift and protein loss strongly affect validity of PV reductions indicated by Hb/Hct and plasma proteins. *Journal of Applied Physiology.* 84: 822-829.

MacDougall, J. D., and Wenger, H. A. (1982). The purpose of physiological testing. In: *Physiological Testing of the Elite Athlete,* MacDougall, J. D., Wenger, H. A. and Green, H. J. (Eds). Movement Publications**:** New York. pp1-2.

Maw, G. J. (1994). Human body-fluid distribution: postural, thermal and exercise stress. *Doctor of Philosophy Dissertation*. Department of Biomedical Science. University of Wollongong, Wollongong, NSW, Australia.

Maw, G. J., Mackenzie, I. L., and Taylor, N. A. S. (1995). Redistribution of body fluids during postural manipulations. *Acta Physiologica Scandinavica.* 155: 157-163.

Maw, G. J., Mackenzie, I. L., and Taylor, N. A. S. (2000). Can skin temperature manipulation, with minimal core temperature change, influence plasma volume in resting humans? *European Journal of Applied Physiology and Occupational Physiology.* 81: 159-162.

McArdle, W. D., Magel, J. R., Gergley, T. J., Spina, R. J., and Toner, M. M. (1984). Thermal adjustment to cold-water exposure in resting men and women. *Journal of Applied Physiology.* 56: 1565-1571.

McCally, M. (1964). Plasma volume response to water immersion: implications for space flight. *Aerospace Medicine.* 35: 130-132.

Michel, C. C. (1984). Fluid movements through capillary walls. In: *Handbook of Physiology, Section 2: The Cardiovascular System,* Renkin, E. M., Michel, C. C. and Geiger, S. R. (Eds). Williams and Wilkins Company**:** Bethesda. pp375-410.

Miki, K., Hajduczok, G., Hong, S. K., and Krasney, J. A. (1986). Plasma volume changes during head-out water immersion in conscious dogs. *American Journal of Physiology.* 251: R582-R590.

Miki, K., Hajduczok, G., Hong, S. K., and Krasney, J. A. (1987a). Extracellular fluid and plasma volumes during water immersion in nephrectomized dogs. *American Journal of Physiology.* 252: R972-R978.

Miki, K., Hong, S. K., and Krasney, J. A. (1987b). Mechanism of the increase in plasma volume during head-out water immersion in dogs. *The Physiologist.* 30: S100-S101.

Miki, K., Klocke, M. R., Hajduczok, G., Hong, S. K., and Krasney, J. A. (1989). Interstitial and intravascular pressures in conscious dogs during head-out water immersion. *American Journal of Physiology.* 257: R358-R364.

Miwa, C., Sugiyama, Y., Mano, T., Iwase, S., and Matsukawa, T. (1997). Sympatho-vagal responses in humans to thermoneutral head-out water immersion. *Aviation, Space, and Environmental Medicine.* 68: 1109-1114.

Muldowney, F. P. (1957). The relationship of total red cell mass to lean body mass in man. *Clinical Science.* 16: 163-169.

Najean, Y., and Deschrywer, F. (1984). The body venous haematocrit ratio and its use for calculating total blood volume from fractional volumes. *European Journal of Nuclear Medicine.* 9: 558-560.

New South Wales Department of Health (1987). *Code of Safe Practice in Clinical Laboratories*. NSW Department of Health, Sydney, Australia.

Norsk, P., Bonde-Petersen, F., and Warberg, J. (1986). Central venous pressure and plasma arginine vasopressin in man during water immersion combined with changes in blood volume. *European Journal of Applied Physiology and Occupational Physiology.* 54: 608-616.

Park, K. S., Choi, J. K., and Park, Y. S. (1999). Cardiovascular regulation during water immersion. *Applied Human Science.* 18: 233-241.

Parving, H.-H., Klebe, J. G., and Ingomar, C. J. (1973). Simultaneous determination of plasma volume and transcapillary escape rate with ^{131}I-labelled albumin and T-1824 in the newborn. *Acta Paediatrica Scandinavica.* 62: 248-252.

Patterson, M. (1999). Effect of heat acclimation on body-fluid regulation. *Doctor of Philosophy Dissertation*. Department of Biomedical Science. University of Wollongong, Wollongong, NSW, Australia.

Pearson, T. C., Guthrie, D. L., Simpson, J., Chinn, S., Barosi, G., Ferrant, A., Lewis, S. M., and Najean, Y. (1995). Interpretation of measured red cell mass and plasma volume in adults: expert panel on radionuclides of the international council for standardization in haematology. *British Journal of Haematology.* 89: 748-756.

Pugh, L. G. C. E. (1964). Blood volume and haemoglobin concentration at altitude above 18,000 ft (5500 m). *Journal of Physiology (London).* 170: 344-354.

Reeve, E. B., Gregersen, M. I., Allen, T. H., and Sear, H. (1953a). Distribution of cells and plasma in the normal and splenectomized dog and its influence on blood volume estimates with P^{32} and T-1824. *American Journal of Physiology.* 175: 195-203.

Reeve, E. B., Gregersen, M. I., Allen, T. H., Sear, H., and Walcott, W. W. (1953b). Effects of alteration in blood volume and venous hematocrit in splenectomized dogs on estimates of total blood volume with P^{32} and T-1824. *American Journal of Physiology.* 175: 204-210.

Regan, J. M. (1998). Human physiological responses to cold-water immersion: acute and repeated exposures. *Doctor of Philosophy Dissertation*. Department of Biomedical Sciences. University of Wollongong, Wollongong, NSW, Australia.

Retzlaff, J. A., Tauxe, W. M., Kiely, J. M., and Stroebel, C. F. (1969). Erythrocyte volume, plasma volume, and lean body mass in man and woman. *Blood.* 33: 649-661.

Risch, W. D., Koubenec, H.-J., Beckmann, U., S, L., and Gauer, O. H. (1978b). The effect of graded immersion on heart volume, central venous pressure, pulmonary blood distribution, and heart rate in man. *Pflügers Archiv.* 374: 115-118.

Risch, W. D., Koubenec, H.-J., Gauer, O. H., and Lange, S. (1978a). Time course of cardiac distension with rapid immersion in a thermo-neutral bath. *Pflügers Archiv - European Journal of Physiology.* 374: 119-120.

Rochelle, R. D., and Horvath, S. M. (1978). Thermoregulation in surfers and nonsurfers immersed in cold water. *Undersea Biomedical Research.* 5: 377-390.

