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Fluid and energy deficits : hydration markers, saliva immunoglobulin A and endurance performance

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FLUID AND ENERGY DEFICITS: HYDRATION MARKERS, SALIVA IMMUNOGLOBULIN A AND ENDURANCE PERFORMANCE

by

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Summary

The purpose of this thesis was to examine the effect of fluid and/or energy deficits on: 1. the validity of plasma, urine and saliva hydration indices to identify acute and chronic changes in hydration status; 2. salivary immunoglobulin A (s-IgA) at rest and after exercise; and 3. endurance performance.

Modest hypohydration (2-3% body mass loss) evoked by acute (1.5 h) and chronic exercise and fluid restriction (48 h) was identified by plasma, urine and saliva osmolality and saliva flow rate. Using upper euhydrated values, recommended by the American College Sports Medicine for plasma and urine osmolality (>290 and 700 $mOsmol \cdot kg^{-1}$, respectively) and a proposed saliva osmolality threshold (>61 $mOsmol \cdot kg^{-1}$), greater than 75% of individuals were correctly identified as hypohydrated following exercise and fluid restriction protocols. Modest hypohydration resulting from fluid and energy restriction was also identified by saliva and urine indices but not by plasma osmolality. Hydration indices were unable to indicate hypohydration associated with energy restriction alone. This highlights the importance of adequate dietary intake when monitoring hydration changes using these markers, particularly plasma osmolality.

s-IgA secretion rate was decreased after 48 hours of combined fluid and energy restriction. This was most probably due to decreased saliva flow rate and impaired synthesis and/or secretion of s-IgA which may increase individuals' susceptibility to infection. Compared with pre time trial (TT) s-IgA was not lower following a 30 minute treadmill TT highlighting a limited effect of this exercise bout and prior fluid and/or energy restriction on s-IgA responses to exercise. Encouragingly, s-IgA was shown to be normalised after six hours of rehydration and refeeding.

TT performance was significantly decreased following 48 hours of energy restriction and combined fluid and energy restriction but not after fluid restriction alone; supporting a limited effect of modest hypohydration on endurance performance in a temperate environment.

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Publications

I was involved in all aspects of protocol design, data collection, data analyses and preparation of manuscripts for publication and the following thesis chapters. However, I also gratefully acknowledge input from the other named authors for each publication. The following is a list of publications arising from the material presented in this thesis.

Full papers

Walsh, N.P., Laing, S.J., Oliver, S.J., Montague, J.C., Walters, R. and Bilzon, J.L.J. (2004). Saliva parameters as potential indices of hydration status during acute dehydration. *Med.Sci.Sports Exerc.*, *36*, 1535-1542.

Oliver, S.J., Laing, S.J., Wilson, S., Bilzon, J.L.J., Walsh, N.P. (2007). Endurance running performance after 48 hours of restricted fluid and/or energy intake. *Med.Sci.Sports Exerc.*, *39*, 316-322.

Oliver, S.J., Laing, S.J., Wilson, S., Bilzon, J.L.J., Walsh, N.P. (2007). Salivary immunoglobulin A response at rest and after exercise following a 48 hour period of fluid and/or energy restriction. *Br.J.Nutr.*, *97*, 1-8.

Abstracts

Oliver, S.J., Walsh, N.P., Laing, S.J., Montague, J.C. and Kerslake, B.P. (2004). New salivary markers of hydration compare favorably with plasma and urine osmolality during acute dehydration. *Med.Sci.Sports Exerc.*, *36*, S239.

Oliver, S.J., Laing, S.J., Wilson, S., Bilzon, J.L.J. and Walsh, N.P. (2005). The effects of a 48 h period of fluid, calorie, or fluid and calorie restriction on saliva IgA responses. *Brain Behav.Immun.*, *19*, 484-485.

Oliver, S., Wilson, S., Laing, S., Jackson, A., Bilzon, J. and Walsh, N. (2005). The effects of a 48 hour period of fluid or combined fluid and calorie restriction on plasma osmolality, urine osmolality and urine specific gravity. *J.Sports Sci.*, *23*, 1173-1174.

Laing, S.J., Wilson, S., Oliver, S.J., Jackson, A.R., Lloyd-Jones, E., Walters, R., Whitham, M., Bilzon, J.L.J. and Walsh N.P. (2005). The effects of a 48 h period of fluid, calorie or fluid and calorie restriction and a 30 min time trial on circulating lymphocyte and T-lymphocyte $CD3^+$, $CD4^+$ and $CD8^+$ counts. *J.Sports Sci.*, *23*, 1172-1173.

Oliver, S.J., Laing, S.J., Wilson, S., Bilzon, J.L.J. and Walsh, N.P. (2006). Saliva indices track hypohydration during 48 hours of fluid or combined fluid and calorie restriction. *Med.Sci.Sports Exerc.*, *38*, S219.

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Thesis Format

A literature review (**Chapter 2**) provides a brief background and proposes the broad aims of the research presented in the thesis. A general methods chapter follows that outlines the common procedures and analyses performed in the subsequent experimental studies (**Chapter 3**). The thesis consists of two independent experimental studies. The first study principally investigates the validity of saliva parameters to track and identify dehydration evoked by exercise and heat stress (**Chapter 4**). The second experimental study is divided into three chapters which investigate the effects of fluid and/or energy restriction on plasma, urine and saliva hydration markers, s-IgA availability and endurance exercise performance (**Chapters 5 - 7**). A general discussion (**Chapter 8**) contains a summary and critical analysis of the main findings of the research programme, highlighting limitations and potential areas for future research. As all chapters are linked, at times there is necessary overlap between chapters. Throughout the thesis, abbreviations are defined at first use. For clarity a list of abbreviations, tables and figures appears prior to **Chapter 1**. **Bold type** is used when referral is required to sections elsewhere within this thesis.

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List of Abbreviations

ACSM	American College of Sports Medicine
ALI	*ad libitum* fluid intake
ANOVA	analysis of variance
BCAA	branched-chain amino acid
BIA	bioelectrical impedance analysis
BIS	bioelectrical impedance spectroscopy
BM	body mass
BML	body mass loss
$CD4^+$	cluster of differentiation 4
cm	centimetre
CNS	central nervous system
CON	control trial
CV	co-efficient of variation
DEXA	dual energy x-ray absorptiometry
ECF	extracellular fluid
EDTA	ethylenediaminetetraacetic acid
EHI	exertional heat illness
ELISA	enzyme-linked immunosorbent assay
ER	energy restriction
FFA	free fatty acid
FI	fluid intake
FR	fluid restriction
F+ER	fluid and energy restriction
g	gram
h	hour
HR	heart rate
HR_{max}	maximal heart rate
HSD	honest significant difference
ICF	intracellular fluid
Ig	immunoglobulin
IL-2	interleukin-2
kg	kilogram

kJ	kilojoule
km	kilometre
L	litre
m	metre
min	minute
mg	milligram
mL	millilitre
mmol	millimole
mOsmol	milliosmole
NBM	nude body mass
NFI	no fluid intake
RH	relative humidity
RMR	resting metabolic rate
RPE	rating of perceived exertion
r	Pearson's correlation coefficient
SD	standard deviation
SEM	standard error of the mean
s-IgA	saliva immunoglobulin-A
SNS	sympathetic nervous system
SV	stroke volume
TBW	total body water
T_{re}	rectal temperature
TT	time trial
TTE	time to exhaustion
URTI	upper respiratory tract infection
$\dot{V}O_{2max}$	maximal oxygen uptake
W	watts
yrs	years
°C	degrees Celsius
µSv	microsievert

CHAPTER ONE

General Introduction

Fluid and/or energy deficits frequently occur in occupational and athletic settings. For example, in military recruits during survival training (Carins & Booth, 2002; Nindl *et al.*, 2002), athletes with eating disorders (Beals & Manore, 1994; Baum, 2006) and athletes making weight for competition (Brownell *et al.*, 1987; Oppliger *et al.*, 2003). Causes include, but are not limited to, restricted access to fluid and food, an inadequate thirst mechanism, diuretics, heat exposure, illness (e.g. vomiting and diarrhoea) and exercise induced sweat loss with inadequate fluid replacement (Consolazio *et al.*, 1968; Caldwell, 1987; Greenleaf, 1992; Oppliger *et al.*, 2003).

Modest hypohydration is associated with compromised heat dissipation which may raise the risk of exertional heat illness (EHI), impaired cardiovascular function, cognitive-motor function and endurance exercise performance (Armstrong *et al.*, 1985; Gopinathan *et al.*, 1988; Sawka, 1992; Casa *et al.*, 2005a). Nevertheless, the effect of modest hypohydration on endurance performance completed in a temperate environment lasting less than 1 hour (h) remains equivocal (Cheuvront *et al.*, 2003). Hypohydration may also impair mucosal immunity and increase susceptibility to upper respiratory tract infection (URTI) by reducing saliva flow rate and limiting the availability of several proteins known to have important anti-viral and anti-bacterial properties (Fox *et al.*, 1985; Tenovuo, 1998; West *et al.*, 2006). The development of a hydration marker able to identify modest levels of hypohydration, would, therefore be useful to indicate individuals who might be susceptible to EHI, impaired exercise and cognitive-motor performance. Furthermore, saliva hydration markers may help to identify those individuals with an increased susceptibility to URTI.

Energy deficits also increase metabolic strain and stimulate the neuro-endocrine system which has been associated with decreased exercise capacity and an impaired immune function (Aragon-Vargas, 1993; Chandra, 1997). The effects of fluid and/or energy deficits on salivary immunoglobulin A (s-IgA) availability in active individuals at rest and after exercise remain unclear (Gleeson *et al.*, 2004b). The presence of other stressors (e.g. psychological and exercise) in previous investigations may account, at least in part, for the previous reductions in s-IgA availability associated with intense training (Gleeson *et al.*, 1999; Carins & Booth, 2002; Gomez-Merino *et al.*, 2003). Although numerous investigations have examined the effects of energy restriction on exercise time to exhaustion (TTE), the independent and combined effects of fluid and energy restriction on endurance performance assessed using a time trial (TT) has not been examined. In particular, the effect of modest hypohydration evoked over a period greater than five hours on endurance performance is unknown.

CHAPTER TWO

Literature Review

Water is fundamental for life. An individual may live without food for 40 days yet fluid deprivation can be fatal within a few days in a hot climate (Cannon, 1918). Water provides a medium for biochemical reactions, is important for cell function, transports nutrients and waste, assists in body temperature regulation and is essential for maintaining blood volume and hence cardiovascular function (Rowell, 1974; Haussinger & Schliess, 1999; Sawka & Montain, 2000). Water balance is therefore imperative for homeostasis (Vokes, 1987).

2.1 Water and electrolyte requirements

Constant fluid and electrolyte exchange occurs between the body and the environment (e.g. dietary fluid and salt intake, urine and sweat loss). Normal water balance or total body water (TBW) should therefore be considered a sinusoidal wave rather than a specific point (Greenleaf, 1992). Throughout this thesis, euhydration will refer to normal TBW, whereas hypohydration will refer to a TBW deficit. Dehydration is used to describe the dynamic process of water loss between euhydration and hypohydration. Likewise, rehydration is used to describe the process of water gain from hypohydration to euhydration (Sawka & Coyle, 1999).

Daily sources of obligatory water loss include: respiratory (0.2-0.4 L), faecal (0.1-0.2 L), sweat (0.4-1.0 L) and urinary losses (0.5-1.5 L). Conversely, a small gain of obligatory water occurs each day as a by-product of metabolism (0.2-0.4 L, (Shirreffs, 2000; Sawka *et al.*, 2005). To replace these water losses a recommended total water intake of 3.7 L per day has been suggested for sedentary to moderately active adult males (Institute of Medicine,

2004b). Not accounting for sweat sodium losses the total obligatory daily sodium losses from urine, skin (non-sweat) and faeces is no more than 0.2 g (Dahl, 1958). Sodium losses are therefore easily matched by very low dietary sodium intakes because the kidneys reabsorb greater than 99% of total filtered load (Institute of Medicine, 2004a). Conversely, excessive sodium intake is excreted in urine (Madias & Adrogue, 2005). The recommended adequate intake of 1.5 g sodium per day is often far exceeded by western dietary intakes. For example, in the United Kingdom, United States and Canada sodium intakes typically range from 1.5 to 5.7 g per day (Henderson *et al.*, 2003; Institute of Medicine, 2004a). Consequently, under most circumstances the normal dietary intake provides in excess of the required sodium to replace even the additional sweat sodium losses in sedentary individuals.

In more active individuals, water turnover rates in excess of 5 L per day have been reported (Leiper *et al.*, 1996; Fusch *et al.*, 1996; Fusch *et al.*, 1998; Ruby *et al.*, 2003). The increase in water turnover rate can predominantly be explained by elevated sweat loss resulting from exercise or in combination with high ambient temperatures. Maximal sweat rates for acute exercise are commonly between 1 and 2 L per hour; however, in some extreme cases sweat rates greater than 3.5 L per hour have been reported in athletic scenarios (Armstrong *et al.*, 1986; Burke & Hawley, 1997). Individuals rarely maintain these maximal sweat rates for long periods of time. Exceptions include those individuals working for numerous hours in hot environments where sweat losses between 10 and 12 L per day have been reported (Robinson & Robinson, 1954). The magnitude of sweat loss incurred is primarily dependent on the environment (temperature and relative humidity, RH) and the exercise intensity and duration (Montain *et al.*, 1998; Buono & Wall, 2000).

In addition, inappropriate clothing, acclimatisation and training may augment sweat rates (Shapiro *et al.*, 1982; Sawka & Coyle, 1999; McLellan *et al.*, 1999).

For many individuals a reduction in dietary salt intake as promoted by national campaigns (e.g. "Salt-watch it", Scientific Advisory Committee on Nutrition, 2003) may be advisable for health benefits (e.g. decreased blood pressure in hypertensive individuals). However, athletes following low salt diets may consume insufficient sodium to replace sweat losses (Maughan *et al.*, 2004). Even a normal dietary salt intake may provide insufficient sodium for individuals with high sweat sodium concentrations or in situations where larger volumes of sweat and sodium are lost (e.g. hot environments and/or prolonged exercise, Luetkemeier *et al.*, 1997; Bergeron, 2003). The average concentration of sodium in sweat is approximately 50 $mmol \cdot L^{-1}$ but may range from 20 to 100 $mmol \cdot L^{-1}$ (Verde *et al.*, 1982; Shirreffs & Maughan, 1997). Sweat sodium concentration varies depending on the rate of sweating, environmental temperature and humidity, state of heat acclimation, diet and hydration status (Robinson *et al.*, 1950; Sawka *et al.*, 1985; Allsopp *et al.*, 1998; Nielsen, 1998). Assuming an average sweating rate (e.g. 1-2 $L \cdot h^{-1}$) and sodium concentration (e.g. 50 $mmol \cdot L^{-1}$) it is not uncommon for active individuals to lose 1.2 to 2.3 g of sodium per hour (Shirreffs & Maughan, 1997; Burke & Hawley, 1997). Individuals may also fail to maintain sodium balance when dietary intake is restricted. For example, military recruits on field exercise (Nindl *et al.*, 2002; Booth *et al.*, 2003), athletes with eating disorders (Beals & Manore, 1994; Baum, 2006) and athletes making weight for competition (Brownell *et al.*, 1987; Oppliger *et al.*, 2003).

2.2 Water regulation and redistribution

TBW is divided into two major compartments; the intracellular and extracellular fluid (ECF). The ECF is divided into two further compartments the interstitial fluid and smaller intravascular or plasma fluid. Constant equilibrium between these compartments ensures the electrolyte composition of interstitial and plasma fluid compartments remains similar in practically all situations and consequently changes in plasma fluid may be considered to represent alterations in ECF as a whole (Vokes, 1987). Extracellular fluid volume and its contents is in constant exchange with the environment and as most cells are freely permeable to water alterations in ECF osmolality cause changes in intracellular fluid (ICF) volume and osmolality. ECF osmolality is therefore critical for cellular function since cells cannot tolerate significant changes in volume (Vokes, 1987). The preservation of ECF volume and subsequently blood volume is important to circulatory control, tissue perfusion, delivery of nutrients and removal of waste products (Madias & Adrogue, 2005). Consequently, a complex system exists to ensure ECF osmolality and volume is maintained near constancy across a broad range of fluid and sodium intakes and losses. The precise regulation of water and electrolyte homeostasis is accomplished by the interaction of neuro-endocrine and renal systems which balance fluid and electrolyte intake and excretion (Antunes-Rodrigues *et al.*, 2004).

Under normal resting circumstances, when food and water are freely available and the environment is thermally comfortable, fluid and electrolyte balance is regulated by thirst, appetite and urinary water losses (Verbalis, 2003). For example, an increase in ECF osmolality increases thirst and vasopressin secretion which in turn stimulates water consumption and reabsorption of water by kidneys. The increased water intake and urinary water conservation increases TBW and ensures ECF osmolality is returned to its previous

level (Madias & Adrogue, 2005). Alternately, absolute ECF volume is determined by the total sodium content (Vokes, 1987). For example, a sodium loss in excess of fluid loss causes an excretion of largely solute-free urinary water and the restoration of ECF osmolality but leads to a reduction in ECF volume (Robinson *et al.*, 1956; Vokes, 1987).

Irrespective of the complex water regulatory system hypohydration frequently occurs in occupational and athletic settings. Common causes of water deficits include, but are not limited to, water deprivation, restricted access to fluid and food, diuretics, heat exposure, illness (e.g. vomiting and diarrhoea) and exercise induced sweat loss with inadequate fluid replacement (Bloom, 1967; Caldwell, 1987; Greenleaf, 1992; Oppliger *et al.*, 2003). Furthermore, despite individuals having access to adequate fluid, hypohydration is inevitable in some athletic and occupational situations. For example, stressors commonly encountered by active individuals including exercise, environmental heat and cold, altitude, water immersion and dehydration have been reported to compromise the sensitivity of the thirst mechanism which may lead to involuntary dehydration (Engell *et al.*, 1987; Greenleaf, 1992; Kenefick *et al.*, 2004). Fluid replacement may also be compromised in active individuals as sweat rate may exceed maximal gastric emptying rates (Gisolfi, 2000). Gastric emptying is further decreased during dehydration, heat stress and high intensity exercise (>80% maximal oxygen uptake, $\dot{V}O_{2max}$, Neufer *et al.*, 1989; Brouns, 1998). In addition, irrespective of fluid intake, failure to match sodium losses with dietary intake has been shown to cause hypohydration as indicated by a reduction in ECF, blood and plasma volume, and decreases in body mass (BM, Robinson *et al.*, 1956; Consolazio *et al.*, 1967; Consolazio *et al.*, 1968). In fact, Robinson and colleagues (1956) termed this type of water deficit "secondary dehydration" as water losses followed the electrolyte deficit.

The method of dehydration and composition of fluid lost determine the type of water deficit and the partitioning of remaining water between body fluid compartments (Sawka, 1992). Since cell membranes are freely permeable to water but only selectively permeable to solutes the distribution of water between fluid compartments depends upon the solute concentration in the ICF and ECF compartments (Vokes, 1987). Consequently if the method used to evoke hypohydration leads to differences in ICF and/or ECF solute losses, the partitioning of water loss between fluid compartments will vary accordingly (Sawka, 1992). Dehydration resulting from predominantly sweat loss results in hypertonic hypovolaemia as sweat is hypotonic compared with the ECF (Senay, Jr. & Christensen, 1965; Costill & Fink, 1974). Osmotic pressure resulting from the increase in ECF osmolality has been shown to mobilise fluid from the intracellular to ECF compartment (Nose *et al.*, 1988).

Importantly for cardiovascular function, the increase in plasma osmolality has been suggested to help defend circulating blood volume as water is mobilised from extravascular sources to the intravascular fluid compartment (Nose *et al.*, 1988). In contrast, dehydration that evokes a more equal loss of electrolytes and fluids (e.g. diuretics) leading to an isotonic hypovolemia (Myhre & Robinson, 1977; Fortney *et al.*, 1981) has been shown to cause a much greater ratio of plasma fluid loss to TBW loss. These findings suggest water redistribution is dependent on the fluid compartment solute concentration and the solute concentration of the lost fluid. Indeed these results imply the lower the sodium concentration of the lost fluid the smaller the decrease in plasma (blood) volume (Nose *et al.*, 1988). These claims are supported by the decreases in sweat sodium concentration and better maintenance of plasma and blood volume reported following heat acclimation (Shapiro *et al.*, 1998; Nielsen, 1998)

2.3 Water deficits and exercise performance

The effects of fluid losses on exercise performance have been studied by either allowing fluid losses to develop during the exercise (dehydration) or by evoking a desired water deficit prior to an exercise bout (hypohydration). These alternate methods for studying the effect of water deficits on performance have different relevance to athletic and occupational settings. In an athletic scenario, the exercise induced dehydration is more relevant for those involved in endurance events where athletes usually begin euhydrated (e.g. marathon runners and tri-athletes), whereas, those investigations that evoke prior hypohydration are more relevant to athletes where fluid losses are restricted intentionally to make or maintain a desired weight (e.g. dancers, judoka, wrestlers, jockeys and light-weight rowers) or where athletes fail to replace fluid losses between exercise sessions (e.g. twice daily practices, Cheuvront *et al.*, 2003).

Fluid loss effects on aerobic exercise have been extensively studied with in excess of 25 investigations assessing the effect of fluid loss on continuous aerobic exercise alone (Cheuvront *et al.*, 2003). As discussed by a number of reviews a decrement in endurance performance depends upon the magnitude of the water deficit, the duration of the exercise bout and the environmental temperature (Sawka, 1992; Sawka & Coyle, 1999; Cheuvront *et al.*, 2003; Coyle, 2004). Irrespective of environmental conditions, studies have reported a decline in exercise capacity with increased hypohydration (Craig & Cummings, 1966; Pinchan *et al.*, 1988). Additionally, numerous investigations have shown increases in physiological strain indices (e.g. core temperature, heart rate, RPE, and thermal sensation) with greater levels of hypohydration in temperate and hot environments (Sawka *et al.*, 1985; Montain & Coyle, 1992b; Armstrong *et al.*, 1997b; Buono & Wall, 2000). As the duration of exercise becomes more prolonged, increased heat production (hyperthermia)

and sweat loss (dehydration) will cause greater strain on the cardiovascular and thermoregulatory systems which is likely to cause progressively greater reductions in endurance performance.