Röcker, L., Taenzer, M., Drygas, W. K., Lill, H., Heyduck, B., and Altenkirch, H. U. (1990). Effect of prolonged physical exercise on the fibrinolytic system. *European Journal of Applied Physiology & Occupational Physiology.* 60: 478-81.

Rowell, L. B. (1993). *Human cardiovascular control*. New York: Oxford University Press.

Sandler, M. P., Kronenberg, M. W., Forman, M. B., Wolfe, O. H., Clanton, J. A., and Partain, C. L. (1984). Dynamic fluctuations in blood and spleen radioactivity: Splenic contraction and relation to clinical radionuclide volume calculations. *Journal of the American College of Cardiology.* 3: 1205-1211.

Sawka, M. N. (1992). Physiological consequences of hypohydration: exercise performance and thermoregulation. *Medicine and Science in Sports and Exercise.* 24: 657-670.

Sawka, M. N., Gonzalez, R. R., Young, A. J., Muza, S. R., Pandolf, K. B., Latzka, W. A., Dennis, R. C., and Valeri, C. R. (1988). Polycythemia and hydration: effects on thermoregulation and blood volume during exercise-heat stress. *American Journal of Physiology.* 255: R456-463.

Schultz, A. L., Hammarsten, J. F., Heller, B. I., and Ebert, R. V. (1953). A critical comparison of the T-1824 dye and iodinated albumin methods for plasma volume measurement. *The Journal of Clinical Investigation.* 32: 107-112.

Senay, L. C., Mitchell, D., and Wyndham, C. H. (1976). Acclimatization in a hot, humid environment: body fluid adjustments. *Journal of Applied Physiology.* 40: 786-96

Sheung, H.-P., and Huggins, R. A. (1979). A review of body composition studies with emphasis on total body water. *American Journal of Clinical Nutrition.* 32: 630-647.

Sjöstrand, T. (1953). Volume and distribution of blood and their significance in regulating the circulation. *Physiological Reviews.* 33: 202-228.

Šrámek, P., Šimecková, M., Janský, L., Šavlíková, J., and Vybíral, S. (2000). Human physiological responses to immersion into water of different temperatures. *European Journal of Applied Physiology and Occupational Physiology.* 81: 436-442.

Stadaeger, C., Johansen, L. B., Warberg, J., Christensen, N. J., Foldager, N., Bie, P., and Norsk, P. (1992). Circulation, kidney function, and volume-regulating hormones during prolonged water immersion in humans. *Journal of Applied Physiology.* 73: 530-538.

Starling, E. (1896). On the absorption of fluids from the connective tissue spaces. *Journal of Physiology (London).* 19: 312-326.

Strauss, M. B., Davis, R. K., Rosenbaum, J. D., and Rossmeisl, E. C. (1951). "Water diuresis" produced during recumbency by the intravenous infusion of isotonic saline solution. *The Journal of Clinical Investigation.* 30: 862-868.

Strong, L. H., Gee, G. K., and Goldman, R. F. (1985). Metabolic and vasomotor insulative responses occurring on immersion in cold water. *Journal of Applied Physiology.* 58: 964-977.

Tajima, F., Sagawa, S., Claybaugh, J. R., and Shiraki, K. (1999). Renal, endocrine, and cardiovascular responses during head-out water immersion in legless men. *Aviation, Space, and Environmental Medicine.* 70: 465-470.

Thomsen, J. K., Fogh-Andersen, N., Bulow, K., and Devantier, A. (1991). Blood and plasma volumes determined by carbon monoxide gas, ^{99m}Tc-labelled erythrocytes, ^{125}I-albumin and the T 1824 technique. *Scandinavian Journal of Clinical Laboratory Investigations.* 51: 185-190.

Tikuisis, P., Jacobs, I., Moroz, D., Vallerand, A. L., and Martineau, L. (2000). Comparison of thermoregulatory responses between men and women immersed in cold water. *Journal of Applied Physiology.* 89: 1403-1411.

Tipton, M. J., Eglin, C. M., and Golden, F. S. C. (1998). Habituation of the initial responses to cold water immersion in humans: a central or peripheral mechanism? *Journal of Physiology (London).* 512: 621-628.

van Beaumont, W., and Rochelle, R. H. (1974). Erythrocyte volume stability with plasma osmolarity changes in exercising man. *Proceedings of the Society for Experimental Biology and Medicine.* 145: 240-243.

Vogelaere, P., Deklunder, G., and Lecroart, J. (1995). Cardiac output variations in supine resting subjects during head-out water immersion. *International Journal of Biometeorology.* 39: 40-45.

Vogelaere, P., Savourey, G., Deklunder, G., Lecroart, J., Brasseur, M., Bekaert, S., and Bittel, J. (1992). Reversal of cold induced haemoconcentration. *European Journal of Applied Physiology and Occupational Physiology.* 64: 244-249.

von Diringshofen, H. (1948). Die wirkugen das hydrostatischen druckes des wassebades auf blutdruk in den kapillaren und die bindegewebsentwassening. *Zeitschrift für Kreislaufforschung.* 37: 382-390.

Wennesland, R., Brown, E., Hopper, J., Hodges, J. L., Guttentag, O. E., Scott, K. G., Tucker, I. N., and Bradley, B. (1959). Red cell, plasma and blood volume in healthy men measured by radiochromium (Cr^{51}) cell tagging and hematocrit: influence of age, somatotype and habits of physical activity on the variance after regression of volumes to height and weight combined. *The Journal of Clinical Investigation.* 387: 1065-1077.

Wyndham, C. H., Benade, A. J., Williams, C. G., Strydom, N. B., Goldin, A., and Heyns, A. J. (1968). Changes in central circulation and body fluid spaces during acclimatization to heat. *Journal of Applied Physiology.* 25: 586-93.

Yamazaki, F., Endo, Y., Torii, R., Sagawa, S., and Shiraki, K. (2000). Continuous monitoring of change in hemodilution during water immersion in humans: effect of water temperature. *Aviation, Space and Environmental Medicine.* 71: 632-639.

Yoshida, T., Nagashima, K., Nakai, S., Yorimoto, A., Kawabata, T., and Morimoto, T. (1999). Attenuation of urinary sodium excretion during cold-air exposure in trained athletes. *Japanese Journal of Physiology.* 49: 193-9.

Young, A. J., Muza, S. R., Sawka, M. N., and Pandolf, K. B. (1987). Human vascular fluid responses to cold stress are not altered by cold acclimation. *Undersea Biomedical research.* 14: 215-228.