Increases in environmental temperature cause a redirection of blood flow and volume to the cutaneous vasculature to aid heat dissipation and prevent hyperthermia (Montain & Coyle, 1992a; Montain & Coyle, 1992b; Buono & Wall, 2000). The redirection of blood volume may decrease aerobic exercise performance by reducing the proportion of cardiac output that perfuses the active musculature and by decreasing the central blood volume thereby reducing venous return, stroke volume (SV) and cardiac output (Sawka & Montain, 2000). The negative effect of increased environmental temperature is supported by investigations that report reduced exercise TTE as temperature increases (Galloway & Maughan, 1997). In addition, compared with respective euhydrated trials, 30 min TT performance was decreased following modest hypohydration (3% body mass loss, BML) in a temperate environment (20°C) but was maintained in a cold environment (2°C, Cheuvront *et al.*, 2005). It might be suggested that the lower skin temperature from exercising in a cold environment caused cutaneous vasoconstriction which ensured central blood volume and cardiac output was maintained despite the modest hypohydration. The negative synergistic interaction of hypohydration (~4% BML) and hyperthermia (~1°C increase in core temperature) has been shown to reduce SV by 20% and cardiac output by 13%; whereas hypohydration and hyperthermia alone, decreased SV by 7 to 8% but cardiac output remained unaltered because of a compensatory increase in HR (Gonzalez-Alonso *et al.*, 1997). For endurance performance this suggests individuals may tolerate either modest hypohydration or hyperthermia alone; however, a combination causing a significant decrease in cardiac output is likely to decrease endurance performance.

The general consensus is that endurance exercise greater than 1 h in duration will be negatively affected by performing in a hot environment (>30°C) following 2% BML or in a temperate environment (~20°C) following 3% BML (Barr, 1999; Cheuvront *et al.*, 2003; Coyle, 2004, **Table 2.1**). Furthermore, given the recent findings of Cheuvront and colleagues (2005) it is possible that modest levels of dehydration may have a limited effect on endurance performance completed in cold environments; however, further studies are required to confirm these findings. The effects of modest water deficits (<3% BML) on endurance performance lasting less than 1 h completed in a temperate environment remain unclear (Sawka, 1992; Barr, 1999; Cheuvront *et al.*, 2003, **Table 2.1**).

One widely cited paper reports significant 7% increases in time to complete 5000 and 10000 m track races in a 16°C environment when athletes were hypohydrated to approximately 2% BML using the diuretic Furosemide (Armstrong *et al.*, 1985). Another study shows that a 3% BML evoked by heat exposure decreases work completed on a cycle ergometer by 8% in a 30 min period in a 20°C environment (Cheuvront *et al.*, 2005). In contrast, similar BML evoked by a combination of exercise and fluid restriction had no significant effect on work completed on a cycle ergometer in a 15 min period in a 21°C environment (McConell *et al.*, 1999) or distance completed on a treadmill in a 30 min period in a 25°C environment (Daries *et al.*, 2000).

Table 2.1: A summary of studies investigating the effect of hypohydration on endurance performance.

Study	Environment °C (RH)	Dehydration procedure	Magnitude of hypohydration %BML	Performance test	Performance results
Armstrong *et al.*, 1985	16 (32)	Diuretics (40 mg Furosemide)	EUH vs. HYP 2.0 for 1500, 1.6 for 5000 and 2.1 for 10000 m	1500 m track race 5000 m track race 10000 m track race	7% increase in time to complete 5000 and 10000 m after HYP vs. EUH. No significant difference between HYP vs. EUH for 1500 m
Burge *et al.*, 1993	N/A	Voluntary FR + ER + EX to achieve 5% BML followed by 1.5 L rehydration solution	HYP = 3.0 EUH = 0.0	2000 m rowing race	5% decrease in power output on HYP vs. EUH
Walsh *et al.*, 1994	32 (60)	EX (CE 70% VO_{2max} for 60 min) + FR	NFI = 1.8 FI = 0.0	CE, TTE at 90% VO_{2max} immediately after EX + FR	31% decrease in TTE on NFI vs. FI
Below *et al.*, 1995	31 (54)	EX (CE 80% VO_{2max} for 50 min) + FR	SFI = 2.0 FI = 0.5	CE, 10 min TT immediately after EX + FR	7% decrease in TT work completed on SFI vs. FI

McConell *et al.*, 1997	21 (43)	EX (CE 70% VO_{2max} for 120 min) + FR	NFI = 3.2 SFI = 1.8 FI = 0.1	CE, TTE at 90% VO_{2max} immediately after EX + FR	48% decrease in TTE on NFI vs. FI only. No difference between FI and SFI or NFI and SFI
McConell *et al.*, 1999	21 (41)	EX (CE 80% VO_{2max} for 45 min) + FR	NFI = 1.9 SFI = 1.0 FI = 0.0	CE, 15 min TT immediately after EX + FR	No difference in TT work completed
Daries *et al.*, 2000	25 (55)	EX (TM 65% VO_{2max} for 90 min) + FR	SFI = 3.3 ALI = 2.7 FI = 1.3	TM, 30 min TT immediately after EX + FR	No difference in TT work completed
Cheuvront *et al.*, 2005	20 (50) & 2 (50)	Heat exposure (3 h 45°C 50% RH)	HYP = 3.0 EUH = 0.0	CE, 30 TT 2 h after HE	8% decrease in work completed on HYP vs. EUH in 20°C. A non significant 3% decrease in work completed on HYP vs. EUH in 2°C.

Abbreviations = RH, relative humidity; N/A, not available; $\dot{V}O_{2\,max}$, maximal oxygen uptake; BML, body mass loss; EX, exercise; CE, cycle ergometer; TM, treadmill; HYP, hypohydration; EUH, euhydration; FR, fluid restriction; ER, energy restriction; FI, fluid intake ≥ sweat loss; SFI, some fluid intake; NFI, no fluid intake; ALI, *ad-libitum* intake; TTE, time to exhaustion; TT, time trial.

As previously described in **Section 2.2**, the different methods used to evoke hypohydration may cause varied redistribution of the remaining fluid between fluid compartments which subsequently may account for the equivocal findings regarding the effects of modest hypohydration on endurance performance (Barr, 1999; Cheuvront *et al.*, 2003). For example, compared with control, peak power output on an incremental cycle test to exhaustion (albeit lasting only 10 to 12 min) was better preserved when hypohydration was evoked by diet restriction and exercise than when hypohydration was achieved by diuretics or sauna exposure (Caldwell *et al.*, 1984). In line with this, endurance performance was compromised when hypohydration was evoked by diuretic administration (Armstrong *et al.*, 1985) and heat exposure (Cheuvront *et al.*, 2005) but not when similar BML was achieved using a combination of exercise and fluid restriction (McConell *et al.*, 1999; Daries *et al.*, 2000). From a practical perspective, an advantage of the latter two studies (McConell *et al.*, 1999; Daries *et al.*, 2000) is that a combination of exercise and fluid restriction represents the type of dehydration commonly occurring in military personnel and athletes performing in temperate conditions.

Clearly, as modest hypohydration is common within active populations more research is required to clarify the effects of modest hypohydration on exercise performance in a temperate environment. Furthermore, all of the aforementioned studies induced hypohydration over a short time period (≤5 h). Therefore, a paucity of information is available about the effects of more prolonged dehydration, similar to that encountered in many occupational and athletic settings, on endurance performance.

2.3.1 Negative effect of a water deficit on exercise performance: Proposed mechanisms

Mechanisms proposed to explain the negative effect of a water deficit on exercise performance include: increased cardiovascular strain, increased heat strain (hyperthermia), altered central nervous system (CNS) function, altered metabolic function or a combination thereof (Barr, 1999; Cheuvront *et al.*, 2003). Typically, dehydration lowers blood volume (hypovolaemia) approximately proportional to the loss in BM (Saltin, 1964; Nadel *et al.*, 1980). Hypovolaemia decreases central blood volume, in turn reducing venous return and SV, leading to an increase in cardiovascular strain (Gonzalez-Alonso *et al.*, 1997). The increased cardiovascular strain is identified by the compensatory increase in heart rate (HR) which ensures cardiac output is maintained. This is a frequent observation in dehydrated versus euhydrated individuals during exercise at a constant workload (Sawka *et al.*, 1985; Buono & Wall, 2000). During exercise, HR is suggested to increase by 5 to 8 beats·min^{-1} for every 1% BML due to dehydration (Coyle, 2004). During submaximal exercise in a temperate environment an increase in HR may compensate for hypohydration and maintain cardiac output; however, environmental heat stress results in competition between the central and peripheral circulation for the limited blood volume which may result in decreased cardiac output and skin blood flow, compromising heat loss and exercise performance (Sawka & Coyle, 1999).

Hypovolaemia also increases heat storage which is mediated by reduced sweating rate (evaporative heat loss) and reduced skin blood flow (dry heat loss) for a given core temperature (Sawka *et al.*, 1985; Montain & Coyle, 1992a; Montain & Coyle, 1992b; Buono & Wall, 2000). As sweat is hypotonic compared with the ECF, hypohydration evoked predominately by sweat loss (e.g. exercise and/or heat exposure) causes hypertonic

hypovolaemia (Senay, Jr. & Christensen, 1965; Costill & Fink, 1974). Independent of hypovolaemia, hypertonicity has also been shown to reduce heat dissipation by reducing sweat sensitivity and increasing the temperature for the onset of sweating (Nielsen, 1974; Sawka *et al.*, 1989). Irrespective of the mechanisms, during exercise, hypohydration has been reported to linearly increase core temperature by 0.08°C in temperate (23°C) and 0.15°C in a hot environment (49°C) for each percent decrease in BM compared with euhydrated trials (Sawka *et al.*, 1985; Buono & Wall, 2000). The ability of the cardiovascular system to meet the competing demands of skin and muscle for blood flow without compromising regulation of blood pressure may be a critical factor in determining exercise capacity (Harrison, 1986). Ultimately, blood pressure is maintained at the expense of skin blood flow which leads to an increased rate of heat storage and rises in core temperature (Gonzalez-Alonso *et al.*, 1995).

Hyperthermia (a core temperature >40°C) is often accompanied by dehydration; however, dehydration is not a necessary prerequisite for hyperthermia (Murray, 1996). For example, during exercise at intensities from 80 to 90% $\dot{V}O_{2\,max,}$ metabolic heat production can raise core temperature by approximately 3°C to hyperthermic levels in less than 30 min without causing significant dehydration. Nonetheless, water deficits occurring prior to or during exercise decrease heat dissipation and augment the elevation in core temperature during exercise (Sawka *et al.*, 1985; Montain & Coyle, 1992b). Exercise performance might be reduced indirectly by hypohydration via hyperthermia as at any given exercise intensity rises in core temperature are greater whilst hypohydrated compared with euhydrated (Montain *et al.*, 1998). Additionally, as hypohydration augments hyperthermia it might be assumed to increase the chance of developing heat related illnesses (e.g. heat syncope, EHI, Claremont *et al.*, 1976; Binkley *et al.*, 2002; Casa *et al.*, 2005a).

Hyperthermia has been suggested to impair exercise capacity by both central (i.e. CNS) and peripheral factors (i.e. muscle metabolism, Hargreaves & Febbraio, 1998; Nielsen *et al.*, 2001). At core temperatures ranging from 39.5 to 40°C the drive for exercise may be reduced due to a negative effect of hyperthermia on motor control centres in the brain (Nielsen *et al.*, 1993; Gonzalez-Alonso *et al.*, 1999; Nielsen *et al.*, 2001). Support for this theory comes from investigations that report a reduction in motor cortical activity measured by electroencephalogram (EEG) to be correlated with hyperthermia during fatiguing exercise (Nybo & Nielsen, 2001; Nielsen *et al.*, 2001). Further research is required to investigate the critical core temperature theory as the narrow prescriptive zone for the critical core temperature (39.5-40°C) is frequently exceeded for prolonged periods in asymptomatic individuals during exercise (Maron *et al.*, 1977; Byrne *et al.*, 2006).

Muscle metabolism has also been suggested to be influenced by dehydration and may provide an additional explanation for the decrease in exercise capacity observed following dehydration (Febbraio *et al.*, 1996; Hargreaves & Febbraio, 1998). Indeed, a 16% increase in muscle glycogen usage was reported during exercise with no fluid intake versus exercise with adequate fluid intake (Febbraio *et al.*, 1996). The increase in muscle glycogen utilisation during exercise when dehydrated, compared with euhydrated, is probably related to an increase in catecholamine production or higher core and muscle temperatures or a combination thereof (Febbraio, 2000). Alternatively, dehydration may increase muscle glycogen utilisation as a consequence of reduced cardiac output and subsequent decreased muscle blood flow and oxygen delivery to the muscle (Gonzalez-Alonso *et al.*, 1998).

2.3.2 Energy restriction and endurance performance

The debilitating effects of energy restriction, with prescribed or *ad libitum* water intake, on endurance performance are well documented (Aragon-Vargas, 1993). Indeed, compared with fed trials, TTE at intensities ranging from 50 to 85% $\dot{V}O_{2\,max}$ has been reported to be decreased by 26 to 63% after energy restriction lasting from 23 to 36 h (Loy *et al.*, 1986; Nieman *et al.*, 1987; Maughan & Gleeson, 1988; Zinker *et al.*, 1990). Causes may include, but are not limited to, muscle and liver glycogen depletion, altered acid-base balance and central fatigue (Gleeson *et al.*, 1988; Maughan & Gleeson, 1988; Zinker *et al.*, 1990; Aragon-Vargas, 1993).

Using a combination of exercise and diet modification, a close relationship between pre-exercise muscle and liver glycogen content and exercise performance has previously been shown, where lower pre-exercise muscle and liver glycogen led to an earlier onset of fatigue (Bergstrom *et al.*, 1967; Karlsson & Saltin, 1971). Fasting and diets low in carbohydrate are also associated with increased metabolic acidosis which may also decrease exercise performance (Greenhaff *et al.*, 1987). An increase in acidity may decrease the contractile muscle mechanism by reducing calcium sensitivity and anaerobic glycolysis via decreased phosphofructokinase activity (Maughan *et al.*, 1997). An increase in acidity may also augment the sensation of pain by stimulating free nerve endings which consequently may lead to an earlier onset of fatigue (Maughan *et al.*, 1997).

Energy restriction associated with increases in branched-chain amino acid (BCAA) metabolism for gluconeogenesis and circulating free fatty acid (FFA) concentrations may also decrease exercise performance via increases in central fatigue (Newsholme & Blomstrand, 2006). Specifically, a greater quantity of the serotonin precursor tryptophan

has been suggested to cross into the brain because increased FFA's displace albumin bound tryptophan and lower plasma BCAA result in less competition for a specific transport mechanism (Davis & Bailey, 1997). Brain serotonin is involved in the control of arousal, sleepiness and mood and might therefore be suggested to affect exercise performance. However, at least in humans, a direct role of serotonin in decreasing exercise performance remains equivocal (Blomstrand, 2006). As it is difficult to blind participants to nutritent deficits the deleterious effects of energy restriction to endurance performance may also simply be a manifestation of human expectations.

2.3.3 Time to exhaustion versus time trial protocols

Time to exhaustion protocols have been criticised for having poor test-retest reliability with mean coefficient of variation (CV) ranging from 20 to 27% (Krebs & Powers, 1989; Jeukendrup *et al.*, 1996) compared with mean CV of less than 5% for TT protocols (Jeukendrup *et al.*, 1996; Schabort *et al.*, 1998). Poor reliability in TTE protocols might be explained by psychological factors (e.g. motivation and boredom) which may be more variable in an "open" versus "known end-point" test (Jeukendrup *et al.*, 1996). Variability of this magnitude causes difficulty when attempting to identify the proportion of change in individual performance attributable to the intervention and not to measurement error. In addition, as pacing strategy is an inherent component of racing, TT endurance performance may be considered more indicative of real-life athletic events compared with TTE protocols. Time trial reproducibility investigations to date have been conducted in well trained elite athletes with experience of racing (Jeukendrup *et al.*, 1996; Schabort *et al.*, 1998). Less experienced non-elite individuals TT performance may be more variable and therefore future research is required to determine the reproducibility of TT performance in non-elite individuals.

To date energy restriction studies have all used TTE protocols. Although numerous investigations have examined the effects of energy restriction on TTE, the effect of energy restriction on TT performance has yet to be assessed. Furthermore, the combined effect of hypohydration and energy restriction remains to be examined.

2.4 Hydration assessment techniques

Modest hypohydration (2-3% BML) is associated with compromised heat dissipation (possibly raising the risk of EHI), cardiovascular function, endurance exercise performance and cognitive-motor function (Armstrong *et al.*, 1985; Gopinathan *et al.*, 1988; Sawka, 1992; Casa *et al.*, 2005a). Currently available hydration assessment techniques vary greatly in their applicability due to differences in validity (i.e. type of hypohydration anticipated and the sensitivity to detect small meaningful changes in hydration status) and practicality (i.e. cost, technical demand and immediacy of result). To be valid, hydration assessment techniques must be shown to track both acute and chronic dehydration. Given the numerous negative consequences of modest hypohydration, a hydration marker should also be sensitive to identify body water losses equivalent to 2 to 3% of BML (Shirreffs, 2000). Furthermore, hydration markers should also reliably identify individual hydration status. For a hydration assessment technique to be truly practical for routine monitoring of individual hydration status it must be inexpensive, provide an immediate result and require little technical equipment or expertise. A valid and practical hydration marker able to identify modest hypohydration would help to indicate individuals that might be susceptible to EHI and impaired exercise and cognitive-motor performance. Hydration techniques include body mass difference, isotope dilution and bioelectrical impedance which assess total fluid volume changes. In addition a number of haematological, urinary and saliva indices may be used to estimate hydration status with a single measure.

2.4.1 Body mass difference

Body mass is frequently used to estimate changes in fluid balance over acute periods (e.g. single exercise bouts). The estimated change in hydration status is usually calculated as the difference in weight during the assessment period expressed as a percentage of the initial BM (i.e. ΔBM/initial BM) x 100). Using the change in BM to estimate a change in hydration status is based upon the assumption that 1 kg weight lost is equal to 1 L of TBW lost. To ensure this assumption is not violated corrections should be made for food and fluid intake and sweat trapped in clothing which will artificially inflate BM leading to underestimations of TBW lost. Following heavy sweating it is therefore imperative to assess nude body mass (NBM) after individuals have towelled. An advantage of determining absolute fluid volumes lost during dehydration, compared with a change in the concentration of a marker (e.g. plasma osmolality), is the required fluid replacement volume for subsequent rehydration can be calculated.

Encouragingly, day-to-day changes in first morning BM have been shown to predominantly reflect changes in hydration status (Grandjean *et al.*, 2000; Grandjean *et al.*, 2003; Cheuvront *et al.*, 2004). These studies have shown that first morning BM measures vary by no more than 0.7% day-to-day in free-living sedentary persons and individuals undergoing daily exercise and heat acclimatisation for periods of up to 14 days (Grandjean *et al.*, 2000; Grandjean *et al.*, 2003; Cheuvront *et al.*, 2004). Based upon these investigations it has been suggested that a change in day-to-day first morning BM of greater than 1% indicates hypohydration (Casa *et al.*, 2005b). For periods greater than 14 days using change in BM to reflect changes in hydration is not well accepted. Changes in BM over longer periods are likely to be affected by gross changes in body composition which may invalidate any estimation of hydration status. The standard against which the

validity of other hydration assessment techniques are compared in acute settings remains NBM change (Armstrong *et al.*, 1994; Shirreffs & Maughan, 1998; Popowski *et al.*, 2001). In fact, if the proper controls are made, NBM change provides a more sensitive estimate of acute changes in TBW than serial measures of TBW by dilution techniques (Gudivaka *et al.*, 1999).

2.4.2 Dilution and bioelectrical impedance hydration assessment techniques

Dilution and bioelectrical impedance techniques enable the assessment of absolute body fluid compartment volumes including: blood, plasma, ECF, ICF and TBW. Like BM, repeated measures are required to assess changes in hydration status. With a reported sensitivity of 0.8 L dilution techniques are popularly regarded as the gold standard for assessing TBW (Armstrong, 2005). Blood and plasma volume can also be measured by isotope or dye dilutions and have been shown to track TBW losses associated with dehydration (Harrison, 1985). Indeed, dehydration lowers blood volume approximately proportional to the loss in BM (Saltin, 1964; Nadel *et al.*, 1980). Unfortunately, cost and both complicated and protracted biochemical analysis make dilution techniques impractical as a routine hydration assessment measure. Indirect measurement of blood and plasma volume change in the short term (hours) only may be estimated by measuring haematocrit and haemoglobin concentration (Dill & Costill, 1974). However, the sampling and analysis must be standardised because haematocrit and haemoglobin have been reported to be altered by changes in posture, use of a tourniquet during blood collection and the method of analysis needed to assess haematocrit (McNair *et al.*, 1979; Harrison, 1985; Maughan *et al.*, 2001).

Bioelectrical impedance analysis (BIA) and bioelectrical impedance spectroscopy (BIS) provide alternative methods to evaluate TBW, ICF and ECF volumes. In a controlled laboratory condition with euhydrated individuals absolute TBW, ICF and ECF volumes determined by BIS have been shown to be correlate strongly with dilution methods ($r >$ 0.9, Armstrong et al., 1997a). However, the difference in individual assessment of TBW between dilution and impedance measures were shown to range from -5.4 to 2.9 L (Armstrong *et al.*, 1997a). More alarmingly, compared with dilution methods, impedance techniques have been shown to underestimate fluid losses during dehydration as BML increases (O'Brien *et al.*, 1999). These investigations suggest that impedance techniques may not possess sufficient sensitivity to identify modest levels of dehydration in individuals. Furthermore, impedance techniques are invalidated by changes in skin temperature, body fluid electrolyte concentration and subject posture making these techniques unsuitable to assess individual hydration status following or during exercise (O'Brien *et al.*, 2002).

2.4.3 Limitation of fluid volume assessment

Hydration assessment techniques that assess absolute fluid volumes are unable to provide an indication of an individual's hydration status with a single measure. Using TBW as an example, a TBW fluid volume assessment of 42 L is arbitrary and does not help to identify an individual's hydration status. Even with a simultaneous BM measure hydration status may not be determined as TBW may vary from 45 to 75% of BM depending on body composition (Wang *et al.*, 1999). Therefore a single measure of absolute TBW does not provide an indication of hydration status. The same criticism is applicable to volumes derived from other body fluid compartments including intravascular, ECF or ICF. Furthermore, thresholds to indicate euhydration and hypohydration may not be determined

for volumes since individual differences in BM and body composition are too varied. Hydration assessment techniques that assess absolute fluid volumes require repeated measures and are therefore limited to assessing changes in hydration status. Hydration assessment techniques that may assess hydration status with a single measure include haematological and urinary concentrations (e.g. plasma and urine osmolality, Shirreffs, 2000; Oppliger & Bartok, 2002).