Zeyl, A., Stocks, J. M., Taylor, N. A. S., and Jenkins, A. B. (2001). Cold-induced decreases in human, circulating leptin, and in the subcutaneous adipose leptin secretion rate. *Proceedings of the Australian Physiological and Pharmacological Society*, Wollongong, Australia. 32 (1: supplement 1), 188P.

Zweens, J., and Frankena, H. (1981). An improved method for the determination of the plasma volume with Evans blue. *Journal of Clinical Chemistry and Clinical Biochemistry.* 19: 919-924.

APPENDICES

APPENDIX A

Evans blue dye column elution technique

The determination of plasma volume with Evans blue dye was first reported by Gregersen *et al.* (1935). The technique is based on Evans blue dye binding with albumin, mixing in the bloodstream, and the volume of dye in plasma is quantified with a spectrophotometer. Subsequently, the technique has been modified to extract interfering substances at the same wavelength (615 nm) where Evans blue dye is maximally absorbed, with the use of a detergent to separate Evans blue dye from albumin and a cellulose pulp matrix to absorb the unbound dye (Allen, 1951; Campbell *et al.* 1958).

Evans blue dye injection equipment

1. 20 gauge teflon cannula and extension tubing.
2. Alcohol swab.
3. 9 ml lithium-heparin monovettes (2).
4. Evans blue dye vial.
5. 5 ml syringe.
6. 20 ml syringe.
7. 21 gauge needle.
8. Sterile 0.9% sodium chloride.
9. Three-way tap.
10. Scientific electronic balance, with precision of 0.0001 g (ER-182A Electronic Balance, A&D Co. Ltd., Tokyo, Japan).
11. Centrifuge.

Preparation of Injection

1. Attach a 21 gauge needle to a 5 ml syringe and record the mass to 4 decimal places.
2. Wipe the rubber stopper on the vial with an alcohol wipe.
3. Aspirate 2.5 ml of Evans blue dye with the syringe and attached needle.
4. Eject all air in the syringe and replace the needle with a new sterile needle.
5. Record the new mass to 4 decimal places.

Pre-injection sample

1. Collect 15 ml of blood into two lithium-heparin monovettes from a catheter in an antecubital fossa vein.
2. Centrifuge the samples for 15 min at 1700 *g*.
3. Extract the plasma and transfer into 5-ml plastic tubes with a cap and refrigerate at 4ºC until extraction later the same day (1 ml for background and 1 ml for preparation of the standard).

Injection

1. Attach a 5 ml syringe of Evans blue dye to a three-way tap connected to extension tubing attached to the catheter in the opposite antecubital fossa vein.
2. Attach a 20-ml syringe filled with sterile 0.9% sodium chloride to the remaining three-way tap port.
3. Inject the contents of the Evans blue dye syringe over one minute and commence the timer at the end of the injection.
4. Flush the syringe and three-way tap several times with the 20 ml syringe of sterile 0.9% sodium chloride over a 15-sec period (as shown in Figure A).
5. Recap the 5-ml Evans blue dye syringe, record the mass and calculate the exact volume of Evans blue dye injected.

Post-injection sample

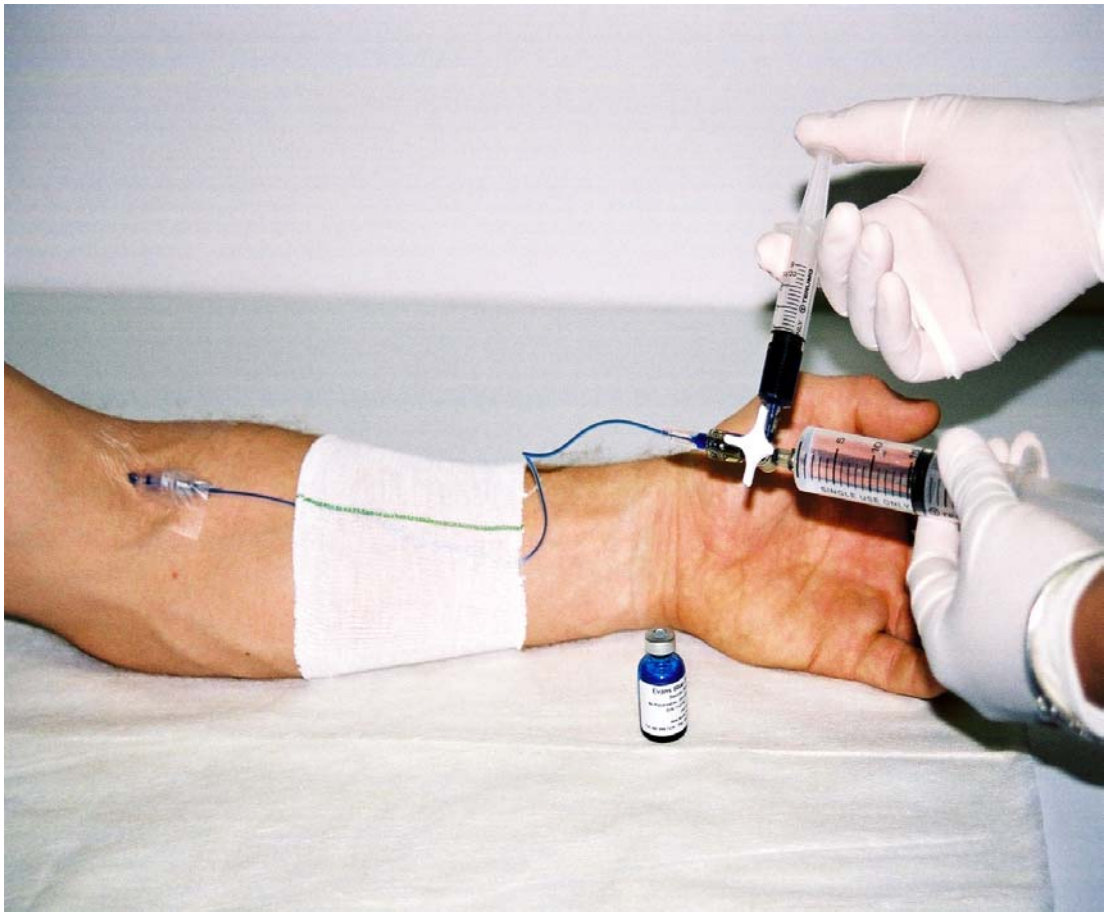
1. At approximately 9 min post-injection, prepare the antecubital fossa vein catheter used for sampling.
2. At exactly 10 min post-injection, withdraw 5 ml of blood into a lithium-heparin monovette.
3. Centrifuge the sample for 15 min at 1700 *g*.
4. Extract the plasma and transfer into a 5-ml plastic tube with a cap and refrigerate at 4ºC until extraction later the same day (1 ml for 10-min test sample).

Column elution technique

Reagents and Equipment

1. Desalting prepacked columns (3) (Sephadex® G-25 M, Amersham Pharmacia Biotech, Buckinghamshire, U. K.).

Figure A: Injection of the Evans blue dye through a three-way tap.



2. Waste beakers (3).
3. 10 ml volumetric flasks (3).
4. 50 ml Erlyenmeyer flasks (3).
5. One-way stopcocks (3).
6. Funnels (3).
7. Multi-pipetter (50 ml barrel).
8. Graduated 1-ml pipetter and disposable tips.
9. Disposable cuvettes.
10. Distilled water.
11. Spectrophotometer (Shimadzu UV-1601, UV-visible spectrophotometer, Tokyo, Japan).
12. 2% Na_2PO_4 buffer: 40 gm Na_2PO_4 + 2000 ml distilled water (made weekly and refrigerated in amber glass bottles).
13. 8% K_2HPO_4 buffer: 8 gm K_2HPO_4 + 100 ml distilled water (made weekly and refrigerated in amber glass bottles).
14. Teepol phosphate: 30 ml Teepol + 1000 ml of 2% Na_2 PO_4 buffer (made weekly and refrigerated in amber glass bottles).
15. Acetone: 1:1 mixture of acetone and distilled water (made daily).
16. Evans blue dye standard: 1 ml of Evans blue dye + 50 ml of distilled water (covered in aluminium foil and refrigerated – can be used for up to six months).

Procedure

1. Drain the liquid on top of the Sephadex columns (3) into a waste beaker and attach a funnel.
2. Cut off the bottom teat and attach one-way stopcock.
3. Align the column in a clamp and situate a drainage beaker under each column.
4. Pour 15 ml of 2% Na_2PO_4 buffer on top of the columns and commence draining through the stopcocks.
5. Cease draining when the buffer is approximately 1 cm above the top frit on the column.
6. Designate 3 Erlyenmeyer flasks as:
    - BLANK: 1 ml pre-injection plasma
    - STANDARD: 1 ml pre-injection plasma mixed with 200 μL Evans blue standard
    - TEST: 1 ml 10-min post-injection plasma

7. Combine 15 ml of Teepol phosphate to each flask and gently swirl, then allow to stand for approximately 2 min.
8. Pour the contents into the funnels, open the stopcocks and drain.
9. Rinse the 50-ml Erlyenmeyer flasks with 5 ml of Teepol phosphate and add this volume to each of the three funnels.
10. Add 10 ml of 2% Na_2PO_4 buffer to the columns when the Teepol phosphate solution has reached the level of the top frit.
11. Allow the buffer to drain until there is approximately 0.5 cm of fluid above the top frit (there will be a blue band evident at the top of the column).
12. Transfer 0.5 ml of 8% K_2HPO_4 buffer, with a graduated 1-ml pipetter, into a 10-ml volumetric flask for each of the three columns and label as BLANK, STANDARD and TEST.
13. Pour 5 ml of the acetone solution into the funnels and adjust the stopcocks to allow one drop per second to pass into the waste beakers. Close the stopcocks when the blue band is just above the level of the top frit.
14. Add another 5 ml of acetone solution to each column and place the 10-ml volumetric flasks under the corresponding columns and open the stopcocks allowing the dye solution to pass into each flask.
15. Top up the columns with at least 12 ml of acetone solution and observe the blue band descending towards the bottom frit.
16. When the 10-ml volumetric flasks are half full, turn off the stopcocks and let the solutions sit for 15 min.
17. After 15 min, open the stopcocks and fill the flasks to the 10-ml mark.
18. Cap the 10-ml volumetric flasks and invert several times to mix the solution.
19. Turn on the spectrophotometer.
20. Transfer 2 ml of the BLANK, STANDARD and TEST solutions into three separate cuvettes, for triplicate measurements.
21. Pipette distilled water into four cuvettes for zeroing the spectrophotometer.
22. Set the spectrophotometer to wavelength 615 nm.
23. Wipe the cuvettes filled with distilled water with lint-free cloth and insert two cuvettes into the spectrophotometer (reference and sample apertures), close the door and zero the spectrophotometer.
24. Wipe the cuvettes filled with the plasma samples with lint-free cloth and read the three samples (triplicate) in the sample aperture and read the absorbance.

25. Calculate the plasma volume (see Section *3.5.3.2*).

References

Allen, T. J. (1951). Extraction of T-1824 in the presence of gross hemolysis and lipaemia. *Proceedings of the Society for Experimental Biological Medicine.* 76: 145-147.

Campbell, T. J., Frohman, B. A., and Reeve, B. M. (1958). A simple, rapid, and accurate method of extracting T-1824 from plasma, adapted to the routine measurement of blood volume. *Journal of Laboratory and Clinical Medicine.* 52: 768-777.

Gregersen, M. I., Gibson, J. J., and Stead, E. A. (1935). Plasma volume determination with dyes: errors in colorimetry; use of the blue dye T-1824. *American Journal of Physiology.* 113: 54-55.

APPENDIX B

Radionuclide method

The procedure used for the determination of erythrocyte volume was first described by Sterling and Gray (1950) and later modified by Spears *et al.* (1974). The present study used procedures identical with those used by Regan (1998), which were slightly modified from Maw (1994). The determination of erythrocyte volume was based on the dilution principle, whereby an unknown volume is determined by comparing a known tracer concentration and volume before and after mixing in the unknown volume.

Reagents and Equipment

1. Sodium-radiochromate ($Na^{51}Cr$) for injection; CJS11, Amersham Pharmacia Biotech, Buckinghamshire, U. K.) 37 $MBq.ml^{-1}$ (Half-life: 27.7 days).
2. Saponin powder (BDH, Merck Pty. Ltd., Victoria, Australia).
3. 100-ml vials of sterile sodium chloride injection BP 0.9% (Astra Pharmaceuticals Pty. Ltd., Ryde, Australia).
4. Distilled water.
5. Sterile sodium chloride 0.9%.
6. Well-type gamma (γ) counter, calibrated and normalised for ^{51}Cr (Wallac 1480 Wizard™ 3" gamma counter, Turku, Finland).
7. Scientific electronic balance, with precision of 0.0001 g (ER-182A Electronic Balance, A&D Co. Ltd., Tokyo, Japan).
8. Centrifuge capable of generating 1700 *g*, calculated as:

$$g = (\frac{n}{1000})^2 \times r \times 1.118$$

where: *g* = centrifugal force;
n = revolutions per min;
r = radius (mm);
1.118 = correction factor.