2.4.4 Haematological indices during acute dehydration

Plasma osmolality may be considered a useful indicator of hydration status as it is the primary physiological stimulus used to regulate water balance (Baylis, 1987). Changes in plasma osmolality and volume alter the secretion of vasopressin which in turn alters urine production in the distal renal tubule (Madias & Adrogue, 2005). The control of vasopressin is principally affected by plasma osmolality as only a 1 to 2% increase is required during dehydration to stimulate vasopressin release from the hypothalamus (Baylis, 1987). Plasma osmolality is closely controlled by homeostatic systems and is controlled around a set point 280 to 290 mOsmol·kg^{-1} (Institute of Medicine, 2004b). This range has recently been recommended by the American College of Sports Medicine (ACSM), in a consensus statement concerned with hydration and physical activity, to indicate euhydration (Casa *et al.*, 2005b). Nonetheless, the average euhydrated plasma osmolality reported by investigations with prior rigorous hydration strategies is 288 mOsmol·kg^{-1} (**Table 2.2**) which is at the upper end of the recommended range for euhydration 280-290 mOsmol·kg^{-1} (Casa *et al.*, 2005b).

Plasma osmolality has also been shown to indicate changes in hydration status. Data from 19 fluid balance studies compiled in a recent review show a strong inverse correlation (r =

-0.76) between change in plasma osmolality and changes in TBW across a wide range of hydration states (minus 12 to 2% TBW change, Institute of Medicine, 2004b). It is important to note the majority of these studies evoked changes in hydration status by a combination of exercise and/or heat stress lasting less than 6 h. Therefore, the strength of the relationship between plasma osmolality and changes in hydration status is relatively unknown when fluid losses are evoked over more prolonged periods (>6 h) or from different methods of dehydration. Plasma osmolality has also been shown to progressively track acute dehydration evoked by exercise and heat stress (Popowski *et al.*, 2001). In fact, Popowski and colleagues (2001) showed plasma osmolality to increase by 5 to 7 $mOsmol \cdot kg^{-1}$ for approximately every 1 to 2% BML suggesting changes in plasma osmolality may be able to quantify the magnitude of dehydration. The sensitivity of plasma osmolality to detect a 1% BML change in hydration status in this study has resulted in it being popularly regarded as a gold-standard measure of hydration status (Popowski *et al.*, 2001; Casa *et al.*, 2005b). Nonetheless, haematological derived measures require invasive blood samples to be collected by a qualified phlebotomist and use of a centrifuge which make these markers inappropriate for monitoring day-to-day hydration status of individuals during training or competition.

2.4.5 Urinary indices during acute dehydration

The maintenance of fluid balance is primarily achieved via alterations in urine output where urine volume and concentration of solutes in urine vary in accordance with changes in hydration status (Madias & Adrogue, 2005). At rest urine volume and concentration are largely controlled by alterations in vasopressin. Individuals on a normal diet excrete between 600 to 800 mOsmol of solute per square metre of body surface area per day (Shirreffs & Maughan, 1998). As the kidneys can dilute and concentrate urine from

approximately 50 up to 1200 mOsmol·kg^{-1} the daily solute load can be excreted in a volume of urine ranging between 500 mL and 13 L per day (Shirreffs & Maughan, 1998). The removal of waste products from the body is essential and for this reason once the maximal concentrating capacity of the kidney is reached fluid deprivation will lead to a decrease in TBW (Shirreffs *et al.*, 2004). Early research with individuals under heat stress supported an obligatory daily urine volume equal to approximately 500 mL (Leithead & Pallister, 1960). However, the collection of daily urine volume is impractical and does not allow for the assessment of an individuals hydration status at a single time point.

Table 2.2: A summary of plasma, urine and saliva hydration indices that represent euhydration.

Study/trial	Euhydration strategy prior to obtaining samples	Number of participants	Plasma osmolality ($mOsmol \cdot kg^{-1}$)	Urine osmolality ($mOsmol \cdot kg^{-1}$)	Urine specific gravity ($g \cdot mL^{-1}$)	Saliva flow rate ($\mu L \cdot min^{-1}$)	Saliva osmolality ($mOsmol \cdot kg^{-1}$)
Sawka *et al.*, 1985	Serial BM measures over 2 weeks used to establish individuals euhydrated BM	8	284 ± 5				
Fortney *et al.*, 1988 4 trials	Instructed to consume fluids frequently day prior and 300 mL 1 h before arriving for sampling	6	286-288 [a]				
Armstrong *et al.*, 1994 2 studies	~500 mL of water consumed on the evening prior and upon waking the morning of sampling	23 11	289 ± 3 286 ± 3	858 ± 219 510 ± 332	1.023 ± 0.006 1.013 ± 0.009		
Armstrong *et al.*, 1997b 2 trials	See above	10	287 ± 6 287 ± 5		1.016 ± 0.002 1.015 ± 0.002		
Melin *et al.*, 1997	3 day standard diet	5	290 ± 3				

Armstrong *et al.*, 1998[b]	Water equal to 35 mL·kg^{-1} of BM consumed the day prior	9	~280	~125	~1.004		
Shirreffs & Maughan, 1998 2 studies	Free living no dietary restriction	11 8		675 ± 232 627 ± 186			
Blannin *et al.*, 1998 3 trials[c]	Similar dietary intake prior to the 3 trials	18				272 ± 49 256 ± 70 229 ± 57	52 ± 2 57 ± 3 57 ± 3
Kovacs *et al.*, 1999 3 trials	Small standard breakfast with plenty of fluid	8	290 ± 2 290 ± 2 291 ± 1				
Popowski *et al.*, 2001	Instructed to consume fluids frequently day prior and 250 mL before arriving for sampling	12	288 ± 4	325 ± 218	1.009 ± 0.006		
Maresh *et al.*, 2001	Water equal to ~30 mL·kg^{-1} of BM consumed the day prior	11	289 ± 3		1.022 ± 0.003		
Walsh *et al.*, 2002 2 trials[c]	Small standard breakfast with ~500 mL of water.	15				441 ± 63 434 ± 73	62 ± 4 61 ± 6

Walsh *et al.*, 2004b	Water equal to 30 mL·kg^{-1} of BM consumed the day prior	12				491 ± 223	51 ± 10
Bartok *et al.*, 2004	A standard meal and water equal to 30 mL·kg^{-1} of BM consumed the evening prior to 1st morning samples	25	288 ± 4	614 ± 192	1.015 ± 0.007		
Oppliger *et al.*, 2005	Instructed to consume fluids frequently day prior and 250 mL before arriving for sampling	12	288 ± 4	211 ± 195	1.008 ± 0.007		

Values are means ± SD unless otherwise stated. Abbreviations = BM, body mass, [a] a range of mean euhydrated values from 4 trials, [b] estimated from figures, [c] means ± SEM.

The majority of investigations have focused on assessing urine concentration either quantitatively (specific gravity and osmolality) or qualitatively (colour). Urine concentration has been shown to be a valid and reliable assessment technique to differentiate between euhydration and hypohydration (Armstrong *et al.*, 1994; Shirreffs & Maughan, 1998). For example, first morning urine osmolality measures were shown to be sensitive to hypohydration equal to approximately 2% BML evoked by exercise and heat stress with restricted overnight fluid intake (Shirreffs & Maughan, 1998). Furthermore, urine osmolality, specific gravity and colour have been shown to track progressive acute dehydration evoked by exercise alone and exercise and heat stress (Armstrong *et al.*, 1994; Armstrong *et al.*, 1998). Urine indices have been shown to lag behind changes in plasma osmolality during acute dehydration lasting 1 to 3 h evoked by exercise and heat stress (Popowski *et al.*, 2001) and rehydration (Kovacs *et al.*, 1999).

The inferior sensitivity of urine markers compared with plasma osmolality might be explained in part by an increased neural involvement in urine production during exercise compared with at rest (Wade, 1996; Zambraski, 1996). During moderate to high intensity exercise, an increase in sympatho-adrenal system activity, indicated by increased catecholamine activity, has been shown to be involved in the reduction of urine output (Zambraski, 1996; Melin *et al.*, 2001). Specifically, an increase in sympathetic nervous system (SNS) activity has been suggested to cause vasoconstriction of the glomerular afferent arterioles which decrease renal blood flow which in turn decreases urine flow (Wade, 1996; Zambraski, 1996). The reduction in urine flow is accompanied by an impaired renal concentrating ability which may be responsible for the inferior sensitivity of urine indices during exercise.

Although urine has been shown to be less sensitive than plasma osmolality to small changes in hydration status (Kovacs *et al.*, 1999; Popowski *et al.*, 2001; Walsh *et al.*, 2004b) a practical advantage of urine indices is the less invasive sampling method. Furthermore, the development of simple handheld devices (i.e. refractometers and osmometers) and the urine colour chart (Armstrong *et al.*, 1994), designed to assess urine specific gravity, osmolality and colour, respectively, identify urine indices as the most practical hydration marker currently available for the field setting. A recent ACSM consensus statement, concerned with hydration and physical activity, reported that euhydrated individuals should have a urine specific gravity of less than 1.020 $g \cdot mL^{-1}$, a pale yellow urine colour (e.g. traditional lemonade) or a urine osmolality of less than 700 $mOsmol \cdot kg^{-1}$ (Casa *et al.*, 2005b). Urine specific gravity and urine osmolality are often used interchangeably (Armstrong *et al.*, 1994); however, the measure of osmolality rather than specific gravity should be used where possible as specific gravity, unlike osmolality, is affected by alterations in sample temperature (Madias & Adrogue, 2005).

2.4.6 Haematological and urinary indices during chronic dehydration

Unfortunately, little is known about the validity of hydration indices during more prolonged dehydration where fluid losses may occur more slowly from a combination of sweat and renal losses. As previously described, the method of dehydration and composition of fluid lost determine the type of water deficit and the partitioning of remaining water between body fluid compartments (Nose *et al.*, 1988; Sawka, 1992). In addition, prolonged exercise may influence haematological and therefore urinary hydration markers independently of changes in hydration. For example, haematological indices may be affected by osmotically active particles (e.g. lactate) that are produced by the active muscles and diffuse into the intravascular compartment (Fortney *et al.*, 1988). Secondly,

compared with other forms of dehydration prolonged exercise causes a redistribution of blood flow and volume to the periphery which may at least be partly responsible for the commonly reported decrease in plasma volume (Harrison, 1985). Additionally, plasma volume decreases after acute dehydration have been shown to be greater immediately post exercise compared with measures obtained 30 and 60 min post exercise despite hypohydration level remaining the same (Nose *et al.*, 1988); suggesting fluid equilibration is delayed following exercise. Thirdly, as previously described during exercise urine production is largely under neural control compared with endocrine control at rest (Wade, 1996; Zambraski, 1996). Given the potential differences in fluid redistribution between dehydration methods and as prolonged exercise may affect haematological and urinary hydration markers independently of changes in hydration it is important to assess the validity of hydration indices over more prolonged periods.

The findings of studies examining the validity of hydration markers to indicate hypohydration over periods greater than 4 h remain unclear (Ship & Fischer, 1997; Shirreffs & Maughan, 1998; Armstrong *et al.*, 1998; Shirreffs *et al.*, 2004). Plasma osmolality, urine osmolality, specific gravity and colour were shown to detect hypohydration equal to 3.7% BML evoked by 2 h of exercise with a further 21 h period of fluid restriction (Armstrong *et al.*, 1998). A more recent study that restricted fluid intake alone for 37 h showed increases in serum and urine osmolality detected hypohydration equal to approximately 1% BML after 13 h (Shirreffs *et al.*, 2004). However, during this investigation urine osmolality was determined on total urine volumes collected between the blood samples at 0, 13, 24 and 37 h and therefore the validity of single urine sampling over a prolonged period is unknown. Moreover, despite further BML at 24 h (~1.8%) a plateau in serum and urine osmolality occurred thereby questioning the utility of these

indices to track progressive chronic dehydration and identify the magnitude of hypohydration evoked by prolonged fluid restriction.

2.4.7 Haematological and urinary indices during fluid and energy restriction

Hydration assessment techniques should also be able to identify hypohydration when energy is restricted simultaneously (e.g. athlete making weight or military recruits on field exercise, Carins & Booth, 2002; Oppliger *et al.*, 2003). Despite *ad libitum* fluid ingestion, energy restriction has been shown to cause significant decreases in ECF volume and BM which are indicative of hypohydration (Consolazio *et al.*, 1967; Consolazio *et al.*, 1968). This hypohydration may be attributed to a decrease in sodium content (Robinson *et al.*, 1956; Consolazio *et al.*, 1968). Specifically, to ensure the restoration of ECF osmolality, a decrease in ECF sodium content leads to the excretion of solute-free urinary water and subsequently a decrease in total plasma and ECF volume (Vokes, 1987). Possible causes of a decrease in sodium content include: inadequate sodium intake, increased natriuresis with energy restriction and sweat sodium losses (Robinson *et al.*, 1956; Bloom, 1967; Kolanowski *et al.*, 1977). These causes are particularly likely to affect active individuals or individuals in hot environments (Luetkemeier *et al.*, 1997; Bergeron, 2003).

To the author's knowledge no investigation to date has examined the validity of hydration indices when hypohydration is accompanied by energy restriction. Nonetheless, a negative salt balance, evoked by dietary salt restriction and sweat losses from exercise, combined with 24 hours of fluid restriction was shown to evoke hypohydration equal to 3.4% BML but cause no significant alteration in plasma chloride concentration (Robinson *et al.*, 1956). Given the association between plasma chloride and plasma osmolality these findings suggest the validity of plasma osmolality to indicate changes in hydration status may be

compromised during energy restriction with a concomitant negative salt balance. In addition a similar negative salt balance combined with excess water intake was associated with a decrease plasma chloride concentration and a copious urine production despite modest hypohydration (~2% BML, Robinson *et al.*, 1956; Pearcy *et al.*, 1956). The interpretation of these findings is difficult as urine concentration was not reported. Nonetheless the copious and probably dilute urine production despite hypohydration questions the validity of urine indices (i.e. volume, concentration and colour) to identify hypohydration associated with energy restriction. In summary, these finding suggest dietary restriction might be expected, particularly in active individuals, to impact upon the validity of haematological and urinary hydration indices.

In summary, plasma and urine osmolality have been shown to track dehydration in an acute setting; however, as they require basic biochemical analysis and cannot reliably quantify the magnitude of fluid loss they provide no practical advantage over measures of NBM change in an acute setting. However, plasma and urine osmolality may be suitable to indicate changes in hydration over a more prolonged period when NBM changes can be influenced by many factors other than hydration status (e.g. food intake, bowel movements, body weight changes associated with substrate metabolism or changes in body composition). More importantly, unlike NBM change, plasma and urine osmolality offer a method of assessing hydration status with a single measure. However, insufficient evidence exists to conclude whether plasma and urine markers are valid to track changes in hydration status and sensitive to identify modest hypohydration (2-3% BML) occurring over prolonged periods (>4 h) when hypohydration is evoked by fluid and/or energy restriction.

2.4.8 Saliva as a potential marker of hydration status

Whole saliva is a complex mix of fluids secreted into the mouth by three major pairs of salivary glands (i.e. submandibular, parotid and sublingual) and by a number of minor salivary glands (Humphrey & Williamson, 2001). On average, unstimulated whole saliva flow rate ranges between 300-400 μL per min (**Table 2.2**); however, saliva flow rate is highly individual: flow rates commonly exceed 1000 μL per min or are reduced to less than 100 μL per min (Dawes, 1987; Edgar, 1992). An unstimulated flow rate lower than 100 μL per min or a 50% reduction in normal resting saliva flow rate indicates salivary gland hypofunction (Sreebny & Valdini, 1987). Percentage contributions of the different glands during unstimulated flow are 65% submandibular, 20% parotid, 7 to 8% sublingual and less than 10% from numerous minor glands (Chicharro *et al.*, 1998). The saliva secreted by the parotid glands is serous (watery) whereas the minor glands produce a mainly mucous secretion and the submandibular and sublingual glands produce mixed serous and mucous saliva.

Stimulated saliva flow rates alter the percentage contribution of each gland with the parotid gland contributing to more than 50% of the total saliva produced (Humphrey & Williamson, 2001). Stimulated saliva may account for up to 90% of total daily saliva volume with the majority of this production occurring around meal times to assist with digestion (Humphrey & Williamson, 2001). However, unstimulated samples remain more indicative of normal resting saliva production. The differences in saliva produced mean methods used to obtain saliva and the type of saliva collected must be standardised throughout investigations. For these reasons it is also unwise to compare studies results where the saliva sample and the method used to obtain the sample are different.

As the predominant fluid constituent of saliva is water (97-99.5%), which enters saliva from plasma across acinar cells (Ship & Fischer, 1997), it follows that reductions in saliva flow rate have been observed following periods of dehydration evoked by water deprivation (Cannon, 1918; Gregersen & Bullock, 1933), desert heat exposure (Adolph, 1947) and hot water immersion (Winsor, 1930). Although largely made on observations of one or two individuals collectively, these studies suggest that a relationship exists between whole body hydration status and saliva flow rate. Saliva flow rate has also been commonly reported to decrease following prolonged exercise (Ford *et al.*, 1997; Ljungberg *et al.*, 1997; Blannin *et al.*, 1998; Bishop *et al.*, 2000; Walsh *et al.*, 2002). Dehydration, at least in part, may be responsible for these reductions in saliva flow rate given that the decreases in flow rate were prevented when individuals consumed sufficient water to offset fluid losses (Ford *et al.*, 1997; Bishop *et al.*, 2000).

A more recent investigation has shown saliva flow rate, saliva osmolality and saliva total protein concentration are able to track progressive dehydration to 3% BML evoked by exercise and heat stress lasting 1 to 3 h (Walsh *et al.*, 2004b). Encouragingly, both saliva osmolality and total protein concentration were shown to identify hypohydration equal to approximately 1% BML which compares well with plasma osmolality (Popowski *et al.*, 2001). Saliva flow rate was shown to have poorer sensitivity than both saliva osmolality and total protein concentration identifying BML equal to 2%. The sensitivity of the saliva parameters was sufficient to fulfil the criteria of a hydration marker (Shirreffs, 2000). Nonetheless, for saliva parameters to be truly accepted as markers of hydration status future investigations are required to confirm these findings and show that saliva parameters are able to correlate with well accepted hydration indices like plasma and urine osmolality. In addition, besides the one investigation in only two participants (Gregersen & Bullock,

1933), no thorough investigation to date has examined the effects of prolonged dehydration (>3 h) on whole saliva indices. Saliva flow rate, albeit parotid, has been shown to decrease in response to a 24 h period of fasting (Ship & Fischer, 1997). Together with the acute exercise findings (Walsh *et al.*, 2004b) theses studies suggest saliva flow rate may be a useful indicator of hydration status over more prolonged periods to indicate changes in hydration status. However, whether whole saliva hydration indices (e.g. saliva osmolality) are sensitive to modest hypohydration and able to track progressive changes in hydration status that may occur over a number of days evoked by combinations of exercise, heat exposure, fluid and food restriction remains unclear.

2.4.9 Saliva control

Salivary gland function is predominantly under the control of the autonomic nervous system with the salivary glands innervated by both the sympathetic and parasympathetic nerves (Chicharro *et al.*, 1998). In general, parasympathetic nerves are considered to govern salivary fluid secretion, whereas sympathetic nerves regulate protein secretion (Teeuw *et al.*, 2004). Parasympathetic stimulation is suggested to evoke copious serous flow whereas an increase in sympathetic activity produces saliva low in volume and high in proteins (Chicharro *et al.*, 1998). Specifically, increased sympathetic activity is suggested to cause vasoconstriction of the blood vessels supplying the gland reducing blood flow and thereby causing decreased saliva production. An increase in SNS activity is therefore most commonly postulated to explain the decrease in saliva flow rate often observed following prolonged exercise (Dawes, 1981; Chicharro *et al.*, 1998).

A role for hypohydration has also been proposed to explain the secretion of smaller amounts of more concentrated saliva (e.g. with increased osmolality and total protein

concentration) observed following prolonged exercise (Ljungberg *et al.*, 1997; Blannin *et al.*, 1998; Bishop *et al.*, 2000; Walsh *et al.*, 2002; Walsh *et al.*, 2004b). In order for water to move from plasma through acinar cells to form primary saliva, a trans-acinar cell sodium gradient must be generated (Ship & Fischer, 1997). During hypertonic hypovolaemia the ECF sodium concentration increases and this is reflected in a graded increase in plasma osmolality with progressive dehydration (Popowski *et al.*, 2001). As a result, a greater sodium concentration must be generated across the salivary acinar cell to drive fluid into the acinar lumen to form primary saliva. An increase in ECF sodium concentration during hypertonic hypovolaemia might therefore result in the production of smaller amounts of more concentrated saliva. These mechanisms may also explain why saliva flow rate is unchanged after prolonged exercise with sufficient water to offset dehydration (Ford *et al.*, 1997; Bishop *et al.*, 2000). Specifically, compared with fluid restriction, water provided to offset fluid losses during prolonged exercise has been shown to reduce sympathetic activity and hypertonic hypovolemia (Montain & Coyle, 1992a; Gonzalez-Alonso *et al.*, 1995; Hargreaves *et al.*, 1996). Further investigations are clearly required to elucidate the mechanism(s) responsible for the reduction in saliva flow rate commonly observed after prolonged exercise and fluid losses.

2.5 Mucosal immunity

Saliva is essential for the maintenance of mucosal integrity and therefore oral health (Mandel, 1989). Saliva flow and composition function in lubrication, buffering and digestion, while also possessing anti-viral and anti-bacterial properties (Tenovuo, 1998; West *et al.*, 2006). Indeed, individuals suffering salivary gland hypofunction suffer an increased incidence of dental caries and oral infections (Fox, 2004). The importance of mucosal secretions cannot be underestimated given that 95% of all infections are initiated

at mucosal surfaces (Bosch *et al.*, 2002). Saliva provides a mechanical washing effect to protect the oral mucosa whilst mucosal secretions (e.g. immunoglobulins, mucins, amylase, lactoferrin) prevent viral replication and bacterial attachment to the mucosal surfaces (McNabb & Tomasi, 1981; Dowd, 1999). Immunoglobulins (Ig) total 5 to 30% of the total proteins in saliva and have several subclasses (i.e. A, M, G and E) the most prevalent being s-IgA (Lamm, 1997). Immunological defence is mainly provided by secretory IgA and to some extent secretory IgM antibodies. These antibodies exert their protective action by the exclusion of antigens at the luminal surface, intracellular virus neutralisation and stromal clearance of antigens (Norderhaug *et al.*, 1999).

2.5.1 IgA synthesis and translocation

The majority (>80%) of mucosal IgA is polymeric, comprising mainly of dimers, which are produced by plasma cells residing in submucosa lymphoid and non-lymphoid tissue (Woof & Mestecky, 2005; Brandtzaeg, 2007). Immunoglobulin antibody production by plasma cells requires activation of B lymphocytes either directly by antigens or indirectly via antigen presenting cells and subsequent cytokine release (Gleeson, 2000). The formation and secretion of polymeric IgA into the oral cavity is a two-step process. First, the polymeric IgA molecule binds with the basolaterally expressed epithelial polymeric immunoglobulin receptor (pIgR, Johansen & Brandtzaeg, 2004). The pIgR is then translocated via endocytosis or transcytosis from the basolateral to apical surface of the glandular cell. At the apical surface the pIgR receptor is cleaved which causes the release of secretory IgA into the lumen. Once in the lumen secretory IgA is able to interact with antigens and protect the mucosal layer.