10. Calibrated autopipette with tips.
11. Safety working cabinet (Grade II: laminar flow with sample/operator protection).
12. Calibrated 250-ml glass flask (1 per trial).
13. Sterile McCartney bottle (1 per trial).

14. Citrate-phosphate-dextrose solution with adenine (10% CPD-A) (Sigma Chemical Co, St. Louis, MO, USA).
15. 7 x 5 ml plastic tubes with caps, suitable for γ counting.
16. 20 ml syringe.
17. 10 ml syringes.
18. 5 ml syringes.
19. 18 gauge drawing up needles.
20. Mixing cannulas.
21. Graduated 1 ml insulin-type syringe with needle.
22. 20 gauge teflon cannula and extension tubing.
23. 9 ml EDTA tube.
24. 3 x urine specimen jars.

Preparation of radionuclide

Erythrocyte volume was measured using $Na^{51}Cr$ tagged to autologous erythrocytes. The dose of $Na^{51}Cr$ for trial one and two was 18.5 kBq and an adjusted dose of 29.6 kBq for the third trial, in order to obtain a sample to background ratio of no less than 2 to 1.

1. Remove 1 ml from a 100 ml vial of sterile 0.9% sodium chloride injection.
2. Add 1 ml of $Na^{51}Cr$ to the sterile sodium chloride vial and mix thoroughly (final concentration 37 $kBq.ml^{-1}$).

Procedure

1. Collect 15 ml of blood from the subject in a syringe.
2. Transfer 5 ml of blood into an EDTA tube, to use as a background reference during radioactivity counting.
3. Dispense the remaining 10 ml of blood into the sterile McCartney bottle, pretreated with 2 ml of 10% CPD-A.
4. Centrifuge the McCartney bottle for 12 min at 1700 *g*.
5. Remove the plasma and buffy coat without disturbing the erythrocytes.
6. Obtain a urine sample from the subject, to use as background reference during radioactive counting.

Erythrocyte labelling

1. Combine approximately 0.5 ml of $Na^{51}Cr$ solution to the erythrocytes in the McCartney

bottle. A correction for radionuclide decay[1] is required to maintain consistent dosing.

2. Incubate the mixture at room temperature for 30 min, to enhance $Na^{51}Cr$ labelling of erythrocytes.
3. Carefully wash the mixture three times with sterile 0.9% sodium chloride, followed by centrifugation at 1700 *g* for 10 min each time.
4. Remove the resultant supernatant and resuspend the preparation with sterile 0.9% sodium chloride to a haematocrit of approximately 50%.

Preparation of injections and standards

1. Transfer the entire contents of the $Na^{51}Cr$-labelled erythrocytes into a sterile 10 ml syringe and record the mass of the syringe.
2. Combine approximately 0.5 ml of $Na^{51}Cr$-labelled erythrocytes to a total of 250 ml in distilled water, for formation of the $Na^{51}Cr$ standard.
3. Record the mass of the syringe and determine the actual dilution.

Injection

1. Administer the $Na^{51}Cr$-labelled erythrocyte through a three-way tap attached to a catheter in the subject's right antecubital fossa vein over a 1 min period.
2. Commence the timer at the end of the injection.
3. Flush the syringe through the three-way tap, with 10 ml of sterile 0.9% sodium chloride.
4. Record the mass of the syringe and calculate the precise volume of the injection by subtracting the previous mass.

Sampling and handling

Collect a 5-ml blood sample into an EDTA tube, 30 min after the end of the injection and at 60 min of immersion. Obtain a urine sample after the blood sample, measure the total volume and retain a sample for radioactivity counting.

1. Centrifuge all blood samples for 15 min at 1700 *g*.
2. Remove the plasma and buffy coat from the samples.
3. Dispense 1 ml of erythrocytes into 5-ml plastic tubes pretreated with 3 drops of 1 mg·1 ml^{-1} saponin in distilled water.

---

[1] $A = A_o / 2^n$; where A_o = radioactivity on the day of product reference, ^{n} = number of days following the initial product reference divided by the half-life ($Na^{51}Cr$ = 27.7 days).

4. Dispense 1 ml of urine into the tubes as above.
5. Cap all tubes and refrigerate at 4°C prior to γ counting.

Standards and backgrounds

1. Dispense 1 ml of the prepared $Na^{51}Cr$ standard into a 5-ml plastic tube pretreated with 3 drops of 1 mg·1 ml^{-1} saponin in distilled water, cap and refrigerate at 4°C.
2. Dispense 1 ml of the background urine sample into a 5-ml plastic tube pretreated with 3 drops of 1 mg·1 ml^{-1} saponin in distilled water, cap and refrigerate at 4°C.
3. Use an empty 5-ml plastic tube pretreated with 3 drops of 1 mg·1 ml^{-1} saponin in distilled water, as the background reference against all samples.

Radioactivity counting

1. The standard, backgrounds, urine and erythrocyte tubes are placed in a rack and counted for 10 min per tube in a well-type γ counter.
2. The erythrocyte volumes are calculated from the erythrocytes collected at 30 min post-injection and at 60 min of immersion, corrected for any $Na^{51}Cr$ urinary loss (see Section *3.5.3.1*).

References

Maw, G. J. (1994). Human body-fluid distribution: postural, thermal and exercise stress. *Doctor of Philosophy Dissertation*. Department of Biomedical Science. University of Wollongong, NSW, Australia.

Regan, J. M. (1998). Human physiological responses to cold-water immersion: acute and repeated exposures. *Doctor of Philosophy Dissertation*. Department of Biomedical Sciences. University of Wollongong, NSW, Australia.

Spears, C. P., Hyatt, K. H., Vogel, J. M., and Langfitt, S. B. (1974). Unified method for serial study of body fluid compartments. *Aerospace Medicine.* 45: 274-278.

Sterling, K., and Gray, S. J. (1950). Determination of the circulating red cell volume in man by radioactive chromium. *The Journal of Clinical Investigation.*29: 1614-1619.

APPENDIX C

Evans blue dye computer programme

The determination of plasma volume by measurement of plasma samples at two different wavelengths permits the correction of interfering substances at the maximal absorbance of Evans blue dye. The method used for the determination of plasma volume was essentially that described by Foldager and Blomqvist (1991), which utilises a computer programme to find the best fitting Evans blue dye disappearance curve and back extrapolate to the injection time. While the computer programme has been copyrighted (Niels Foldager, Copenhagen, Denmark), the holder permits the use of a single back-up copy. The program is written in Turbo Pascal (version 3.0) for an IBM computer and provides menu-driven facilities for data correction, graphic visualisation of the disappearance curve and print-outs (Foldager and Blomqvist, 1991).