Each pIgR participates in only one round of translocation (Cox *et al.*, 2007). That is, for every polymeric IgA molecule one pIgR is required for translocation. Accordingly, both the release of IgA antibody by the plasma cell and the availability of pIgR for translocation are potential rate limiting factors in the secretion of saliva secretory IgA. The crucial importance of pIgR for IgA translocation is highlighted by the absence of secretory IgA in knockout mice deficient of these genes (Shimada *et al.*, 1999). In rats pIgR transport of IgA and therefore salivary IgA levels have been shown to be increased in an almost immediate way by the stimulation of either the parasympathetic or sympathetic nerves (Carpenter *et al.*, 1998). Whereas autonomic stimulation may cause rapid changes in translocation of IgA, slower effects have been reported for a number of cytokines and hormones (Norderhaug *et al.*, 1999; Kaetzel, 2005).

Depending on the clinical state of the gingival the monomeric fraction of s-IgA accounts for between 13 and 17% of total IgA in whole saliva (Brandtzaeg, 1973). It has been estimated that up to 77% of monomeric IgA in saliva is derived from serum (Delacroix & Vaerman, 1983). Serum IgA is produced by bone marrow and is 90% monomeric (Woof & Mestecky, 2005). This serum derived IgA is suggested to enter the oral cavity paracellularly in crevicular fluid (Brandtzaeg *et al.*, 1970). This paracellular entry may increase under conditions of cellular sympathomimetic stimulation and/or mechanical damage of mucosal layer (Garrett, 1998; Brandtzaeg, 2007).

2.5.2 Saliva IgA and upper respiratory tract infection in active individuals

Greater volumes of training are associated with a greater number of URTI (Heath *et al.*, 1991). A decrease in s-IgA has been implicated as a possible causal factor in the increased susceptibility to URTI during heavy training in athletes (Gleeson *et al.*, 1999; Fahlman &

Engels, 2005) and military personnel (Carins & Booth, 2002; Tiollier *et al.*, 2005). Numerous studies report decreased s-IgA following prolonged exercise (Tomasi *et al.*, 1982; Mackinnon *et al.*, 1987; Steerenberg *et al.*, 1997; Gleeson *et al.*, 2000) and therefore it might be proposed that the overall exercise training volume in the above training studies is most likely responsible for the decrease in s-IgA and subsequent increase in URTI.

Very few exercise immunology studies have documented the nutritional status of the individuals and therefore the likelihood of any confounding effect of nutritional influences on immune suppression cannot be readily determined (Gleeson *et al.*, 2004b). This is surprising given that athletes and military recruits, especially during periods of intensified training, may consume inadequate nutrients (Barr & Costill, 1992; Edwards *et al.*, 1993; Thompson *et al.*, 1995; Carins & Booth, 2002). For example, an energy deficit equal to approximately 10000 $kJ \cdot d^{-1}$ (~2500 $kcal \cdot d^{-1}$) for five consecutive days was reported in highly trained female swimmers (Trappe *et al.*, 1997). Indeed, Beals and Manore (1994) suggested the sub clinical eating disorder "anorexia athletica" is associated with increased infection. Furthermore, intense military training involving fluid and energy restriction has been shown to decrease s-IgA concentration compared with prior training levels (Carins & Booth, 2002; Gomez-Merino *et al.*, 2003). The decreased s-IgA concentration possibly contributed to the raised URTI incidence reported amongst recruits during training (Carins & Booth, 2002). However, the presence of other stressors previously shown to decrease s-IgA concentration (e.g. arduous exercise, chronic psychological stress, Tomasi *et al.*, 1982; Jemmott *et al.*, 1983) may account, at least in part, for these findings.

2.5.3 Saliva IgA during fluid and energy restriction

Saliva IgA has been reported to be lower in moderately malnourished children compared with well fed children (Watson *et al.*, 1985; Johansson *et al.*, 1994) but remain unaltered after an eight day fast in healthy adults (Johansson *et al.*, 1984). These discrepant findings may be partly explained by differences in saliva collection methods (i.e. parotid *vs.* whole and stimulated *vs.* unstimulated), the method used to express s-IgA (i.e. as a concentration or relative to protein) and the time of day when samples were collected. Studies either did not report the time of day when saliva samples were collected or gave a broad time frame for collection (e.g. 09:00-13:00 h, Johansson *et al.*, 1994). This is a limitation as s-IgA concentration has since been shown to exhibit diurnal variation with a significant decrease during the morning hours reaching a plateau 6 h after awakening (Hucklebridge *et al.*, 1998).

Nutrient restrictions have the potential to weaken many aspects of immune function because macronutrients are involved in cell metabolism and protein synthesis and micronutrients are involved in immune cell replication and antioxidant defences (Chandra, 1997). Decreased fuel availability may also impair immune function through raised sympatho-adrenal activation in response to stress (e.g. exercise, energy restriction) and resulting increases in stress hormones (e.g. cortisol, catecholamines) that are known to have immuno-suppressive effects (Gleeson *et al.*, 2004a). Indeed, increases in noradrenaline and cortisol have been reported following 15 h of fasting (Pequignot *et al.*, 1980) and during severe energy restriction (Fichter *et al.*, 1986), respectively. Elevated plasma cortisol has also been observed during prolonged exercise with fluid restriction (Bishop *et al.*, 2004).

Specifically, s-IgA may be decreased following a period of increased sympatho-adrenal activation as the administration of glucocorticoid *in vivo* had an inhibitory effect, taking several hours, on *in vitro* B lymphocyte antibody synthesis in humans (Saxon *et al.*, 1978). Additionally, the administration of dexamethasone, a synthetic glucocorticoid, reduced secretory IgA levels in rats (Wira *et al.*, 1990). Furthermore, pIgR transport of IgA to the mucosal surface has been shown to be affected by nutrient avaliability. In mice intestinal IgA and pIgR expression were shown to be decreased following energy restriction (Ha & Woodward, 1998). Whilst *in vitro* the pIgR expression has been shown to be dependent upon the adequate presence of vitamin A (Sarkar *et al.*, 1998). In contrast, sympathetic nerve stimulation has been shown to cause a rapid increase pIgR mediated translocation of IgA in rats (Carpenter *et al.*, 1998) and the paracellular transport of serum derived IgA may increase under conditions of cellular sympathomimetic stimulation (Garrett, 1998). Some of the above findings should be interpreted with caution as aspects of the mucosal system differ considerably between rodents and humans (Bosch *et al.*, 2002). The effect of raised sympatho-adrenal activity on s-IgA in humans is therefore unclear.

Hypohydration might also impact upon oral health by reducing the protective washing effect of saliva and limiting the availability of several proteins at mucosal surface (e.g. Ig, mucins, lysozyme, α-amylase) known to have important antiviral and antibacterial properties (Tenovuo, 1998; West *et al.*, 2006). In fact hypohydration evoked by a 24 h period without fluid or food has been shown to decrease saliva flow rate (Ship & Fischer, 1997). Saliva flow rate appears to be an influential factor in protection against oral pathogens and as such it has been recommended that s-IgA be reported as a secretion rate (flow rate x concentration) to reflect the total availability of s-IgA at the oral surface and correct for changes in hydration status (Walsh *et al.*, 1999). In line with this, individuals

who suffer from dry mouth syndrome (xerostomia) are known to suffer an increased incidence of URTI (Fox *et al.*, 1985). Whether a prolonged period of fluid and/or energy restriction alters the s-IgA response at rest or after a subsequent bout of exercise in active individuals remains unknown.

2.6 Thesis objectives

With this information in mind the broad objectives of the following experiments was to investigate: 1. the validity of haematological, urinary and salivary hydration markers during modest dehydration evoked by acute exercise in the heat and during a 48 h period of fluid and/or energy restriction; 2. the effect of a 48 h period of fluid, energy or combined fluid and energy restriction on s-IgA responses at rest and after exercise in active individuals; and 3. the independent and combined effects of fluid and energy restriction lasting 48 h on 30 min self-paced treadmill TT performance in temperate conditions.

CHAPTER THREE

General Methods

3.1 Ethical approval

Approval for all studies was obtained from the local Ethics Committee (*School of Sport, Health and Exercise Sciences, University of Wales, Bangor*). The nature and purpose of each study was fully explained both verbally and in writing to each volunteer (**Appendix A**). Each participant was made fully aware that they were free to withdraw from the study at any time and completed an informed consent form (**Appendix B**). Participants also completed a general health questionnaire (**Appendix C**). Participants were only eligible if they reported the absence of illness in the six weeks prior, had no significant oral, dental or systemic disease and were not taking any prescription or non-prescription medication at the time of participating in the studies. All volunteers were non-smokers.

3.2 Anthropometry and body composition

Height was recorded using a wall stadio-meter (*Bodycare Ltd, Warwickshire, UK*) and NBM by digital platform scales accurate to the nearest 50 g (*Model 705, Seca, Hamburg, Germany*). To monitor NBM changes during exercise induced dehydration (**Chapter 4**) and fluid and/or energy restriction protocols (**Chapters 5 - 7**) participants removed clothing and were towel dried prior to being weighed. Body composition (i.e. body fat percentage) was assessed by dual energy x-ray absorptiometry (DEXA, *Hologic QDR1500, software version 5.72, Bedford, USA*). For this procedure participants removed all metallic objects and dressed in t-shirt and shorts only. Dual energy x-ray absorptiometry omits an effective dose of radiation equal to 3.6 μSv or approximately one fortieth of a chest x-ray (Njeh *et al.*, 1999).

3.3 Maximal oxygen uptake ($\dot{V}O_{2max}$)

For the determination of $\dot{V}O_{2max}$ and trial workloads participants performed a continuous incremental exercise test to volitional exhaustion on either a stationary cycle ergometer (**Chapter 4**, *Monark 814e, Varberg, Sweden*) or a treadmill (**Chapters 5 – 7**, *Powerjog, Sport Engineering Ltd, Birmingham, UK*). For cycle ergometer tests, following a 5 min warm-up period at 70 W participants began cycling at 175 W, with increments of 35 W every 3 min until fatigue. For treadmill tests, on completion of a 5 min warm-up period at 9 $km \cdot h^{-1}$ participants began running at 10 $km \cdot h^{-1}$ at a 1.0% gradient with 2 $km \cdot h^{-1}$ increments every 3 min until 14 $km \cdot h^{-1}$ was reached, after which the gradient was increased by 2.5% every 3 min increment until fatigue. During all $\dot{V}O_{2max}$ tests expired gas was analysed continuously using an on-line breath-by-breath system (*Cortex Metalyser 3B, Biophysik, Leipzig, Germany*) to determine the volumes of expired gas, oxygen and carbon dioxide. Criteria for attaining $\dot{V}O_{2max}$ included the participant reaching volitional exhaustion, a HR within 10 $beats \cdot min^{-1}$ of age predicted HR_{max} and a respiratory exchange ratio greater than or equal to 1.15 (Bird & Davison, 1997).

3.4 Sample collection and analysis

Blood, saliva and urine samples were collected at least 15 min after fluid consumption and at least 1 h after the participants had eaten. In addition, blood and saliva samples were obtained after participants had remained seated for 10 min.

3.4.1 Blood

Blood samples were collected, without venestasis by venepuncture from an antecubital vein, into two separate vacutainer tubes (*Becton Dickinson, Oxford, UK*), containing the anticoagulants ethylenediaminetetraacetic acid (EDTA) and lithium heparin. Blood in the

tube containing EDTA was used to determine haemoglobin concentration using a haematology analyser (*Beckman Coulter Gen S, Fullerton, USA*). Haematocrit (heparinised blood) was determined in triplicate using the capillary method in accordance with (Maughan *et al.*, 2001) and plasma volume changes were estimated (Dill & Costill, 1974). The remaining blood in the EDTA tube and blood in the lithium heparin tube was immediately centrifuged (1500 *g* for 10 min at 5°C: *Hettich Rotina 35R, Bach, Germany*), plasma aspirated and stored at -80°C for further analysis.

Plasma concentrations of FFA (EDTA plasma) and glucose (heparinised plasma) were determined using spectrophotometric kits (**Chapters 6 & 7**, *Randox, County Antrim, UK*). Plasma concentrations of lactate were also determined using spectrophotometric kits (**Chapter 7**, heparinised plasma, *Randox, County Antrim, UK*). The intra-assay co-efficient of variation (CV) was 7.0, 6.0 and 6.6% for FFA, glucose and lactate concentrations respectively. Plasma cortisol (**Chapter 6**, *DRG Diagnostics, Marburg, Germany*), adrenaline and noradrenaline concentrations (**Chapter 4**, *Labor Diagnostica Nord, Nordhorn, Germany*) were determined on EDTA plasma using enzyme-linked immunosorbent assay (ELISA) kits. The intra-assay CV was 3.1, 6.9 and 9.8% for cortisol, adrenaline and noradrenaline concentrations, respectively. All plasma metabolite concentrations were corrected for changes in plasma volume (Dill & Costill, 1974).

Osmolality measurements were determined on heparinised plasma using a freezing point depression Osmometer (**Chapters 4 & 5**, *Model 3 MO, Advanced Instruments, Massachusetts, USA*). The intra-assay CV for plasma osmolality was 0.8%. A separate pilot investigation reported no difference in the osmolality of blood samples determined from lithium heparin plasma or serum; however, compared with lithium heparin plasma or

serum the addition of the anticoagulant EDTA to blood samples caused a significant increase in osmolality (**Appendix D**).

3.4.2 Saliva

Unstimulated whole saliva samples were collected using pre-weighed salivette (**Chapters 4 & 5**, *Sarstedt, Leicestershire, UK*) or pre-weighed universal tubes (**Chapter 6**, dribble method). All saliva samples were collected while the subject sat quietly in the laboratory in temperate conditions. Immediately after thoroughly rinsing the mouth with sterile water each subject was asked to swallow in order to empty the mouth before saliva was collected. The salivette saliva sample was collected by the participants placing the cotton swab (diameter 1 cm, length 2.5 cm) under the tongue for exactly 2 min. The dribble saliva sample was collected for exactly 2 min by the participant leaning forward and passively drooling into a universal container. All saliva samples were collected with minimal orofacial movements. Saliva volume was estimated by weighing the salivette or universal tube immediately after collection to the nearest mg, and saliva density was assumed to equal 1 $g \cdot mL^{-1}$ (Cole & Eastoe, 1988). From this, the saliva flow rate was determined by dividing the volume of saliva by the collection time (**Chapters 4 – 6**). Saliva was then aspirated into eppendorfs and stored at -80°C for further analysis.

All further analysis was performed after thawing. Saliva osmolality measurements were made using a freezing point depression Osmometer with an intra-assay CV of 1.4% (**Chapters 4 & 5**). Saliva total protein concentration was measured on a spectrophotometer using a commercially available kit (**Chapter 4**, *Kit No. 610, Sigma, Poole, UK*). The intra-assay CV was 4.0% for total protein concentration. Saliva IgA concentration was determined by ELISA using an IgA monomer derived from human serum as a standard

(**Chapter 6**, *Probiomics Ltd, Eveleigh, Australia*). The intra-assay CV was 3.2%. The s-IgA secretion rate was calculated by multiplying the saliva flow rate by s-IgA concentration.

3.4.3 Urine

All urine passed was collected into 2 L plastic containers. A 3 mL sample of each urine sample at collection times was aspirated into two eppendorfs and frozen at -80°C for further analysis. The remaining urine sample was added and pooled with relevant urine collection for 24 h volume determination. Urine osmolality measurement was made using a freezing point depression Osmometer with an intra-assay CV of 2.0% (**Chapters 4 & 5**). Urine colour was determined by a urine colour scale (**Chapter 5**, Armstrong *et al.*, 1994).

3.5 Statistical analysis

The primary outcome measures of this thesis were the saliva parameters (saliva flow rate, osmolality, total protein and IgA). The sample size was estimated to be twelve using previous data examining the effects of dehydration on saliva flow rate, osmolality and total protein concentration (Walsh *et al.*, 2004b) and effect of energy restriction on IgA (Watson *et al.*, 1985). The effect size for changes in saliva parameters were greater than 1.1. Alpha and power levels were set at 0.05 and 0.8, respectively, both of which are standard estimates (Jones *et al.*, 2003). To allow for drop out, fifteen (**Chapter 4**) and thirteen (**Chapters 5 - 7**) participants were recruited.

In each experimental study participants completed all trials and subsequently repeated measures analysis of variance (ANOVA) was used to identify main effects and interactions in the dependent variables (e.g. hydration markers, IgA, TT performance). See statistical

analysis section of each experimental chapter for further detail. In cases where the assumptions of sphericity and normality were violated appropriate adjustments to the degrees of freedom were made to the ANOVA. Where significant main effects and interactions were found by ANOVA *Post hoc* Tukey's HSD and Bonferroni corrected *t*-tests were used where appropriate. Unless otherwise stated all data are presented as mean and SD. Significance was accepted at $P < 0.05$. Analyses were performed on Statistical Package for Social Sciences (*Version 11* **Chapter 4,** *Version 12* **Chapters 5 - 7,** *Illinois, USA*).

CHAPTER FOUR

Saliva Parameters as Potential Indices of Hydration Status during Acute Dehydration

4.1 Summary: The purpose of this study was first to assess the validity of saliva flow rate, osmolality and total protein concentration by comparing changes in these parameters with changes in plasma osmolality during an acute dehydration period. Second, the utility of saliva parameters was compared with urine osmolality. Third, the involvement of dehydration and neuro-endocrine regulation in the decrease in saliva flow rate during exercise was examined by comparing flow rate and catecholamine responses during prolonged exercise with and without fluid ingestion. Fifteen trained males exercised with no fluid intake (NFI) until BML of 1.1, 2.1 and 3.0% and on another occasion with fluid intake (FI) to offset fluid losses. Plasma and urine osmolality increased during the NFI ($P < 0.01$). Saliva flow rate decreased ($P < 0.01$), saliva total protein concentration ($P < 0.01$) and saliva osmolality increased from pre-exercise (50 mOsmol·kg^{-1}) to 3.0% BML (105 mOsmol·kg^{-1}) during NFI ($P < 0.01$). Saliva flow rate, saliva osmolality, saliva total protein concentration and urine osmolality correlated strongly with plasma osmolality during dehydration (r = -0.78, 0.87, 0.91, and 0.83, respectively, $P < 0.01$). During the FI trial saliva flow rate and osmolality remained unchanged. Plasma catecholamine concentration increased during exercise ($P < 0.01$) with no significant difference between trials. In conclusion, saliva osmolality and total protein concentration appear to be as sensitive as urine osmolality to track hydration changes during hypertonic hypovolaemia identifying dehydration by 2.1% BML. These results also support a role for dehydration in the decrease in saliva flow rate during prolonged exercise.

4.2 Introduction

Unstimulated whole saliva parameters have recently been identified as potential non-invasive markers of hydration status (Walsh *et al.*, 2004b); however, these findings are yet to be confirmed. Moreover, for saliva parameters to be considered as markers of hydration status they must be shown to correlate strongly with plasma osmolality, a widely accepted hydration index, which is known to quantitatively reflect changes in hydration during dehydration evoked by exercise and heat stress (Sawka *et al.*, 1985; Popowski *et al.*, 2001). Additionally, the utility of saliva parameters have yet to be compared during dehydration to urine osmolality a regularly used non-invasive hydration marker (Armstrong *et al.*, 1994). The main purpose of the present study was to assess the validity of saliva parameters to track changes in hydration status by comparing changes in these parameters to changes in plasma and urine osmolality during progressive acute dehydration. It was hypothesised that saliva flow rate, saliva osmolality and saliva total protein concentration would track dehydration and correlate strongly with plasma osmolality. It was also hypothesised saliva parameters would identify the same magnitude of dehydration as urine osmolality.

In contrast to the often proposed neuro-endocrine role (increase in SNS activity, Dawes, 1981; Chicharro *et al.*, 1998) a role for hypohydration (Walsh *et al.*, 2004b) has recently been proposed to explain the secretion of smaller amounts of more concentrated saliva after exercise (e.g. increased osmolality and total protein concentration). As the predominant fluid constituent of saliva is water, which enters saliva from plasma across acinar cells, it follows that hypohydration may cause salivary gland hypofunction (Ship & Fischer, 1997). In order for water to move from plasma through acinar cells to form primary saliva, a trans-acinar cell sodium gradient must be generated. During hypertonic

hypovolaemia the ECF sodium concentration increases and this is reflected in a graded increase in plasma osmolality with progressive dehydration (Sawka *et al.*, 1985; Popowski *et al.*, 2001). As a result, a greater sodium concentration must be generated across the salivary acinar cell to drive fluid into the acinar lumen to form primary saliva. An increase in ECF sodium concentration during hypertonic hypovolaemia might therefore result in the production of smaller amounts of more concentrated saliva. If changes in the ECF sodium concentration influence saliva output as described then a progressive increase in plasma osmolality during hypertonic hypovolaemia should be associated with the production of smaller amounts of more concentrated saliva.

An increase in SNS activity may also explain the decrease in saliva flow rate during prolonged exercise by causing vasoconstriction of the blood vessels to the salivary glands (Dawes, 1981; Chicharro *et al.*, 1998). However, β-blockade by propranolol had no effect on the saliva flow rate response to sub-maximal exercise designed to elicit cardiovascular activity consistent with β-adrenergic activation (Winzer *et al.*, 1999). Furthermore, studies using combined exercise and fluid restriction (Bishop *et al.*, 2000) and exercise in the heat (Ford *et al.*, 1997) show large reductions in saliva flow rate that were prevented when participants received sufficient water to offset fluid losses during exercise. These studies suggest a likely role for dehydration in the decrease in saliva flow rate with prolonged exercise. Therefore, the involvement of dehydration and neuro-endocrine regulation in the decrease in saliva flow rate during exercise were examined by comparing saliva flow rate and catecholamine responses during prolonged exercise with and without fluids. It was hypothesised that the decrease in saliva flow rate following prolonged exercise is caused by dehydration rather than neuro-endocrine regulation.

4.3 Methods

Participants. Fifteen healthy trained males (age, 23 ± 3 yrs; height, 180 ± 4 cm; BM, 79.3 ± 3.9 kg; body fat, 15 ± 4% and $\dot{V}O_{2\,max}$, 57.3 ± 9.5 $mL \cdot kg^{-1} \cdot min^{-1}$) volunteered to participate in the study. All participants gave written informed consent before the study, which received local Ethics Committee approval.

Preliminary measurements. Prior to the experimental trials, body composition and $\dot{V}O_{2\,max}$ was estimated by DEXA and a continuous incremental exercise test on a cycle ergometer, respectively (for details see **Chapter 3**). Additionally, following the determination of the trial workload, each participant cycled at 60% $\dot{V}O_{2\,max}$ in the heat (30°C and 70% RH) without fluids until 3% BML to estimate individual sweating rate and exercise duration for the experimental protocol.