Equipment

1. 20 gauge teflon cannula and extension tubing.
2. Alcohol swab.
3. 9-ml Lithium-heparin monovettes (5).
4. Evans blue dye vial.
5. 5 ml syringe.
6. 20 ml syringe.
7. 21 gauge needle.
8. 20 gauge drawing up needle.
9. Sterile 0.9% sodium chloride.
10. Distilled water.
11. 250 ml flask.
12. 50 ml volumetric flask.
13. Three-way tap.
14. Scientific electronic balance, with precision of 0.0001 g (ER-182A Electronic Balance, A&D Co. Ltd., Tokyo, Japan).
15. Centrifuge.
16. Cuvettes.
17. Spectrophotometer (Shimadzu UV-1601, UV-visible spectrophotometer, Tokyo, Japan).
18. Computer programme (Foldager and Blomqvist, 1991).

Evans blue dye programme constants

1. 40-ml of blood is obtained without stasis from an antecubital fossa vein and transferred to

four lithium-heparin tubes for centrifugation at 1700 *g* for 20 min.

2. Pipette the remaining plasma and transfer to 5-ml plastic vials with a cap.
3. Turn on the spectrophotometer at set to a wavelength of 620 nm.
4. Fill 3 cuvettes with distilled water for zero calibration of the spectrophotometer.
5. Wipe the cuvettes filled with distilled water with a lint-free cloth and insert two cuvettes into the spectrophotometer (reference and sample apertures), close the door and zero the spectrophotometer.
6. Repeat this process with the remaining cuvette to ensure accuracy with the zeroing process.
7. Transfer the plasma to 15 cuvettes, wipe each cuvette with lint-free cloth and measure the absorbance in duplicate using the sample aperture.
8. Repeat the zeroing procedure at a wavelength of 740 nm and measure the absorbance of the plasma samples in duplicate.
9. Plot the 620 nm absorbances on the x-axis and the 740 nm absorbances on the y-axis and calculate the slope and y-intercept of the regression line.
10. Record the slope as BLANK A and the y-intercept as BLANK B.

Evans blue dye computer programme specific extinction

1. Attach a 21-gauge needle to a 5-ml syringe and record the mass to 4 decimal places.
2. Wipe the rubber stopper on the vial with an alcohol wipe.
3. Aspirate 2 ml of Evans blue dye with the syringe and attached needle.
4. Eject all air in the syringe and replace the needle with a new sterile needle.
5. Record the new mass to 4 decimal places.
6. Empty the syringe into a 250-ml flask, fill to the mark with distilled water and mix thoroughly.
7. Calculate the exact concentration based on the empty and dye-filled syringe masses.
8. Attach a 20-gauge drawing up needle onto to a 5-ml syringe and record the mass to 4 decimal places.
9. Aspirate 5 ml of the 250-ml Evans blue dye solution and replace the needle with a new sterile needle and record the mass to 4 decimal places.
10. Transfer this volume to a 50-ml volumetric flask, add 10 ml of plasma (obtained from the constants) and fill to 50 ml with sterile 0.9% sodium chloride.
11. Calculate the exact concentration based on the mass of the syringes and record this as the standard concentration.

12. Turn on the spectrophotometer at set to a wavelength of 620 nm.
13. Fill 3 cuvettes with distilled water for zero calibration of the spectrophotometer.
14. Wipe the cuvettes filled with distilled water with a lint-free cloth and insert two cuvettes into the spectrophotometer (reference and sample apertures), close the door and zero the spectrophotometer.
15. Repeat this process with the remaining cuvette to ensure accuracy with the zeroing process.
16. Transfer the standard concentration to 3 cuvettes and measure the absorbance in duplicate.
17. Repeat the zeroing procedure at a wavelength of 740 nm and measure the absorbance of the standard concentration in duplicate.
18. Calculate the corrected 620 nm absorbance with the formula:

    Corrected 620 nm = 620 nm ABS – (BLANK A * 740 nm ABS + BLANK B)

    where: ABS = absorbance.
19. Calculate the specific extinction (SPEC EXT) with the formula:

    SPEC EXT = corrected 620 nm / standard concentration (units·mg^{-1}·ml^{-1}).

Preparation of Injection

1. Attach a 21-gauge needle to a 5-ml syringe and record the mass to 4 decimal places.
2. Wipe the rubber stopper on the vial with an alcohol wipe.
3. Aspirate 2.5 ml of Evans blue dye with the syringe and attached needle.
4. Eject all air in the syringe and replace the needle with a new sterile needle.
5. Record the new mass to 4 decimal places.

Pre-injection sample

1. Collect 5 ml of blood into a lithium-heparin monovette from a catheter in an antecubital fossa vein.
2. Centrifuge the sample for 15 min at 1700 *g*.
3. Extract the plasma and transfer into a 5-ml plastic tube with a cap and refrigerate at 4^{o}C until analysis later the same day.

Injection

1. Attach the 5-ml syringe of Evans blue dye to a three-way tap connected to extension tubing of the catheter in the opposite antecubital fossa vein.

2. Attach a 20-ml syringe filled with sterile 0.9% sodium chloride to the other three-way tap port.
3. Inject the contents of the Evans blue dye syringe over 1 minute and commence the timer at the end of the injection.
4. Flush the syringe and three-way tap several times with the 20-ml syringe of sterile 0.9% sodium chloride over a 15-sec period (as shown in Figure A).
5. Recap the 5-ml Evans blue dye syringe, record the mass and calculate the exact volume of Evans blue dye injected.

Post-injection sample

1. Prepare the antecubital vein catheter used for sampling at 4 min post-injection.
2. At exactly 5, 7, 10 and 15 min post-injection withdraw 5 ml of blood into separate lithium-heparin monovettes, flushing with 2 ml of sterile 0.9% sodium chloride between samples.
3. Centrifuge the samples for 15 min at 1700 *g*.
4. Extract the plasma and transfer into 5-ml plastic tubes with a cap and refrigerate at 4^{o}C until analysis later the same day.