Experimental procedures. Participants reported to the laboratory on two occasions, separated by seven days, at 08:00 h following an overnight fast. On arrival participants were euhydrated having drunk water equal to 35 $mL \cdot kg^{-1}$ of BM the previous day. Dressed in shorts and shoes, participants cycled on a stationary cycle ergometer (*Monark 814e, Varberg, Sweden*) in an environmental chamber (*Delta Environmental Systems, Chester, UK*) maintained at a dry bulb temperature of 30°C and 70% RH. The exercise was intermittent consisting of 10 min periods of cycling at 60% $\dot{V}O_{2max}$ in the environmental chamber interspersed with 5 min rest periods in a temperate laboratory. During the 5 min rest period participants NBM was determined. Participants repeated the intermittent exercise on one occasion with NFI until progressive BML of 1, 2 and 3% and on another occasion with FI as water to offset fluid losses. The order of trials was randomised (n = 8 participants completed the NFI trial first). The exercise duration, which was the same in

both trials, and the volume of water required to offset fluid losses on the FI trial were determined during preliminary measurements. During the FI trial, participants received equal volumes of water immediately after exercise was begun and at 15 min intervals thereafter. Participants received no food during the NFI or FI trial. Heart rate (*Polar Electro, Kempele, Finland*) and rectal temperature (T_{re}, *Edale, Cambridge, UK)* were monitored continuously during exercise to ensure that participants did not exceed 180 beats·min^{-1} or 39.5°C respectively. Saliva, blood and urine samples were collected at pre-exercise, 1, 2 and 3% BML on the NFI trial and pre and post-exercise on the FI trial. Additional saliva, blood and urine samples were collected at 75, 135 and 195 min post-exercise on the NFI trial. After exercise on the NFI trial, participants were given a volume of carbohydrate-electrolyte solution (6% carbohydrate and 25 mmol·L^{-1} sodium) equivalent to 25% BML to consume between 0 to 15, 15 to 30 and 30 to 45 min in both the first and second hour of recovery (total drink volume = 150% BML, Shirreffs *et al.*, 1996).

Analytical methods. Haematological parameters including haemoglobin, haematocrit, plasma volume change, plasma osmolality and plasma adrenaline and noradrenaline concentrations; urine parameters including osmolality; saliva parameters including flow rate, osmolality and total protein concentration were measured as described in **Chapter 3**.

Statistical analysis. The NFI trial data were examined using one-way repeated measures ANOVA (7 sample collections). A two-way repeated measures ANOVA was used to compare NFI and fluid intake trial data at pre and post-exercise (2 trials x 2 sample collections).

For each participant a Pearson's correlation coefficient was calculated between plasma osmolality (criterion) and a predictor variable (e.g. urine osmolality, saliva flow rate). Each Pearson's correlation coefficient was then converted using Fisher's Zr transformation to result in a more normal distribution. The mean Fisher's Zr was converted into the Pearson's correlation coefficient (mean *r*) using appropriate statistical tables (Thomas & Nelson, 1996). Based on the sample size and number of Pearson's correlations conducted, an adjustment was made to the value at which the mean correlation coefficient was deemed significant (Shavelson, 1988). Differences between mean correlation coefficients were determined using an adjusted z score equation (Meng *et al.*, 1992).

4.4 Results

At 1, 2 and 3% target BML participants reached an average BML of 1.1 ± 0.1 (range = 0.9-1.3%), 2.1 ± 0.1 (range = 1.9-2.3%) and 3.0 ± 0.1% (range = 2.7-3.2%) on the NFI trial. At post-exercise on the FI trial a BML of 0.2 ± 0.4% was recorded. It took on average 38 ± 9 min to reach 1.1% BML, an additional 33 ± 14 min to reach 2.1% BML and an additional 29 ± 9 min to reach 3.0% BML. Participants lost on average 2.2 ± 0.1 kg BM to achieve 3.0% BML in 100 ± 24 min on the NFI trial. There was no significant difference in T_{re} at pre-exercise on the NFI trial compared with the FI trial (37.1 ± 0.2 and 37.1 ± 0.2°C, respectively). During exercise on NFI and FI trials T_{re} increased progressively ($P < 0.01$). Compared with pre-exercise on the NFI trial T_{re} was significantly elevated immediately post-exercise ($P < 0.01$) but was not significantly different from immediately post-exercise on the FI trial (NFI, 38.5 ± 0.4; FI, 37.9 ± 0.3°C). Fluid provision during the FI trial blunted the significant increase in HR observed on the NFI trial during exercise (final exercising HR; NFI, 173 ± 10; FI, 155 ± 11 beats·min^{-1}, $P < 0.05$).

4.4.1 Plasma osmolality and plasma volume change

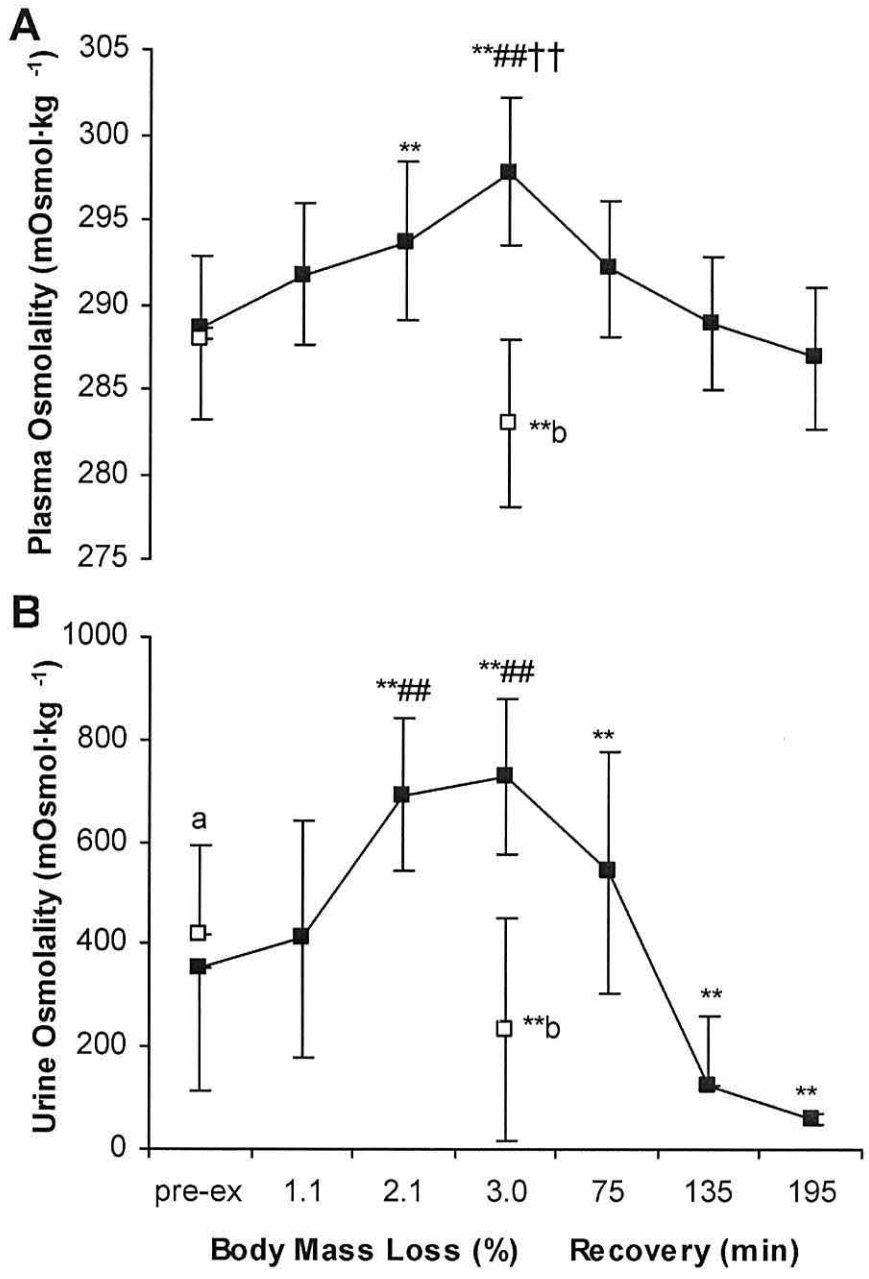
At pre-exercise on the NFI trial plasma osmolality was 289 ± 4 (range 284-297) mOsmol·kg^{-1}. Plasma osmolality increased during progressive dehydration on the NFI trial and was significantly greater than pre-exercise by 2.1% BML ($P < 0.01$, **Figure 4.1A**). At 3.0% BML on the NFI trial plasma osmolality reached 298 ± 1 (range 293-306) mOsmol·kg^{-1}. The rehydration protocol re-established pre-exercise plasma osmolality levels by 75 min post-exercise on the NFI trial. Plasma osmolality was not significantly different at pre-exercise on the FI trial compared with the NFI trial. On the FI trial, post-exercise plasma osmolality decreased compared with pre-exercise ($P < 0.01$) and was significantly lower than on the NFI trial at post-exercise ($P < 0.01$). Plasma volume

decreased by 5.0 ± 2.0% at 3.0% BML on the NFI trial ($P < 0.01$) whereas it did not change significantly on the FI trial (+0.2 ± 3.2%).

4.4.2 Urine osmolality

At pre-exercise on the NFI trial urine osmolality was 353 ± 237 (range 89-704) $mOsmol \cdot kg^{-1}$. Urine osmolality increased during progressive dehydration on the NFI trial and was significantly greater than pre-exercise by 2.1% BML ($P < 0.01$, **Figure 4.1B**). At 3.0% BML on the NFI trial urine osmolality reached 728 ± 233 (range 451-955) $mOsmol \cdot kg^{-1}$. It is noteworthy that two participants could not provide a urine sample at 2.1% BML and three participants could not provide a urine sample at 3.0% BML on the NFI trial. All of the 12 urine samples collected at 3.0% BML gave greater osmolality readings compared with pre-exercise. Urine osmolality remained significantly elevated at 75 min post-exercise ($P < 0.01$) and was significantly lower than pre-exercise at 135 and 195 min post-exercise on the NFI trial ($P < 0.01$). Urine osmolality was significantly greater at pre-exercise on the FI trial compared with the NFI trial ($P < 0.05$). On the FI trial, post-exercise urine osmolality decreased compared with pre-exercise ($P < 0.01$) and was significantly lower than on the NFI trial at post-exercise ($P < 0.01$).

Figure 4.1: Plasma (A) and urine (B) osmolality during progressive acute dehydration (NFI ■) and with sufficient fluids to offset fluid losses (FI □).



Values are mean ± SD (n = 15). a *vs.* NFI trial pre exercise, b *vs.* NFI trial post exercise, ** *vs.* pre exercise, ## *vs.* 1.1% BML, †† *vs.* 2.1% BML; $P < 0.01$. Abbreviations: BML, body mass loss; NFI, no fluid intake; FI, fluid intake.

4.4.3 Saliva flow rate

At pre-exercise on the NFI trial saliva flow rate was 435 ± 320 (range 71-993) $\mu L \cdot min^{-1}$. Saliva flow rate decreased during progressive dehydration on the NFI trial and was significantly lower than pre-exercise at 3.0% BML ($P < 0.01$, **Figure 4.2A**). At 3.0% BML on the NFI trial saliva flow rate decreased to 165 ± 43 (range 13-587) $\mu L \cdot min^{-1}$. At 3.0% BML, saliva flow rate was lower than pre-exercise in 12 out of 15 samples. The rehydration protocol re-established pre-exercise saliva flow rate levels by 75 min post-exercise on the NFI trial. Saliva flow rate was not significantly different at pre-exercise on the FI trial compared with the NFI trial. Fluid provision during the FI trial prevented the decrease in saliva flow rate observed on the NFI trial. At post-exercise, saliva flow rate was significantly greater on the FI trial than on the NFI trial ($P < 0.01$).

4.4.4 Saliva osmolality

At pre-exercise on the NFI trial saliva osmolality was 50 ± 11 (range 38-71) $mOsmol \cdot kg^{-1}$. Saliva osmolality increased during progressive dehydration on the NFI trial and was significantly greater than pre-exercise by 2.1% BML ($P < 0.01$, **Figure 4.2B**). At 3.0% BML on the NFI trial saliva osmolality reached 105 ± 41 (range 55-200) $mOsmol \cdot kg^{-1}$. At 3.0% BML, saliva osmolality was higher than pre-exercise in all samples. The rehydration protocol re-established pre-exercise saliva osmolality levels by 75 min post-exercise on the NFI trial. Saliva osmolality was not significantly different at pre-exercise on the FI trial compared with the NFI trial. Fluid provision during the FI trial prevented the increase in saliva osmolality observed on the NFI trial. At post-exercise, saliva osmolality was significantly lower on the FI trial than on the NFI trial ($P < 0.01$).

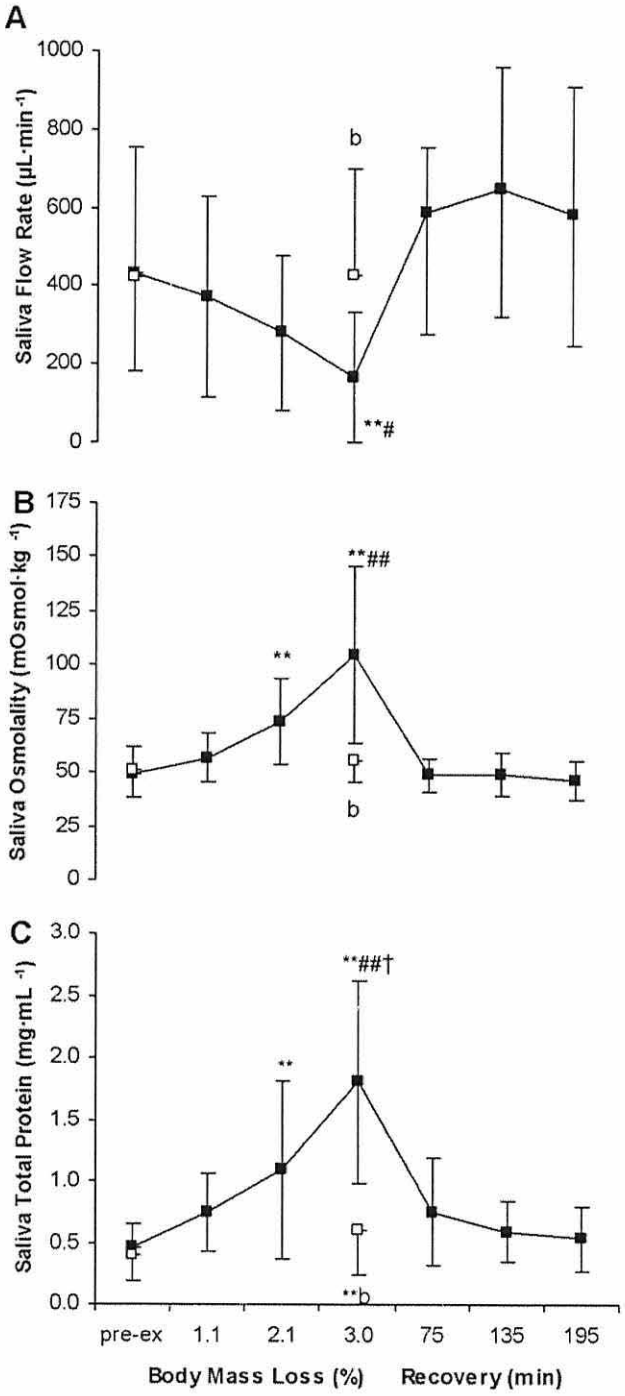
4.4.5 Saliva total protein concentration

At pre-exercise on the NFI trial saliva total protein concentration was 0.47 ± 0.19 (range 0.24-0.82) $mg \cdot mL^{-1}$. Saliva total protein concentration increased during progressive dehydration on the NFI trial and was significantly greater than pre-exercise by 2.1% BML ($P < 0.01$, **Figure 4.2C**). At 3.0% BML on the NFI trial saliva total protein concentration reached 1.80 ± 0.82 (range 0.85-3.22) $mg \cdot mL^{-1}$. At 3.0% BML, saliva total protein concentration was higher than pre-exercise in all samples. The rehydration protocol re-established pre-exercise saliva total protein concentration levels by 75 min post-exercise on the NFI trial. Saliva total protein concentration was not significantly different at pre-exercise on the FI trial compared with the NFI trial. Fluid provision during the FI trial blunted but could not prevent an increase in saliva total protein concentration ($P < 0.01$). At post-exercise, saliva total protein concentration was significantly lower on the FI trial than on the NFI trial ($P < 0.01$).

4.4.6 Correlational comparisons

Saliva flow rate, saliva osmolality, and saliva total protein concentration and urine osmolality correlated strongly with plasma osmolality during dehydration on the NFI trial ($r = -0.78$; $r = 0.87$; $r = 0.91$, $r = 0.83$, respectively, $P < 0.01$). The correlation coefficients for saliva osmolality, saliva total protein concentration and urine osmolality with plasma osmolality were greater than the corresponding correlation coefficient between saliva flow rate and plasma osmolality ($P < 0.01$).

Figure 4.2: Saliva flow rate (A) osmolality (B) and total protein concentration (C) during progressive acute dehydration (NFI ■) and with sufficient fluids to offset fluid losses (FI □).

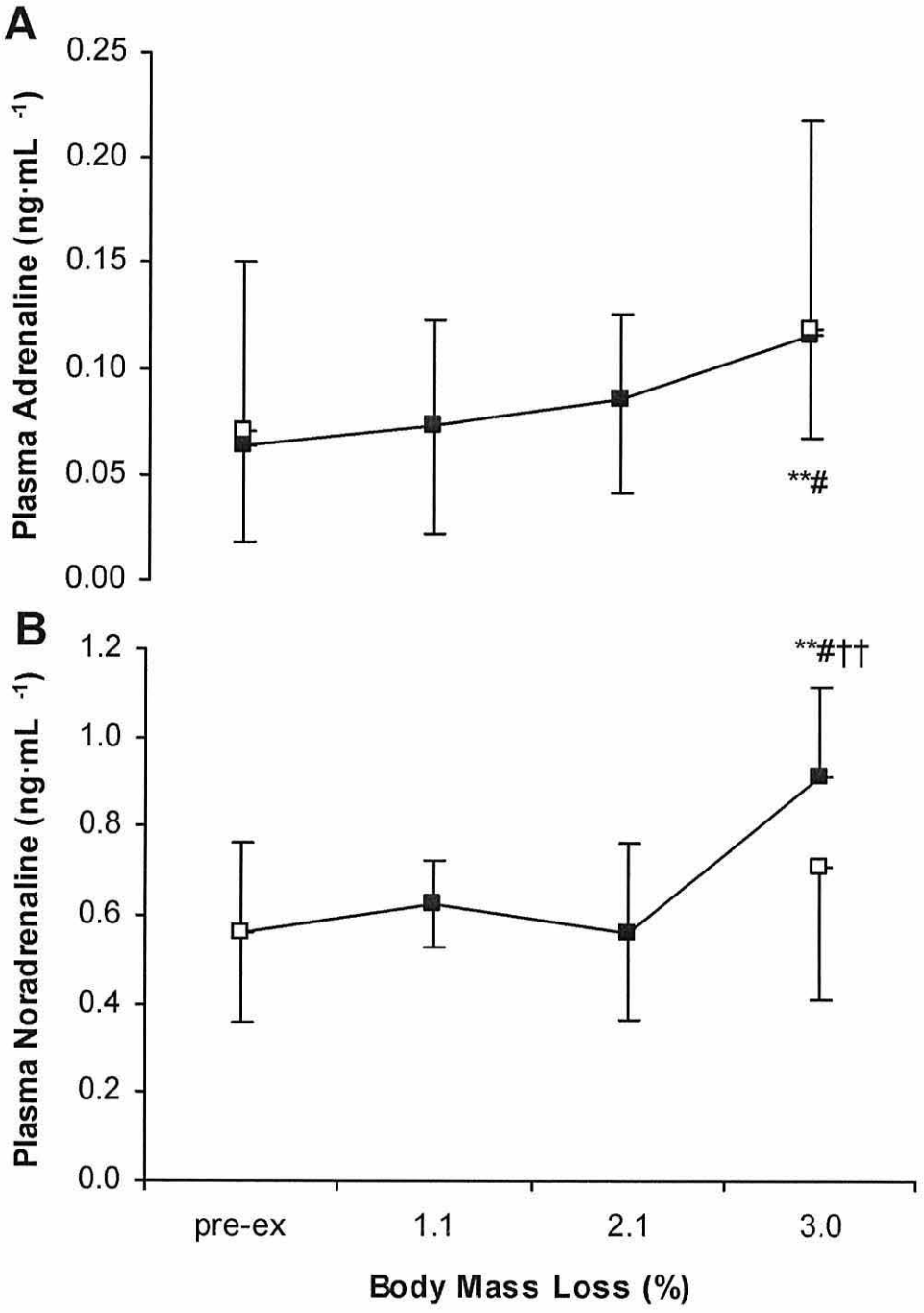


Values are mean ± SD (n = 15). b *vs.* NFI trial post exercise, ** *vs.* pre exercise, ## *vs.* 1.1% BML; $P < 0.01$. # *vs.* 1.1% BML, † *vs.* 2.1% BML; $P < 0.05$. Abbreviations: BML, body mass loss; NFI, no fluid intake; FI, fluid intake.

4.4.7 Plasma catecholamines

At pre-exercise on the NFI trial plasma adrenaline concentration was 0.064 ± 0.046 (range 0.017-0.154) $ng \cdot mL^{-1}$. Plasma adrenaline concentration, corrected for plasma volume change, increased during progressive dehydration on the NFI trial and was significantly greater than pre-exercise at 3.0% BML only ($P < 0.01$, **Figure 4.3A**). At 3.0% BML on the NFI trial plasma adrenaline concentration reached 0.116 ± 0.048 (range 0.063-0.213) $ng \cdot mL^{-1}$. Plasma adrenaline concentration was also significantly greater at post-exercise on FI trial compared with pre-exercise ($P < 0.01$, **Figure 4.3A**) but was not significantly different to post exercise on NFI. At pre-exercise on the NFI trial plasma noradrenaline concentration was 0.564 ± 0.179 (range 0.368-0.892) $ng \cdot mL^{-1}$. Plasma noradrenaline concentration, corrected for plasma volume change, was significantly greater than pre-exercise at 3.0% BML only ($P < 0.01$, **Figure 4.3B**). At 3.0% BML on the NFI trial plasma noradrenaline concentration was 0.916 ± 0.229 (range 0.643-1.338) $ng \cdot mL^{-1}$. Plasma noradrenaline concentration was not significantly different at post exercise on NFI and FI trials.

Figure 4.3: Plasma adrenaline (A) and noradrenaline (B) during progressive acute dehydration (NFI ■) and with sufficient fluids to offset fluid losses (FI □).



Values are mean ± SD (n = 8). ** *vs.* pre exercise, †† *vs.* 2.1% BML; $P < 0.01$. # *vs.* 1.1% BML; $P < 0.05$. Abbreviations: BML, body mass loss; NFI, no fluid intake; FI, fluid intake.

4.5 Discussion

The main purpose of the present study was to identify whether saliva flow rate, osmolality and total protein concentration are sensitive non-invasive markers of hydration status. In extension to previous research (Walsh *et al.*, 2004b) the sensitivity of saliva parameters to track changes in hydration status were compared with plasma and urine osmolality, two regularly used hydration markers (Shirreffs, 2000). In support of the hypothesis the results show a reduction in saliva flow rate and an increase in saliva osmolality and total protein concentration during acute dehydration (**Figure 4.2**). Furthermore, the strong relationships between plasma osmolality and saliva osmolality (mean $r = 0.87$), saliva total protein concentration (mean $r = 0.91$) and saliva flow rate (mean $r = -0.78$) show that changes in these saliva parameters reflect changes in hydration status during hypertonic hypovolaemia. The changes in saliva osmolality and total protein concentration are comparable with plasma and urine osmolality and identify a BML of 2.1% therefore satisfying the established criterion for a marker of acute dehydration (Shirreffs, 2000).

Plasma osmolality, urine osmolality, saliva osmolality and saliva total protein concentration increased during dehydration in all participants. Changes in saliva flow rate during dehydration were less sensitive to whole body water losses, with only 12 out of 15 participants exhibiting a decrease in flow rate at 3.0% BML. From a practical perspective, it is notable that three participants could not provide a urine sample at 3.0% BML on the NFI trial highlighting a potential limitation of urine markers.

Large inter-individual variation was observed in the saliva parameters, particularly saliva flow rate, as indicated in the large standard deviations, at pre-exercise (euhydration) and during both dehydration and rehydration (**Figure 4.2A**). Large inter-individual variation in

parotid saliva flow rate has also been documented where mean ± SEM euhydrated parotid flow rate in young adults was 69 ± 30 $\mu L \cdot min^{-1}$ (Ship & Fischer, 1997). In the present study unstimulated whole saliva flow rate ranged from 71 to 993 $\mu L \cdot min^{-1}$ at euhydration and 13 to 587 $\mu L \cdot min^{-1}$ at 3.0% BML on the NFI trial. Clearly, defining a range for saliva flow rate to represent a state of euhydration in all individuals would be very difficult with such large inter-individual variation. If the mean ± SD was assumed to represent a reasonable range, euhydration might be determined by a saliva osmolality of less than 61 $mOsmol \cdot kg^{-1}$ (euhydrated mean ± SD, 50 ± 11 $mOsmol \cdot kg^{-1}$). At pre-exercise, 3 of the 15 participants would be incorrectly classified as dehydrated for saliva osmolality. For comparison, using the urine osmolality euhydration threshold of less than 700 $mOsmol \cdot kg^{-1}$ recommended by the ACSM (Casa *et al.*, 2005b), two participants would be incorrectly classified as dehydrated at pre-exercise. At 3.0% BML 3 of the 15 participants would be incorrectly classified as euhydrated for saliva osmolality and 5 (of only 12 samples) for urine osmolality. For saliva parameters to have practical use as markers of whole body hydration status it is recommend that euhydrated readings be determined for each individual.

Other factors that may limit the usefulness of saliva parameters as potential markers of whole body hydration status include a possible short-term effect of neural control on saliva flow rate and composition (Dawes, 1981) and a possible short-term effect of food intake and oral hygiene (e.g. brushing teeth) on saliva flow rate (Lavelle, 1988). It is encouraging that unstimulated whole saliva flow rate and osmolality do not show significant diurnal variation (Niedermeier *et al.*, 2000; Walsh *et al.*, 2004b) and remain unchanged immediately after and 15 min after fluid consumption (**Appendix E**). These findings agree

with those showing no change in unstimulated parotid saliva flow rate immediately after drinking up to 1 L of water (Shannon & Chauncey, 1967).

Until now, a role for neuro-endocrine regulation has been widely acknowledged to explain the decrease in saliva flow rate during prolonged exercise (Dawes, 1981; Chicharro *et al.*, 1998). The second purpose of this investigation was to examine the involvement of dehydration and neuro-endocrine regulation (increase in sympathetic activity) in the decrease in saliva flow rate during prolonged exercise by assessing responses in saliva flow rate and plasma catecholamines to prolonged exercise with and without fluid restriction. In support of our hypothesis the results show that performing prolonged exercise with sufficient fluids to offset fluid losses prevents the decrease in saliva flow rate but does not prevent the increase in plasma catecholamines.

In agreement with the present study, the plasma adrenaline response to 2 h of exercise in the heat (35°C) was similar when exercise was performed with no fluids (4.9% BML) or with fluids equivalent to 95% of losses (Gonzalez-Alonso *et al.*, 1995). However, the same authors noted significantly greater plasma noradrenaline concentration after 2 h of exercise with no fluids compared with when fluids were provided. In contrast, no significant difference in plasma noradrenaline concentration between the NFI and FI trial at post-exercise was reported in the present study. This discrepancy may be explained by the fact that the exercise in the previous study (Gonzalez-Alonso *et al.*, 1995) was continuous in nature and performed in a hotter environment (35°C *vs.* 30°C here) resulting in a significant core temperature difference of +1.2°C on the no fluid trial (final core of 39.4 ± 0.2 °C) compared with the fluid trial. In the present study a non-significant core temperature difference of +0.6°C on the NFI trial (final core of 38.5 ± 0.4°C) compared with the FI trial

was observed (final core of 37.9 ± 0.3°C). Indeed, the magnitude of the plasma noradrenaline response to exercise is known to be associated with the magnitude of the rise in core temperature during exercise (Febbraio *et al.*, 1996).

To summarise, the present study shows that performing prolonged exercise with sufficient fluids to offset fluid losses prevents the decrease in saliva flow rate but does not prevent the increase in plasma catecholamines. These results provide further support for a role of dehydration in the decrease in saliva flow rate during prolonged exercise.

The mechanism(s) by which dehydration evoked such large reductions in saliva flow rate (-62% at 3.0% BML during the NFI trial) in the present study remains unclear. Reduction of body fluids during dehydration may cause salivary gland hypofunction because the predominant fluid constituent of saliva is water, which enters saliva from plasma across a trans-acinar sodium gradient (Ship & Fischer, 1997). An increase in ECF sodium concentration during hypertonic hypovolaemia (Sawka *et al.*, 1985; Popowski *et al.*, 2001) may account for the production of smaller amounts of more concentrated saliva (e.g. with increased osmolality) during dehydration in the present study. This mechanism may also account for why saliva flow rate and osmolality did not change during exercise on the FI trial when fluid intake prevented the increase in plasma osmolality. An increase in ECF sodium concentration may also explain the decrease in saliva flow rate observed during hot water immersion (Winsor, 1930) and prolonged exercise with a fluid deficit (Ford *et al.*, 1997; Bishop *et al.*, 2000; Walsh *et al.*, 2004b) where a hypertonic hypovolaemia most likely occurred. Alternatively, as vasopressin responses to exercise are closely coupled to plasma osmolality (Montain *et al.*, 1997), a direct effect of vasopressin on saliva production (possibly by increasing water reabsorption at the striated ducts) might also

explain the saliva flow rate responses to exercise with and without fluids in the present study (Lavelle, 1988).

In conclusion, this study highlights saliva osmolality and total protein concentration as valid and sensitive indices of acute dehydration (hypertonic hypovolaemia). However, for saliva markers to be of use to track changes in hydration status in an applied setting they must also be shown to be valid and sensitive to chronic dehydration. Finally, these results support a role for dehydration in the decrease in saliva flow rate during prolonged exercise.

CHAPTER FIVE

Markers of Hydration Status during 48 Hours of Restricted Fluid and/or Energy Intake

5.1 Summary: The purpose of this study was to investigate the effects of a 48 h period of fluid, energy or combined fluid and energy restriction on the validity of plasma, urine, and saliva hydration markers. Thirteen recreationally active males participated in four randomised 48 h trials. On control (CON) participants received their estimated energy (12154 ± 833 $kJ \cdot d^{-1}$, mean ± SD) and water (3912 ± 500 $mL \cdot d^{-1}$) requirements. On fluid restriction (FR) participants received their energy requirements and 193 ± 50 $mL \cdot d^{-1}$ water to drink and on energy restriction (ER) participants received their water requirements and 1214 ± 172 $kJ \cdot d^{-1}$. Fluid and energy restriction (F+ER) was a combination of FR and ER. Participants performed a 1.5 h treadmill walk at 50% $\dot{V}O_{2max}$ on day one and two of each trial. At 48 h, BML was 0.6 ± 0.4% (CON); 3.2 ± 0.5% (FR); 3.4 ± 0.3% (ER) and 3.6 ± 0.3% (F+ER). Hydration indices were unchanged during CON and ER. On FR, Plasma and urine osmolality increased from 293 ± 5 and 490 ± 81 $mOsmol \cdot kg^{-1}$ at 0 h to 302 ± 5, and 1048 ± 80 $mOsmol \cdot kg^{-1}$ by 24 h, respectively ($P < 0.01$) and saliva osmolality increased from 54 ± 10 at 0 h to 73 ± 16 $mOsmol \cdot kg^{-1}$ by 48 h ($P < 0.01$). On F+ER, plasma osmolality was unchanged, urine osmolality increased to 1023 ± 113 $mOsmol \cdot kg^{-1}$ by 24 h and saliva osmolality increased to 68 ± 5 $mOsmol \cdot kg^{-1}$ by 48 h ($P < 0.01$). In conclusion, plasma osmolality identified hypohydration during 48 h of fluid restriction but not when fluid and energy were restricted simultaneously. In contrast, urine osmolality, urine colour, saliva flow rate and saliva osmolality identified hypohydration during 48 h of fluid restriction and combined fluid and energy restriction. Nonetheless, plasma, urine and saliva indices were unable to identify hypohydration associated with energy restriction alone.

5.2 Introduction

Haematological (e.g. plasma osmolality), urinary (e.g. urine osmolality) and salivary indices (e.g. saliva osmolality) have been shown to track acute dehydration (1-3 h) evoked by exercise and heat stress (Armstrong *et al.*, 1994; Popowski *et al.*, 2001; Walsh *et al.*, 2004b). However, the validity of hydration indices during more prolonged dehydration where fluid losses occur more slowly from a combination of sweat and renal losses is unclear. It is important to assess the validity of hydration indices over more prolonged periods as the dehydration method and composition of fluid lost have been reported to determine the type of water deficit (e.g. hypertonic, isotonic) and influence the partitioning of remaining water between body fluid compartments (Nose *et al.*, 1988; Sawka, 1992).

One recent study that restricted fluid intake alone for 37 h reported increases in serum and urine osmolality after 13 h (~1% BML, Shirreffs *et al.*, 2004). However, despite further BML at 24 h (~1.8%) a plateau in serum and urine osmolality occurred which questions the validity of these indices to identify the magnitude of hypohydration. In addition, urine osmolality was determined on total urine volume collected between blood sampling at 0, 13, 24 and 37 h and as a result the validity of single serial urine osmolality measurements during prolonged dehydration is unknown. Euhydrated ranges recently proposed by the ACSM for plasma osmolality and urine indices (Casa *et al.*, 2005b) could, subject to validation, be of practical use to identify individuals as euhydrated or hypohydrated. However, these recommendations are largely based upon dehydration evoked by acute exercise and heat stress and therefore it is important to assess the validity of plasma and urine indices to identify changes in hydration status evoked by alternative dehydration methods. Moreover, no study to date has examined the effects of prolonged dehydration (>3 h) on whole saliva indices.

Energy restriction even with *ad libitum* water intake has been shown to decrease ECF volume and BM which are indicative of hypohydration (Consolazio *et al.*, 1967; Consolazio *et al.*, 1968). This hypohydration may be attributed to a negative sodium balance (Robinson *et al.*, 1956; Consolazio *et al.*, 1968) which could result from inadequate sodium intake, increased natriuresis (Bloom, 1967; Kolanowski, 1977), sweat sodium losses, or a combination thereof. These causes are likely to be exacerbated in active individuals or in hot environments (Luetkemeier *et al.*, 1997; Bergeron, 2003).

To date no study has examined the validity of hydration indices when hypohydration is accompanied by energy restriction. Nonetheless, a negative salt balance, evoked by dietary salt restriction and sweat losses from exercise, combined with 24 h of fluid restriction was shown to evoke hypohydration equal to 3.4% BML but cause no significant alteration in plasma chloride concentration (Robinson *et al.*, 1956). A similar negative salt balance combined with water intake was associated with a decrease in plasma chloride concentration and copious urine production despite modest hypohydration (~2% BML, Robinson *et al.*, 1956; Pearcy *et al.*, 1956). These findings question the validity of plasma osmolality, given that chloride accounts for a large proportion of plasma osmolality (~105 of 280 $mOsmol \cdot kg^{-1}$ of euhydrated plasma osmolality), and urine indices to identify hypohydration accompanied by energy restriction. Furthermore, the proposed relationship between plasma osmolality and saliva hydration markers (Walsh *et al.*, 2004a) suggests that saliva flow rate and osmolality may also be unable to identify hypohydration associated with energy restriction.

Therefore, the purpose of this investigation was to assess the validity of plasma, urine and saliva hydration markers during a 48 h period of restricted fluid and/or energy intake. It was hypothesised that plasma, urine and saliva hydration markers would track and identify hypohydration evoked by fluid restriction alone but not when hypohydration was accompanied by energy restriction.

5.3 Methods

Participants. Thirteen recreationally active healthy males (age, 21 ± 3 yrs; height, 179 ± 6 cm; BM, 74.7 ± 7.9 kg; body fat, 17 ± 5%; $\dot{V}O_{2max}$, 50.9 ± 4.3 $mL \cdot kg^{-1} \cdot min^{-1}$) volunteered to participate in the study. All participants gave written informed consent before the study, which received local Ethics Committee approval.

Preliminary measurements. Prior to the experimental trials, $\dot{V}O_{2max}$ was estimated by means of a continuous incremental exercise test on a treadmill (Bird & Davison, 1997). On a separate day, 7 to 10 days prior to beginning the experimental trials, participants returned to the laboratory for individual energy expenditure estimation and familiarisation. Participants arrived euhydrated at 08:00 h after an overnight fast, having consumed water equal to 40 $mL \cdot kg^{-1}$ of BM the previous day. On arrival and after voiding, anthropometric measurements of height and NBM were collected. Following these measures, body composition was estimated by DEXA and resting metabolic rate (RMR) was estimated for 10 min as previously recommended (Reeves *et al.*, 2004) using a portable breath-by-breath system (*Metamax 3B, Biophysik, Leipzig, Germany*). After breakfast, participants performed a brisk uphill 1.5 h treadmill walk at 50% $\dot{V}O_{2max}$ (mean speed and gradient, 6.4 ± 0.4 $km \cdot h^{-1}$ and 4 ± 2%, respectively) during which energy expenditure was estimated by indirect calorimetry (*Cortex Metalyser 3B, Biophysik, Leipzig, Germany*). For short periods during the day participants wore the portable breath-by-breath system to estimate the energy expenditure incurred during habitual living in the laboratory environment (e.g. light office work and watching T.V.). This additional energy expenditure data was used, along with the RMR data, to estimate the energy intake required for the experimental trials. In addition, during this 24 h period fluid requirements were estimated by assessing changes in NBM at hourly intervals. Physical activity was standardised throughout the familiarisation

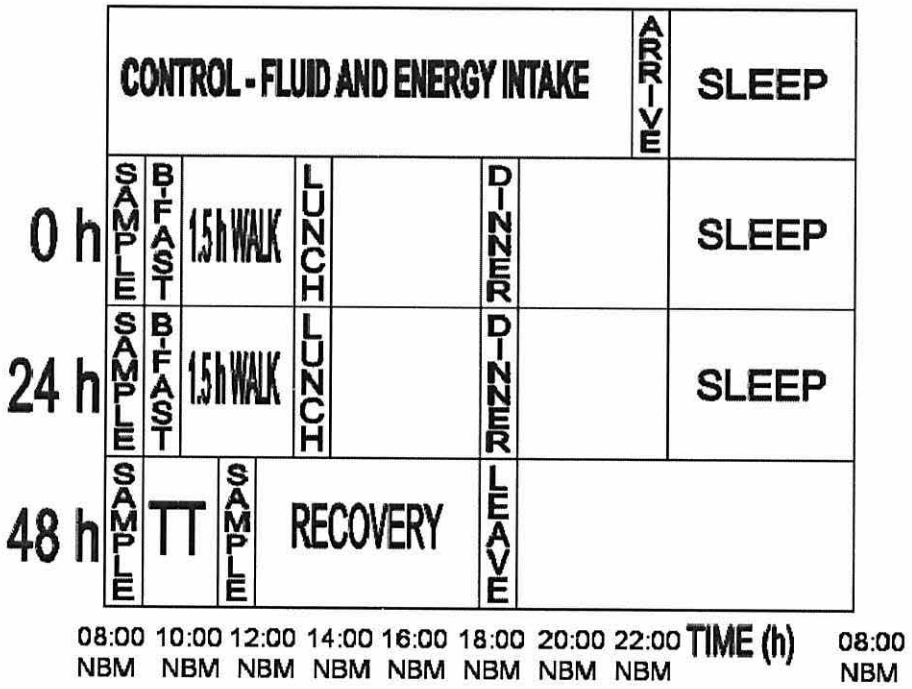
and all experimental trials by recording 24 h step counts with pedometers (*Digi-walker SW-200, Yamax, Tokyo, Japan*). This type of pedometer has been shown to have exceptional validity and reliability for ambulatory activities (Bassett, Jr. *et al.*, 1996).

Experimental procedures. Separated by 7 to 10 days, participants were required to complete four experimental trials in a random order (**Table 5.1**). The four dietary interventions included a control trial (CON), a fluid restriction trial (FR), an energy restriction trial (ER) and a fluid and energy restriction trial (F+ER). Dietary composition was estimated using software (*Dietmaster, Version 4.0, Swift Computer Systems, Surrey, UK*). On the day prior to the experimental trial, to control nutritional and hydration status, participants were provided with their estimated energy requirements (11346 ± 711 $kJ \cdot d^{-1}$ of which 49, 36, 15% were carbohydrate, fat and protein, respectively) and water equal to 40 $mL \cdot kg^{-1}$ of BM. Total energy intake was provided as three prepared meals breakfast, lunch and dinner with participants instructed to consume the meals between 09:00-09:30, 13:00-13:30 and 17:00-17:30 h, respectively. To ensure water was consumed at regular intervals throughout the day, water was divided equally between six bottles with instructions to consume one bottle with each meal and a further bottle following each meal. Participants were also instructed to consume only the food and water provided and to refrain from exercise the day prior to each trial.

Participants arrived at the laboratory at 22:00 h the evening prior to each trial. On the evening prior to each trial participants slept for 8 h in a temperate laboratory (19.7 ± 0.6°C, 59 ± 7% RH). The intervention began at 08:30 h the following morning after participants had voided and after a NBM was obtained. Thereafter, during waking hours NBM was recorded at two-hourly intervals. Euhydration was verified by ensuring all participants'

urine osmolality was less than 700 mOsmol·kg^{-1} (Casa *et al.*, 2005b). Participants were seated for 10 min after which baseline (0 h) blood and saliva samples were obtained. Further collections of first morning urine, blood and saliva were made after 24 h (08:30 h, day 2) and 48 h (08:30 h, day 3, **Figure 5.1**). To simulate active populations (e.g. military recruits on field exercise) participants performed a brisk uphill 1.5 h treadmill walk at a set workload equivalent to 50% $\dot{V}O_{2max}$ after breakfast on day one and two of each trial. Water was consumed equal to fluid losses on CON and ER walks whereas no fluids were provided on FR and F+ER walks. Sweat loss on the 1.5 h walks was calculated from NBM change corrected for fluid intake and urine loss (Cheuvront *et al.*, 2002). After providing 48 h samples of urine, blood and saliva participants performed a self-paced 30 min treadmill TT; the details of the protocol and data from which are described and presented in **Chapter 7**. Further samples were obtained immediately post-TT which was followed by a 6 h rehydration and refeeding period (see **Chapter 6** for a description). All walks and TT were performed in an air conditioned laboratory (19.7 ± 0.6°C, 59 ± 7% RH).

Figure 5.1: Schematic of trial events.



Abbreviations: NBM, nude body mass; TT, time trial.

Table 5.1: Nutrient intake for a 24 h period.

	CON	FR	ER	F+ER
Fluid				
Fluid consumed (mL)	3912 ± 500	960 ± 70	3893 ± 484	962 ± 73
Water to drink (mL)	3145 ± 476	193 ± 50	3816 ± 481	885 ± 70
Water in food (mL)	767 ± 39	767 ± 39	77 ± 4	77 ± 4
Energy				
Energy consumed (kJ)	12154 ± 833	12154 ± 833	1214 ± 84	1214 ± 84
Carbohydrates (g)	387 ± 28	387 ± 28	39 ± 3	39 ± 3
Fat (g)	119 ± 9	119 ± 9	12 ± 1	12 ± 1
Protein (g)	104 ± 5	104 ± 5	10 ± 1	10 ± 1
Sodium (g)	3.3 ± 0.3	3.3 ± 0.3	0.3 ± 0.0	0.3 ± 0.0

Values are mean ± SD. Abbreviations: CON, control; FR, fluid restriction; ER, energy restriction; F+ER, fluid and energy restriction. Macronutrient composition was the same across all trials and 50, 36, 14% were carbohydrate, fat and protein, respectively.

Analytical methods. Haematological parameters including haemoglobin, haematocrit, plasma volume change and osmolality; urine parameters including osmolality and colour; saliva parameters including flow rate and osmolality were measured as described in **Chapter 3**.