Spectrophotometry

1. Transfer the plasma from the pre-injection, 5, 7, 10 and 15 min post-injection samples into duplicate cuvettes.
2. Turn on the spectrophotometer at set to a wavelength of 620 nm.
3. Fill 3 cuvettes with distilled water for zero calibration of the spectrophotometer.
4. Wipe the cuvettes filled with distilled water with a lint-free cloth and insert two cuvettes into the spectrophotometer (reference and sample apertures), close the door and zero the spectrophotometer.
5. Repeat this process with the remaining cuvette to ensure accuracy with the zeroing process.
6. Wipe the plasma filled cuvettes with lint-free cloth and read the samples in duplicate.
7. Repeat the zeroing procedure at a wavelength of 740 nm and measure the absorbance of the plasma samples in duplicate.
8. Wipe the cuvettes with lint-free cloth and read the samples in duplicate.
9. Repeat steps 4 - 8 and mean the absorbances determined at 620 and 740 nm, respectively.

Evans blue dye computer programme

1. Start the Evans blue dye computer programme from a Windows based computer and follow the prompts for the input of predetermined BLANK A, BLANK B and specific extinction values.
2. Enter the variables:
    - Evans blue dye batch number
    - Evans blue dye injection concentration
    - Type of spectrophotometer
    - F-cell ratio (if determined previously, otherwise default is 0.91)
    - Correction for trapped plasma (if determined previously, otherwise default is 0.96).
3. These variables are stored and used as constants in the plasma volume determination of individual analyses.
4. Enter the name of the study, subject and the date.
5. Enter the volume of Evans blue dye injected, the times of plasma sampling and the absorbance at 620 and 740 nm. The haematocrit can be entered, if known, for the indirect calculation of total blood volume.
6. Corrections to any of the above variables can be made after visualising the dye disappearance curve.
7. Press print from the menu to obtain details of the input data and the calculated plasma volume.

References

Foldager, N., and Blomqvist, C. G. (1991). Repeated plasma volume determination with the Evans blue dye dilution technique: The method and a computer program. *Computers in Biology and Medicine.* 21: 35-41.

APPENDIX D

Subject consent form

AUSTRALIAN CATHOLIC UNIVERSITY

Statement of Consent

TITLE OF RESEARCH PROJECT: HYDROSTATIC AND THERMAL INFLUENCES ON INTRAVASCULAR VOLUME DETERMINATION DURING IMMERSION: QUANTIFICATION OF THE F-CELL RATIO

NAMES OF INVESTIGATORS (if staff): DR JODIE STOCKS
DR NIGEL TAYLOR

NAMES OF RESEARCHER (students) : MR CHRISTOPHER GORDON

I ..... (the participant) have read (or, where appropriate, *have had read to me) and understood the information provided in the Letter to the Participants and any questions I have asked have been answered to my satisfaction. I agree to participate in this activity, realizing that I can withdraw at any* time.

I agree that research data collected for the study may be published or provided to other researchers in a form that does not identify me in any way.

NAME OF PARTICIPANT .....
(block letters)

SIGNATURE .....

DATE.....

NAME OF PRINCIPAL INVESTIGATOR (if staff) .....
(block letters)

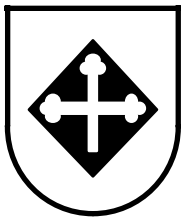
(or NAME OF RESEARCHER (if student) .....)
(block letters)

SIGNATURE .....

DATE.....

APPENDIX E

Subject information package



AUSTRALIAN CATHOLIC UNIVERSITY

TITLE OF PROJECT:
HYDROSTATIC AND THERMAL INFLUENCES ON INTRAVASCULAR VOLUME DETERMINATION DURING IMMERSION: QUANTIFICATION OF THE F-CELL RATIO

NAMES OF INVESTIGATORS:
DR JODIE STOCKS
DR NIGEL TAYLOR

NAME OF RESEARCHER (student): CHRISTOPHER GORDON (Master of Health Science in Human Movement [Research] candidate)

PURPOSE

It is the purpose of this investigation to examine the accuracy of the measurement technique used to determine the volume of blood (red cell volume and plasma volume) in the body during seated rest, and warm- and cold-water immersion.

PROCEDURES

You will take part in three tests, with two weeks between each test. Subjects will be exposed to: (i) seated control period in air (60 min), (ii) immersion in warm water (60 min, 34.5^{o}C), and (iii) immersion in cold water (60 min, 18^{o}C), not necessarily in that order. During the immersion trials you will wear swimming costumes and will be seated and immersed to the level of your chest with both arms above the water level. Tests will be conducted at the same time of day. Prior to testing on the first day, you will be required to undertake a questionnaire.

Measuring blood volume

The principles of radionuclide and dye dilution will be employed to measure the volume of blood in your body. Since the blood volume is made up of the red cell volume and the plasma volume compartments, we will use two different tracers to measure the volume of both compartments of the blood volume. A known amount of a radionuclide tracer (^{51}Chromium (^{51}Cr)) and a dye tracer (Evans blue dye) will be injected into your blood through an arm vein to allow us to determine the red cell volume and plasma volume

respectively. Once the two tracers have had time to circulate in your body, measurement of the red cell and plasma volumes will require us to take a sample of blood from an arm vein. The technique for measuring red cell volume with ^{51}Cr is that recommended by the International Committee for Standardisation in Haematology (ICSH, 1980), and developed by the current laboratory in collaboration with the Illawarra Regional Hospital. Evans blue dye is a harmless dye which has been used for over 50 years to determine plasma volume by binding to protein within the plasma following its injection into the blood stream. No documented cases relating to cancer with Evans blue dye have occurred. Evans blue dye can tint the skin a blue or green colour but this is extremely rare at the administered dose ($\approx$0.3 mg·kg^{-1}), and the effect is only temporary. An already established technique will be used in the current investigation (Greenleaf *et al.*, 1979; Foldager and Blomqvist, 1991).

A 20-ml sample of your blood will be required for the preparation of the radionuclide tracer injection. This will be drawn at the Thermal Physiology Laboratory upon arrival at the laboratory, prior to each test. Standard precautions, in accordance with the N.S.W. Department of Health's Code of Safe Practice in Clinical Laboratories (1987), will be followed for blood handling. The blood will be handled under sterile conditions, in a biological safety cabinet in the Laboratory, using sterile disposable equipment. The radionuclide and dye preparations will be injected through a catheter positioned in your forearm or hand by a nurse. Following the injections, a second catheter will be inserted into the opposite arm. This is necessary since blood sampling must be performed from the opposite arm to the injection site. It will reduce the number of needles to two in each trial, and will eliminate any problems with locating a vein when you are cold during the cold-water immersion trial.