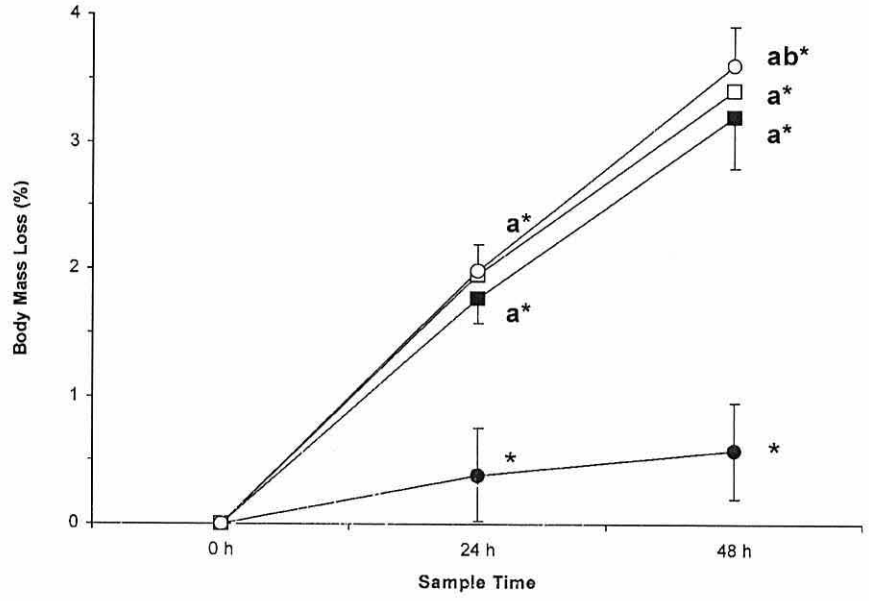
Statistical analysis. A one-way fully repeated measures ANOVA was performed on pre-experimental BM and trial pedometer counts. Two-way fully repeated ANOVA's were performed on BML, plasma, urine and saliva indices. Pearson product correlation (r) was calculated between urine osmolality and colour.

5.4 Results

5.4.1 Physical activity, body mass loss and sweat loss

Mean experimental trial physical activity ranged between 18459 and 19230 steps·d^{-1} and was not significantly different between the trials ($P > 0.05$). At baseline (0 h) BM was not significantly different between trials (CON, 73.4 ± 7.2; FR, 73.9 ± 7.4; ER, 73.9 ± 7.4; F+ER, 73.7 ± 7.6 kg, $P > 0.05$). During the 48 h period on CON, BM was relatively well maintained (-0.6 ± 0.4%, **Figure 5.2**). The 48 h period on FR, ER and F+ER evoked linear BML that was significantly different from CON by 24 h ($P < 0.01$). Following the TT, BML was; 1.7 ± 0.4% on CON, 4.1 ± 0.5% on FR, 4.3 ± 0.3% on ER and 4.4 ± 0.4% on F+ER ($P < 0.01$). Average 1.5 h walk sweat losses was signifcantly greater on CON (1.0 ± 0.3 L, $P < 0.01$) compared with FR (0.7 ± 0.2 L), ER (0.8 ± 0.2 L) and F+ER (0.7 ± 0.2 L).

Figure 5.2: The effects of a 48 h period of fluid restriction (■), energy restriction (□), fluid and energy restriction (○) and control (●) on body mass loss (%).



Values are mean ± SD. a *vs.* CON, b *vs.* FR, * *vs.* 0 h, $P < 0.01$. Overlapping means have same significance value.

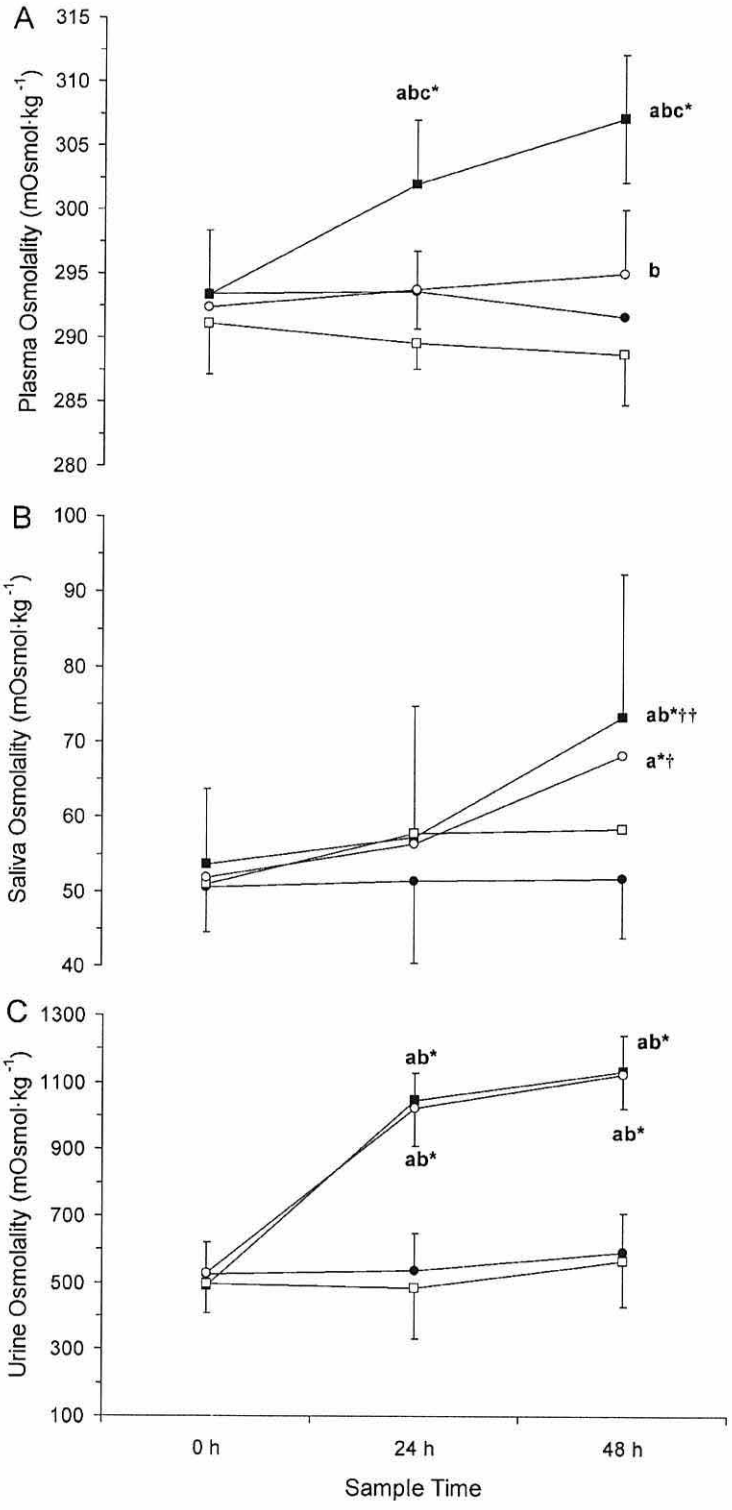
5.4.2 Plasma osmolality and plasma volume change

Plasma osmolality increased on FR by 24 h compared with all other trials ($P < 0.01$) but remained unaltered during the 48 h period on CON, ER and F+ER (**Figure 5.3A**). Post-TT plasma osmolality increased significantly compared with 48 h equalling 303 ± 4, 314 ± 4, 297 ± 6 and 303 ± 6 mOsmol·kg^{-1} on CON, FR, ER and F+ER, respectively ($P < 0.01$). At 48 h, plasma volume was unaltered on CON and FR (-1.4 ± 5.7 and -0.9 ± 4.9%, $P > 0.05$) and decreased on ER and F+ER (-5.2 ± 3.2 and -5.1 ± 4.5%, $P < 0.05$). Compared with 48 h, plasma volume decreased as a result of the TT although this only reached significance on FR (CON, -5.8 ± 3.9; FR, -6.3 ± 3.5 ($P < 0.05$); ER, -4.9 ± 4.6; F+ER, -4.8 ± 3.7%).

5.4.3 Saliva osmolality and flow rate

Saliva osmolality increased on FR and F+ER by 48 h ($P < 0.01$) whilst remaining unaltered on CON and ER (**Figure 5.3B**). Post-TT saliva osmolality was significantly increased compared with 48 h equalling 66 ± 13, 107 ± 25, 80 ± 20 and 95 ± 26 mOsmol·kg^{-1} on CON ($P < 0.05$), FR, ER and F+ER ($P < 0.01$), respectively. Saliva flow rate decreased on FR and F+ER by 48 h ($P < 0.01$) remaining unaltered on CON and ER (**Table 5.2**). Post-TT saliva flow rate was significantly decreased compared with 48 h equalling 266 ± 177, 167 ± 192 and 103 ± 96 μL·min^{-1} on CON, ER and F+ER, respectively ($P < 0.05$). On FR, saliva flow rate decreased to 95 ± 79 μL·min^{-1} post-TT but this was not significantly different compared with 48 h.

Figure 5.3: The effects of 48 h period of fluid restriction (■), energy restriction (□), fluid and energy restriction (○) and control (●) on plasma (A), saliva (B) and urine osmolality (C).



Values are mean ± SD. a *vs.* CON, b *vs.* ER, c *vs.* F+ER, * *vs.* 0 h, †† *vs.* 24 h; $P < 0.01$. † *vs.* 24 h; $P < 0.05$.

5.4.4 Urine osmolality, colour and volume

Compared with CON and ER, urine osmolality increased ($P < 0.01$) similarly on FR and F+ER reaching a plateau by 24 h (**Figure 5.3C**). Urine osmolality remained unchanged on CON and ER during the 48 h. Compared with 48 h, Post-TT urine osmolality remained unchanged on CON and F+ER (707 ± 109 and 1024 ± 86 mOsmol·kg^{-1}, respectively), was increased on ER (758 ± 111 mOsmol·kg^{-1}, $P < 0.01$) and decreased on FR (982 ± 109 mOsmol·kg^{-1}, $P < 0.01$). Urine colour identified the same pattern of results as osmolality (**Table 5.2**). Pearson product correlation indicated a strong relationship between urine osmolality and colour ($r = 0.83$, $P < 0.01$). Urine volume over the 48 h period decreased as a result of FR and F+ER (1597 ± 174 and 1196 ± 141 mL, respectively) compared with CON (4804 ± 77 mL, $P < 0.01$). Urine loss was greater during the 48 h on ER (6457 ± 747 mL) compared with CON ($P < 0.01$).

Table 5.2: The effects of a 48 h period of fluid restriction (FR), energy restriction (ER) fluid and energy restriction (F+ER) and control (CON) on saliva flow rate and urine colour.

		0 h	24 h	48 h
Saliva Flow Rate	CON	458 ± 346	441 ± 310	441 ± 312
($\mu L \cdot min^{-1}$)	FR	510 ± 421	372 ± 368	$169 \pm 128^{ab*\dagger}$
	ER	484 ± 358	488 ± 440	330 ± 332
	F+ER	452 ± 319	319 ± 219^{b}	$265 \pm 184^{a*}$
Urine Colour	CON	4 ± 1	4 ± 1	5 ± 1
(1-8,	FR	4 ± 1	$7 \pm 1^{abb*}$	$7 \pm 0^{abb*}$
Armstrong *et al.*, 1994)	ER	4 ± 1	5 ± 1	5 ± 1
	F+ER	4 ± 1	$7 \pm 1^{abb*}$	$7 \pm 0^{abb*}$

Values are mean ± SD. a *vs.* CON, bb *vs.* ER, * *vs.* 0 h, † *vs.* 24 h; $P < 0.01$. b *vs.* ER; $P < 0.05$. Abbreviations: CON, control; FR, fluid restriction; ER, energy restriction; F+ER, fluid and energy restriction.

5.5 Discussion

In support of our hypothesis these results show that plasma osmolality, urine osmolality, urine colour, saliva flow rate and saliva osmolality identified hypohydration during a 48 h period of fluid restriction. Furthermore plasma, urine and saliva markers did not indicate hypohydration on ER alone. It is widely acknowledged that much of the BML observed during the first 72 h of energy restriction is attributable to hypohydration (Consolazio *et al.*, 1967; Consolazio *et al.*, 1968). Indeed, urine output and BML were much greater after 48 h on ER compared with CON. Furthermore, plasma osmolality, often referred to as a gold-standard marker of hydration (Popowski *et al.*, 2001; Casa *et al.*, 2005b), did not detect hypohydration when fluid and energy were restricted simultaneously. In contrast, and contrary to our hypothesis, urine and saliva markers identified hypohydration during 48 h of combined fluid and energy restriction. These results highlight the importance of adequate dietary intake when monitoring hydration using these hydration markers: particularly when using plasma osmolality.

Plasma osmolality exceeded the proposed 290 mOsmol·kg^{-1} euhydration threshold (Popowski *et al.*, 2001; Casa *et al.*, 2005b) in 73% of samples at 0 h. This was despite all 0 h urine osmolality readings indicating euhydration (<700 mOsmol·kg^{-1}, Casa *et al.*, 2005b) and similar BM at 0 h in each of the four trials. To ensure euhydration, participants consumed water equal to 40 mL·kg^{-1} in the 24 h prior to each trial; incidentally, this is a greater amount of water than has previously been prescribed to ensure euhydration (Armstrong *et al.*, 1994; Popowski *et al.*, 2001; Walsh *et al.*, 2004a). It is also noteworthy that a similar daily water intake (42 mL·kg^{-1} of BM) ensured that mean plasma, urine and saliva osmolality remained unchanged throughout the 48 h period on CON and BML was less than 0.6% which compares favourably with typical daily variations in BM in

euhydrated individuals (Grandjean *et al.*, 2000; Grandjean *et al.*, 2003; Cheuvront *et al.*, 2004). As such, the present findings do not support using plasma osmolality of 290 $mOsmol \cdot kg^{-1}$ as an upper threshold for euhydration.

Unlike the previous 37 h fluid restriction study (Shirreffs *et al.*, 2004), where plasma osmolality reached a plateau by 24 h, plasma osmolality increased incrementally during the 48 h on FR and in response to a further 0.9% BML evoked by the TT. Plasma osmolality identified hypohydration equal to approximately 2% BML on FR which suggests the sensitivity of plasma osmolality over more prolonged periods of fluid restriction compares favourably with that reported during acute exercise and heat exposure (Popowski *et al.*, 2001; Walsh *et al.*, 2004a). In contrast, plasma osmolality did not increase during 48 h on F+ER despite individuals receiving the same small volume of total fluid as FR. More specifically, plasma osmolality increased by less than 3 $mOsmol \cdot kg^{-1}$ in 70% of individuals during 48 h on F+ER whereas 100% of individuals increased by more than 5 $mOsmol \cdot kg^{-1}$ during 48 h on FR. In addition, plasma osmolality remained unchanged during 48 h on ER suggesting the marker is unable to identify hypohydration associated with energy restriction alone. These results are the first to indicate the importance of adequate dietary intake for the validity of plasma osmolality as a hydration marker.

Urine osmolality was also unable to identify hypohydration during the 48 h period evoked by energy restriction alone. However, urine osmolality may be considered a valid hydration marker during fluid restriction alone or when fluid and energy restriction are restricted simultaneously. From a practical perspective, 100% of individuals on FR and F+ER would be correctly identified as hypohydrated by 24 h using the 700 $mOsmol \cdot kg^{-1}$ threshold (Casa *et al.*, 2005b). Despite further significant increases in BML at 48 h and

post-TT on FR and F+ER there was no further increase in urine osmolality. This most probably represents the maximal concentrating capacity of the kidneys (~1200 mOsmol·kg^{-1},Shirreffs *et al.*, 2004). Consequently, practitioners should consider the limitation of urinary indices to quantify the magnitude of hypohydration. From a practical perspective it is important to highlight the findings of urine colour which were shown to be strongly correlated (r = 0.83) with and exhibit the same pattern of results as urine osmolality.

Raised plasma osmolality most likely accounts for the observed increase in urine osmolality during the 48 h on FR through increases in circulating vasopressin (Vokes, 1987). Given the widely accepted role for plasma osmolality in the control of circulating vasopressin (Baylis, 1987; Vokes, 1987) the mechanism for raised urine osmolality on F+ER with no change in plasma osmolality is less clear. Increases in circulating vasopressin, of the order necessary for increases in urine osmolality, have been shown following relatively modest degrees of hypotension and hypovolaemia (~5%) and during hypoglycaemia (blood glucose <2.2 mmol·L^{-1}, Baylis, 1987). As the current study showed reductions in plasma volume of ~5% during the 48 h on F+ER but blood glucose did not fall below 4 mmol·L^{-1} (**Table 6.1**) a role for hypoglycaemia is less likely.

The inadequate sodium intake combined with sweat sodium loss most probably accounts for the unaltered plasma osmolality on ER and F+ER (Robinson *et al.*, 1956; Vokes, 1987). A mean sweat sodium concentration of 24 mmol·L^{-1} has previously been measured, by whole-body wash-down technique, for a 1.5 h walk at 50% $\dot{V}O_{2max}$ in temperate environment (20°C, 50% RH, Patterson *et al.*, 2000). Assuming this mean sweat concentration and using the 1.5 h walk sweat rates from ER and F+ER the estimated 1.5 h walk sodium losses (mean 0.4-0.5 g) exceeded the restricted daily sodium intake (mean 0.3

g, **Table 5.1**). Irrespective of hypohydration, a negative ECF sodium balance leads to the excretion of solute-free water to ensure the restoration of ECF osmolality (Vokes, 1987). In accordance with previous findings the copious and dilute urine production on ER (6.5 *vs.* 4.8 L on CON, $P < 0.01$) can be suggested as a consequence of the negative sodium balance (Robinson *et al.*, 1956; Pearcy *et al.*, 1956). Additionally, large hypotonic fluid loads ingested during rehydration have been shown to cause copious and dilute urine to be produced despite significant hypohydration (Kovacs *et al.*, 1999).

To our knowledge, this is the first study to show that saliva osmolality and flow rate can identify hypohydration during 48 h of fluid restriction or fluid and energy restriction. Saliva osmolality and flow rate identified hypohydration equal to ~3% BML which compares favourably to acute exercise and heat exposure (Walsh *et al.*, 2004a; Walsh *et al.*, 2004b) and fulfils the criteria of a hydration marker (Shirreffs, 2000). The decrease in whole saliva flow rate on FR and F+ER concurs with a decrease in parotid saliva flow rate after a 24 h period without fluid or food (Ship & Fischer, 1997). Unlike plasma osmolality, the validity of saliva indices was unaffected by the addition of energy to fluid restriction on F+ER. Saliva osmolality increased and flow rate decreased on ER yet these changes were not significant suggesting that these saliva indices are unable to identify hypohydration associated with energy restriction alone. Saliva osmolality maybe considered more sensitive than saliva flow rate as it detected further acute dehydration resulting from the TT and showed less inter-individual variation.

Pending the confirmation of a hypohydration threshold, saliva osmolality may become a useful non-invasive hydration marker. If we tentatively use the previously determined euhydrated mean for saliva osmolality (<61 mOsmol·kg^{-1}, **Chapter 4**) 81% of individuals

would have been correctly classified as euhydrated at baseline and 66 and 100% of individuals would be identified as hypohydrated at 48 h and post TT on FR. Ideally though, we recommend that euhydrated readings for saliva osmolality be determined for each individual. We recognise the practical limitation of determining osmolality using the salivette collection method as it requires a centrifuge. Encouragingly, we have recently demonstrated that osmolality readings using the salivette method correlate strongly (r = 0.83) with a simpler dribble method, which does not require a centrifuge, across a range of hydration states (**Appendix F**). Compared with collecting urine, collecting saliva is less intrusive and in some scenarios may prove a more convenient form of sampling.

The physiological mechanism(s) responsible for the reduction in saliva flow rate remains unclear. We have previously hypothesised that an increase in plasma sodium (osmolality) during dehydration may account for the production of smaller amounts of more concentrated saliva (e.g. decreased saliva flow rate and increased osmolality, Walsh *et al.*, 2004a). In order for water to move from plasma through acinar cells to form primary saliva a trans-acinar cell sodium gradient must be generated (Ship & Fischer, 1997). Dehydration may cause the ECF to reflect an increased salt concentration and accordingly, a greater salt concentration will have to be generated across the salivary acinar cell in order to drive fluid into the acinar lumen.

Our previous hypothesis is supported by saliva and plasma responses on FR. However, the decrease in saliva flow rate and increase in saliva osmolality on F+ER with no increase in plasma osmolality questions a direct effect of plasma sodium in decreasing saliva flow rate. Alternatively, other responses to dehydration including an increase in circulating vasopressin and up-regulated renin-angiotensin-aldosterone and SNS activity (Melin *et al.*,

1997; Huch *et al.*, 1998) may cause vasoconstriction of the blood vessels to the salivary glands, thereby limiting water availability to the saliva gland and ultimately reducing saliva flow rate (Lavelle, 1988; Chicharro *et al.*, 1998; Talke *et al.*, 2003). Future research should attempt to elucidate the mechanism(s) responsible for changes in saliva flow rate and osmolality during acute and prolonged dehydration.

In conclusion, plasma osmolality, often referred to as a gold-standard marker of hydration, identifies hypohydration during a 48 h period of fluid restriction but does not detect hypohydration when fluid and energy are restricted simultaneously. In contrast, urine osmolality, urine colour, saliva flow rate and saliva osmolality identify hypohydration during 48 h of fluid restriction or combined fluid and energy restriction. Nonetheless, plasma, urine and saliva indices were unable to identify hypohydration associated with energy restriction alone. These results highlight the importance of adequate dietary intake when monitoring hydration status: particularly when using plasma osmolality.

CHAPTER SIX

Salivary IgA Response at Rest and after Exercise following 48 Hours of Restricted Fluid and/or Energy Intake

6.1 Summary: The purpose of this study was to investigate the effects of a 48 h period of fluid, energy or combined fluid and energy restriction on s-IgA responses at rest and after a 30 min treadmill TT. Thirteen healthy males participated in four randomised 48 h trials. On the control trial (CON) participants received their estimated energy (12154 ± 833 $kJ \cdot d^{-1}$, mean ± SD) and water (3912 ± 500 $mL \cdot d^{-1}$) requirements. On fluid restriction (FR) participants received their energy requirements and 193 ± 50 $mL \cdot d^{-1}$ water to drink and on energy restriction (ER) participants received their water requirements and 1214 ± 84 $kJ \cdot d^{-1}$. Fluid and energy restriction (F+ER) was a combination of FR and ER. After 48 h, participants performed a self-paced 30 min treadmill running TT followed by rehydration (0-2 h) and refeeding (2-6 h). Unstimulated saliva was collected at 0, 24, 48 h, post-TT, 2 h and 6 h post-TT. Saliva flow rate and s-IgA remained unchanged during the 48 h on CON and ER. However, 48 h on FR decreased saliva flow rate (64%) which most probably accounted for the increase in s-IgA concentration ($P < 0.01$). Despite a decrease in saliva flow rate (54%) s-IgA concentration did not increase on F+ER resulting in a decreased s-IgA secretion rate by 24h (0 h, 20 ± 5; 24 h, 12 ± 6 $\mu g \cdot min^{-1}$, $P < 0.01$). Post-TT s-IgA secretion rate was not lower compared with 48 h on any trial indicating a limited effect of the TT. Saliva-IgA secretion rate returned to within 0 h values by 6 h post-TT on F+ER. In conclusion, a 24 to 48 h period of combined fluid and energy restriction decreased s-IgA secretion rate but normalisation occurred within 6 h with rehydration and refeeding.