Following the measurement of your resting blood volume, the experimental protocol will begin. You will either stay seated for the control trial, or enter the immersion tank and adopt a seated posture for commencement of the immersion trial in warm or cold water. Thereafter, blood samples will be drawn at minutes 49 and 60. A second Evans blue dye injection will be injected at 50 min of immersion. The second injection is necessary since the dye is not stable within the plasma volume beyond 10-min post injection. Therefore, dye injections are necessary for the pre- and post-trial plasma volume determinations. Following each sample, the catheter will be flushed with a sterile saline solution to keep the needle patent. The total volume of blood drawn for each immersion trial will amount to 54 ml. The experimenter responsible for drawing blood has been trained for the task by medical staff in the Haematology Department of Wollongong Hospital.

Plasma and urine assays

Urine voids will be collected from you in a container used in privacy, twice during baseline and at the completion of the trial.

Body Temperature

Your core temperature and heart rate will be monitored throughout each trial. Core temperature will be monitored using oesophageal and rectal thermistors. Oesophageal temperature will be measured at the level of the heart, which is considered to be the optimal position of the probe.

RISKS AND INCONVENIENCES TO THE PARTICIPANT

The handling of blood is not without risk. However, standard precautions, in accordance with the N.S.W. Department of Health's Code of Safe Practice in Clinical Laboratories (1987), will be followed for blood handling. The blood will be handled under sterile conditions, in a biological safety cabinet in the Thermal Physiology Research Laboratory, using sterile disposable equipment. The tracers to measure your blood volume will be injected by a registered nurse, through a catheter located in a forearm or hand vein. You will feel a small pinch when the catheter is inserted but it will cause only minimal, if any discomfort while in the vein. Blood sampling will be carried out by the nurse or Dr Stocks (trained in this procedure at Wollongong Hospital).

Use of Radionuclides

The technique for measuring red cell volume with ^{51}Cr is that recommended by the International Committee for Standardisation in Haematology (ICSH, 1980). The method has been used extensively in the current laboratory (Maw *et al.*,1995,1996; Regan, 1998; Patterson; 1998).

The nuclide will conform to the Specifications for the Quality Control of Pharmaceutical Preparations published by the World Health Organisation (ICSH, 1980). The ^{51}Cr will be tagged to 5 ml of your own red blood cells, under sterile conditions in the Applied Physiology Laboratory at the University of Wollongong. You are assured of receiving your own blood during the reinfusion process as this procedure will only be performed on one subject at any one time. Up to 8 μCi of radiochromate will be tagged to your red blood cells during each trial. This dose is considerably less than the levels recommended for the study of red cell volume (0.2 μCi per kg of body mass) by the ICSH (1980). The dose received by the organ critically targeted by chromium (spleen) will be less than one-tenth that imposed by specific spleen clinical studies (Carey *et al.*, 1983).

Use of Evans blue dye

Evans Blue dye is a bluish green tetrasodium salt which is very soluble in water. It is a harmless dye which has been used as a diagnostic agent for over 50 years to determine plasma volume. This is accomplished by injecting a known quantity of Evans blue dye into the blood stream where it binds to plasma albumin. After allowing time for thorough mixing within the body, a blood sample is taken to allow the determination of the dye concentration in the plasma. Plasma volume will be measured using Evans blue dye. No adverse effects, either acute or chronic, have ever been reported using Evans blue dye. A NASA Ames assay has demonstrated no toxic or mutagenic effects (NASA, 1985). Thus, no data exists to suggest any measurable risk associated with the use of Evans Blue in human subjects. Large doses of the dye may stain the skin but this is temporary and disappears over a period of weeks. However, the proposed dosage of Evans Blue dye in this study should not result in staining of the skin in any subjects.

Use of oesophageal and rectal probes to measure core temperature

The oesophageal probe will be inserted by a trained technician (either Dr Stocks or Dr Taylor). Rectal temperature will be measured using a thermistor, that you will self-position, in privacy, 12 cm beyond the anal sphincter. Both of these procedures are routine in the current laboratory and have not been associated with any medical complications. Discomfort will be minimal during probe insertion, while in place, and during removal.

Thermal Stress

Water temperatures in this experiment will range from 18^{o}C to 35^{o}C. In each trial, subjects will be clothed in a swimming costume and will be advised to drink plenty of fluids prior to and on completion of each trial. However, due to the focus of the experiment, no drinking will be allowed during the actual trials.

TIME REQUIREMENTS FOR THE STUDY

You will be required on three separate occasions over a six-week period for approximately three hours (nine hours total for the study).

POTENTIAL BENEFITS FOR THE INDIVIDUAL AND SOCIETY

The participant will be able to get a direct measure of their plasma and red cell volumes and how they react under immersion and cold stress. The study provides an appropriate introduction to research. This study will improve our understanding of the plasma volume response during water immersion. If it is found that the current plasma volume measurement technique is in error, it will have important implications for the study of plasma volume responses to external stress in humans.

FREEDOM OF CONSENT

Participation in this project is entirely voluntary. You are free to deny consent before or during the experiment. In the latter case such withdrawal of consent should be performed at the time you specify, and not at the end of a particular trial. Your participation or withdrawal of consent will not influence your present or future involvement with Australian Catholic University or the University of Wollongong. In the case of student involvement, it will not influence grades awarded by the Universities. You have the right to withdraw from any experiment, and this right shall be preserved over and above the goals of the experiment.

INQUIRIES

Questions concerning the procedures or rationale used in this investigation are welcome at any time. Please ask for clarification of any point which you feel is not explained to your satisfaction. Any questions regarding this project may be directed to Mr Christopher Gordon or Dr Jodie Stocks (School of Human Movement, Australian Catholic University, phone: 02-9739-2339), or ultimately to the Head of School of Human Movement, Mr Peter Wiebe (phone: 02-9739-2049).

ETHICS APPROVAL

This study has been approved by the University Research Projects Ethics Committee at Australian Catholic University and the Human Research Ethics Committee at the University of Wollongong.

COMPLAINTS

In the event that you have any complaint about the way you have been treated during the study, or a query that the Investigators have not been able to satisfy, you may contact the Australian Catholic University Human Research Ethics Committee:

Deputy Chair, University Human Research Ethics Committee
C/O Office of Research
Australian Catholic University
25A Barker Rd
Strathfield. NSW. 2135.
Tel: 02 9739 2250
Fax: 02 9739 2240

Any complaint made will be treated in confidence, investigated fully and the participant informed of the outcome.

PROJECT PARTICIPATION

If you agree to participate in this project, you should sign both copies of the Informed Consent form, retain one copy for your records and return the other copy to the Investigator.