6.2 Introduction

Intense military training involving severe fluid and energy restriction has been shown to decrease s-IgA concentration (Carins & Booth, 2002; Gomez-Merino *et al.*, 2003) which possibly contributed to the raised URTI incidence among recruits (Carins & Booth, 2002). These findings may also be partly attributable to arduous exercise (Tomasi *et al.*, 1982) and/or chronic psychological stress (Jemmott *et al.*, 1983) which have also independently been shown to decrease s-IgA. Additionally, these studies (Carins & Booth, 2002; Gomez-Merino *et al.*, 2003) did not report s-IgA as a secretion rate as recommended (Walsh *et al.*, 1999) and the lack of experimental control is also a limitation. Saliva IgA has also been reported to be decreased in moderately malnourished children compared with well fed children (Watson *et al.*, 1985; Johansson *et al.*, 1994). In contrast s-IgA was unaltered during an eight day fast in healthy adults (Johansson *et al.*, 1984). The discrepant findings may be partly explained by differences in saliva collection methods, the method used to express s-IgA and the time of day when samples were collected, as s-IgA has been shown exhibit significant diurnal variation (Hucklebridge *et al.*, 1998).

Decreased nutrient availability may affect s-IgA responses through raised stress hormones (e.g. cortisol) or an increase in sympathetic nervous activity. Elevated plasma cortisol has been observed during prolonged exercise with fluid restriction (Bishop *et al.*, 2004) and after severe energy restriction (Fichter *et al.*, 1986). Glucorticoids have been shown to have a delayed inhibitory effect, taking several hours, on *in vitro* B lymphocyte antibody synthesis in human subjects (Saxon *et al.*, 1978) and reduce mucosal IgA levels in rats (Wira *et al.*, 1990). In contrast, sympathetic nerve stimulation of rat submandibular gland has been shown to increase translocation of IgA almost immediately (Carpenter *et al.*, 1998).

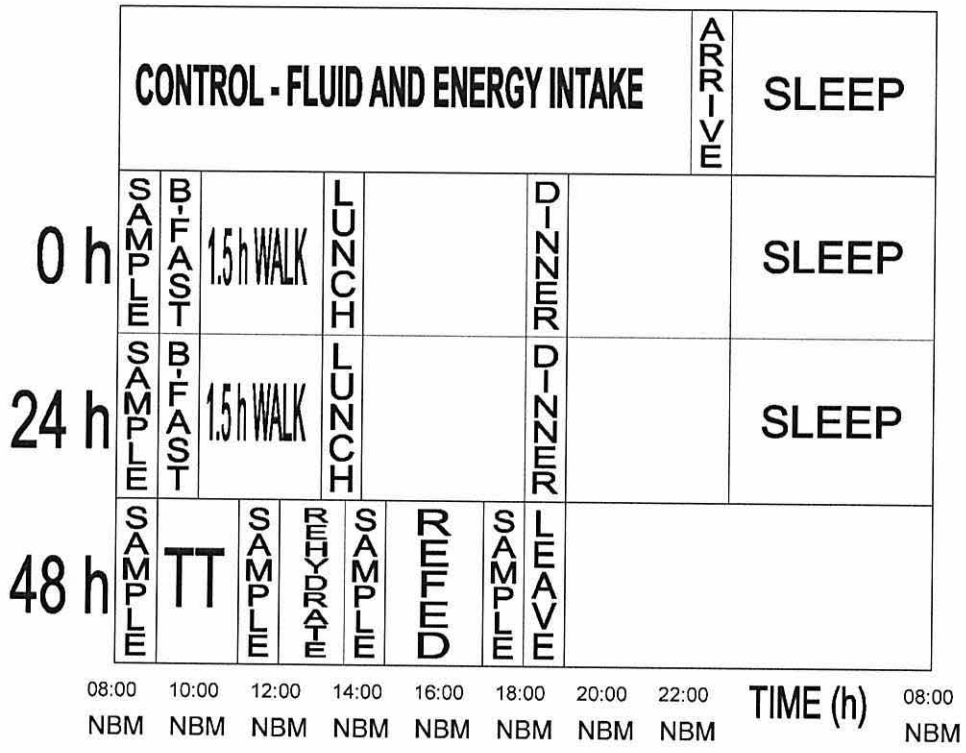
Dehydration evoked by a 24 h period without fluid or food has been shown to decrease saliva flow rate (Ship & Fischer, 1997) which may impair oral health by limiting the availability of several proteins (e.g. IgA, lysozyme and α-amylase) known to have important anti-microbial properties (Tenovuo, 1998; Rantonen & Meurman, 2000; West *et al.*, 2006). Indeed, saliva flow rate appears to be an influential factor in protection against oral pathogens and as such it has been recommended that s-IgA be reported as a secretion rate (flow rate x concentration) to reflect the total availability of s-IgA at the oral surface and correct for changes in hydration status (Walsh *et al.*, 1999). In line with this, individuals who suffer from dry mouth syndrome (xerostomia) are known to suffer an increased incidence of URTI (Fox *et al.*, 1985).

Whether prolonged fluid and/or energy restriction alters the s-IgA response to subsequent exercise remains unknown. Therefore, the purpose of this study was to determine the effects of a prolonged (48 h) period of fluid, energy or combined fluid and energy restriction on s-IgA responses at rest and after exercise. It was hypothesised that fluid or energy restriction would decrease s-IgA availability both at rest and after exercise and that the effects of combined fluid and energy restriction would be additive.

6.3 Methods

For details on *participants*, *preliminary measurements*, *experimental trials* and *procedures* see **Chapter 5**. A summary of the trial events is provided in **Figure 6.1**. For *analytical methods* see **Chapter 3**. In addition to previously described *experimental trial* and *procedures* further blood and saliva samples were obtained following a 30 min treadmill TT, 2 h post-TT and 6 h post-TT. No fluids were consumed during the treadmill TT. During the first 2 h of recovery, fluid was provided as a citrus flavoured electrolyte only solution (50 $mmol \cdot L^{-1}$ sodium, *Science in Sport, Blackburn, UK*). The total volume of beverage consumed was equal to 100% BML or up to 29 $mL \cdot kg^{-1}$ of BM. In accordance with previous recommendations to ensure maximal gastric emptying the rehydration solution was divided into one priming bolus (40% of total volume up to a maximum of 11 $mL \cdot kg^{-1}$ of BM) consumed in the first 15 min of rehydration with a further six smaller equal boluses (10% of total volume equal to a maximum 3 $mL \cdot kg^{-1}$ of BM) consumed at 15 min intervals (Mitchell *et al.*, 1994). During hours 2-3 and 4-5 of recovery, participants consumed a total of 8164 ± 557 kJ (49, 36, 15% were carbohydrate, fat and protein, respectively) divided equally into two meals. Water was available *ad libitum* during these two meals.

Figure 6.1: Schematic of trial events.



Abbreviations: NBM, nude body mass; TT, time trial.

Statistical analysis. A one-way fully repeated measures ANOVA was performed on pre-experimental BM measures and trial physical activity. Two-way fully repeated ANOVA's were performed on BML, plasma and saliva parameters.

6.4 Results

6.4.1 Body mass loss and recovery fluid intake

After 48 h, BML on FR, ER and F+ER was significantly greater than CON (**Figure 5.2**). Following the TT, final BML was; 1.7 ± 0.4% on CON, 4.1 ± 0.5% on FR, 4.3 ± 0.3% on ER and 4.4 ± 0.4% on F+ER ($P < 0.01$). The 6 h recovery protocol re-established 0 h BM on CON and FR (-1.0 ± 0.4 and -1.0 ± 0.6, respectively) although significant BML was still evident on ER and F+ER (0.9 ± 0.7 and 0.5 ± 0.6, respectively, $P < 0.01$). Fluid provided during the 2 h rehydration period was 1363 ± 322 mL on CON, 2056 ± 203 mL on FR, 2048 ± 204 mL on ER and 2037 ± 192 on F+ER. The *ad libitum* water intake during recovery meals (2-3 and 4-5 h post) was 830 ± 258 mL on CON, 1031 ± 343 mL on FR, 818 ± 464 mL on ER and 914 ± 443 mL on F+ER.

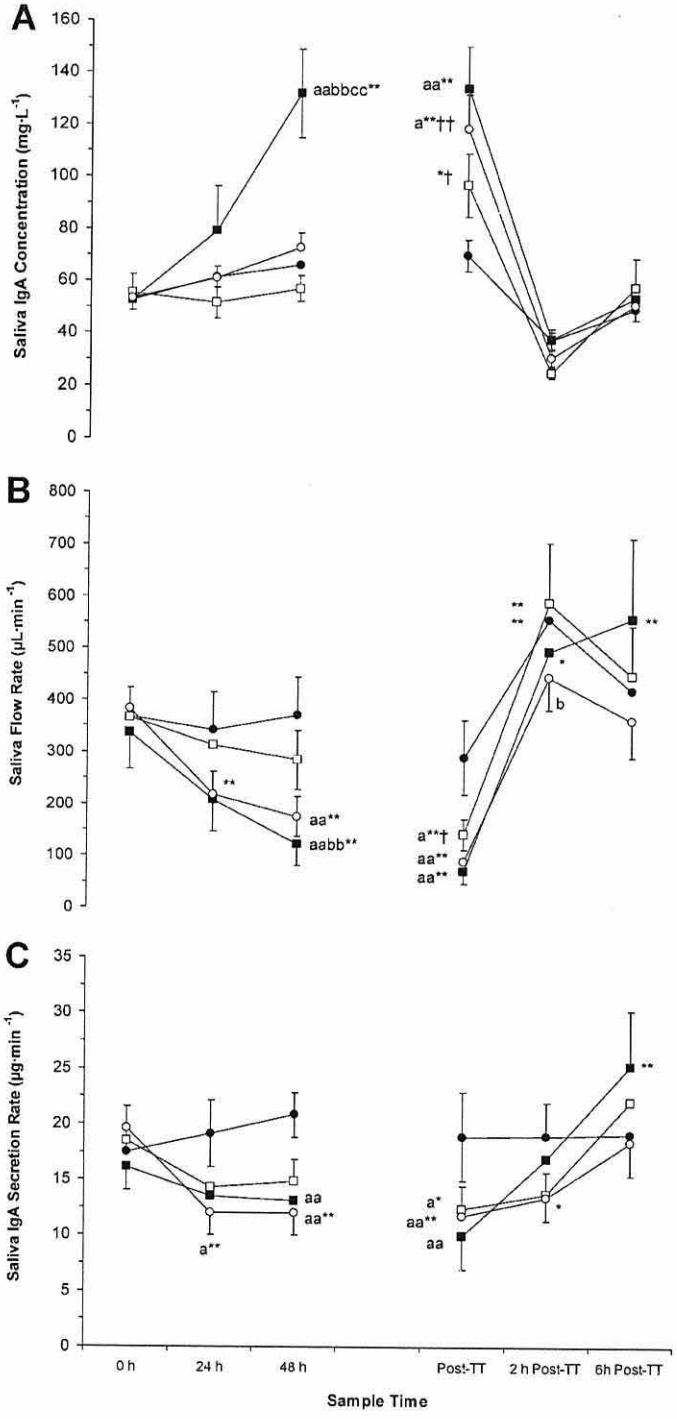
6.4.2 Saliva responses

Due to insufficient saliva volume for s-IgA analysis at all time points in all participants the data presented here is from the eight participants where a complete data set was obtained. Saliva IgA concentration, saliva flow rate and s-IgA secretion rate remained unaltered during the 48 h period on CON and ER (**Figure 6.2**). Saliva IgA concentration increased on FR by 48 h and was greater than all other trials at this time ($P < 0.01$, **Figure 6.2A**). The increase in s-IgA concentration on FR is most likely attributable to the decrease in saliva flow rate ($P < 0.01$, **Figure 6.2B**). In contrast, a similar decrease in saliva flow rate ($P < 0.01$) during 48 h on F+ER was not accompanied by a significant increase in s-IgA concentration: this resulted in a reduced s-IgA secretion rate at 24 and 48 h on F+ER ($P < 0.01$, **Figure 6.2C**).

Following the 30 min TT, saliva flow rate was significantly lower than 0 h on FR, ER and F+ER ($P < 0.01$) and lower than pre-TT (48 h) on ER ($P < 0.05$). Post-TT s-IgA concentration was significantly greater than 0 h on ER ($P < 0.05$), FR and F+ER ($P < 0.01$) and greater than pre-TT (48 h) on ER ($P < 0.05$) and F+ER ($P < 0.01$). Saliva IgA secretion rate was lower post-TT compared with 0 h on ER and F+ER ($P < 0.05$) but was not lower at any time post-TT compared with pre-TT (48 h) on any trial. This suggests a limited effect of a 30 min maximal bout of running on s-IgA secretion rate even under conditions of severe fluid and/or energy intake restriction.

The 2 h rehydration protocol re-established s-IgA concentration to within 0 h values on all trials: this is most likely attributable to the increase in saliva flow rate observed at 2 h post-TT. Following the 2 h rehydration period, s-IgA secretion rate had returned to within 0 h values on CON, FR and ER but remained lower than 0 h on F+ER ($P < 0.05$). Saliva IgA secretion rate returned to within 0 h values on F+ER by 6 h post-TT after rehydration and refeeding. The increased s-IgA secretion rate on FR at 6 h post-TT is probably attributable to the elevated saliva flow rate at this time ($P < 0.01$).

Figure 6.2: The effects of 48 h period of fluid restriction (■), energy restriction (□), fluid and energy restriction (○) and control (●) on saliva IgA concentration (A), saliva flow rate (B) and saliva IgA secretion rate (C).



Values are mean ± SEM, (n = 8). aa *vs.* CON, bb *vs.* ER, cc *vs.* F+ER, ** *vs.* 0 h, †† *vs.* 48 h; $P < 0.01$. a *vs.* CON, b *vs.* ER, c *vs.* F+ER, * *vs.* 0 h, † *vs.* 48 h; $P < 0.05$.

6.4.3 Plasma volume change, free fatty acids, glucose and cortisol

Plasma volume did not change significantly during the 48 h period (CON, -1.4 ± 5.7; FR, -0.9 ± 4.9%; ER -5.2 ± 3.2; F+ER, -5.1 ± 4.5%) but decreased post-TT (CON, -5.8 ± 3.9; FR, -6.3 ± 3.5 ($P < 0.05$); ER, -4.9 ± 4.6; F+ER, -4.8 ± 3.7%, $P < 0.05$). Plasma volume was increased on all trials following the 2 h rehydration protocol (CON, 6.4 ± 4.7; FR, 7.6 ± 4.3; ER, 9.0 ± 6.5; F+ER, 10.5 ± 5.3%, $P < 0.05$) and 6 h recovery period (CON, 10.2 ± 4.6; FR, 13.4 ± 6.1; ER, 14.2 ± 5.0; F+ER, 14.3 ± 5.6%, $P < 0.01$). Plasma FFA concentration increased on energy restriction trials by 24 h (ER and F+ER, $P < 0.05$, **Table 6.1**) and was significantly greater than CON and FR from this point onwards ($P < 0.05$). Plasma glucose concentration was lower than CON on ER and F+ER by 48 h ($P < 0.05$). Following the TT, plasma glucose concentration was significantly greater on CON and FR compared with 0 h, ER and F+ER ($P < 0.05$). Plasma cortisol concentration did not alter significantly throughout the 48 h period but was greater on ER and F+ER compared with CON and FR following the TT and the 2 h rehydration period ($P > 0.05$). Diurnal variation most likely accounts for the decrease in plasma cortisol concentration at 6 h post (17:00 h) compared with 0 h (08:30 h) on all trials.

Table 6.1: The effects of a 48 h period of fluid (FR), energy (ER) or combined fluid and energy (F+ER) restriction and a 30 min treadmill time trial on plasma free fatty acid, glucose and cortisol concentration.

		CON	FR	ER	F+ER
FFA	0 h	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.2	0.4 ± 0.2
($mmol \cdot L^{-1}$)	24 h	0.6 ± 0.2	0.5 ± 0.1	1.6 ± 0.7^{ab*}	1.7 ± 0.5^{ab*}
	48 h	0.6 ± 0.2	0.6 ± 0.2	1.8 ± 0.6^{ab*}	2.0 ± 0.7^{ab*}
	Post-TT	1.1 ± 0.3*†	1.1 ± 0.3*†	2.2 ± 0.4^{ab*}	2.4 ± 0.6^{ab*}
Glucose	0 h	5.1 ± 0.6	5.0 ± 0.6	5.3 ± 0.5	5.1 ± 0.8
($mmol \cdot L^{-1}$)	24 h	5.3 ± 0.6	5.4 ± 0.6	4.4 ± 0.7	4.4 ± 0.8
	48 h	5.4 ± 0.6	5.5 ± 0.8	4.2 ± 0.8^{ab}	4.1 ± 0.6^{ab}
	Post-TT	8.2 ± 2.3*†	7.9 ± 1.7*†	4.5 ± 1.1^{ab}	4.4 ± 0.8^{ab}
	2 h Post	5.8 ± 0.9	5.7 ± 0.8	5.5 ± 1.0	5.3 ± 0.8
	6 h Post	6.6 ± 0.9*†	6.4 ± 0.9*	7.2 ± 1.1*†	7.2 ± 1.5*†
Cortisol	0 h	494 ± 87	455 ± 59	479 ± 60	502 ± 58
($nmol \cdot L^{-1}$)	24 h	528 ± 86	502 ± 82	554 ± 89	544 ± 62
	48 h	516 ± 80	500 ± 86	559 ± 116	564 ± 76
	Post-TT	459 ± 104	448 ± 97	571 ± 148^{ab*}	556 ± 138^{ab}
	2 h Post	263 ± 61*†	284 ± 62*†	377 ± 98^{ab*†}	379 ± 123^{ab*†}
	6 h Post	246 ± 51*†	186 ± 49*†	247 ± 63*†	243 ± 93*†

Values are mean ± SD, (n = 13). a *vs.* CON, b *vs.* FR.* *vs.* 0 h, † *vs.* 48 h; $P < 0.05$. Abbreviations: CON, control; FR, fluid restriction; ER, energy restriction; F+ER, fluid and energy restriction; FFA, free fatty acid.

6.5 Discussion

These results support the hypothesis that 48 h of combined fluid and energy restriction (~11000 kJ and ~2.9 L fluid deficit each day) decrease s-IgA availability (i.e. s-IgA secretion rate). These results do not however, support the hypotheses that 48 h of either fluid or energy restriction alone decrease the availability of s-IgA. In addition, s-IgA secretion rate was not lower at any time following the TT compared with pre-TT (48 h) which suggests a limited effect of a 30 min maximal exercise bout on s-IgA availability irrespective of prior nutritional status. Saliva IgA availability remained significantly depressed on F+ER following rehydration but normalised rapidly after refeeding.

Saliva IgA concentration was significantly increased following 48 h on FR compared with all other trials which might be considered representative of an enhanced mucosal response. However, expressing s-IgA as a secretion rate indicates a 19% reduction in s-IgA availability on FR, albeit this did not reach significance ($P = 0.20$). This decrease in s-IgA secretion rate is most likely attributable to dehydration (3.2% BML) and the associated decrease in saliva flow rate (Gregersen & Bullock, 1933; Ship & Fischer, 1997). Despite similar reductions in saliva flow rate on FR (64%) and F+ER (54%) s-IgA concentration was unaltered during 48 h on F+ER, resulting in a significant 39% decrease in s-IgA secretion rate at 48 h. These results highlight the importance of expressing s-IgA as a secretion rate thereby correcting for changes in hydration status (Walsh *et al.*, 1999). Removal of concentrating and dilution effects of saliva flow rate ensures a true indication of s-IgA availability at the oral mucosa.

The reduced s-IgA availability on F+ER at 48 h is likely to be attributable to a combination of decreased saliva flow rate and impaired local synthesis and/or translocation of s-IgA;

this may render the individual more susceptible to URTI (Gleeson *et al.*, 1999; Carins & Booth, 2002). In previous athletic and occupational studies the stressor(s) responsible for the reported decrease in s-IgA availability and increased URTI remain unclear because any one of a combination of stressors (e.g. arduous exercise, psychological stress, sleep deprivation, fluid and energy restriction) may have impacted upon s-IgA availability (Gleeson *et al.*, 1999; Carins & Booth, 2002). These results are the first to demonstrate, under controlled laboratory conditions, a role for fluid and energy restriction in the reduction in s-IgA availability in active healthy individuals. The mechanism(s) by which fluid and energy restriction impairs local synthesis and/or translocation of s-IgA remains unclear.

The present results do not support a role for decreased plasma glucose (Gleeson *et al.*, 2004a) or increased plasma cortisol (Saxon *et al.*, 1978; Wira *et al.*, 1990) in the impairment of s-IgA synthesis and/or translocation as both remained unchanged during the 48 h period compared with 0 h on the energy restriction trials. The restricted protein intake at least in part may be responsible for the impaired local synthesis and/or translocation of s-IgA as protein deficiency is associated with impaired cell replication and the production of proteins (i.e. Ig and cytokines, Chandra, 1997; Gleeson *et al.*, 2004a). Specifically, cluster of differentiation four positive ($CD4^+$) helper lymphocytes and interleukin-2 (IL-2) secretion have been shown to be reduced following energy restriction; both of which are thought essential for the induction and regulation of IgA synthesis (Savendahl & Underwood, 1997; Walrand *et al.*, 2001). Although we have not reported changes in circulating IL-2, a significant decrease in circulating blood $CD4^+$ helper lymphocytes during ER and F+ER was observed (Data published elsewhere, Laing *et al.*, 2005). The present study cannot also exclude other potent modulators of the immune system which

include the autonomic nervous system, other hormones (e.g. catecholamines) and cytokines which are known to be altered during energy restriction and may potentially influence s-IgA production and translocation (Pequignot *et al.*, 1980; Proctor & Carpenter, 2002; Gleeson *et al.*, 2004a; Kaetzel, 2005).

The decrease in saliva flow rate during the 48 h period on FR and F+ER corresponds with previous reports of reductions in whole saliva flow rate after water deprivation lasting 48 to 72 h (Gregersen & Bullock, 1933) and reductions in parotid flow rate after a 24 h period without food and water (Ship & Fischer, 1997). The exact physiological mechanism(s) responsible for the reduction in saliva flow rate associated with dehydration is unclear. It had been hypothesised that an increase in plasma sodium (osmolality) during dehydration may account for the production of smaller amounts of more concentrated saliva (Ship & Fischer, 1997; Walsh *et al.*, 2004b). However, the decrease in saliva flow rate on F+ER with no concomitant change in plasma osmolality questions a direct effect of plasma osmolality in decreasing saliva flow rate (**Figure 6.2**). Alternatively, other responses to dehydration including an increase in circulating vasopressin and up-regulated renin-angiotensin-aldosterone and SNS activity (Melin *et al.*, 1997; Huch *et al.*, 1998) may cause vasoconstriction of the blood vessels to the salivary glands, thereby limiting water availability to the saliva gland and ultimately reducing saliva flow rate (Lavelle, 1988; Chicharro *et al.*, 1998; Talke *et al.*, 2003). Besides decreasing the availability of s-IgA, decreases in saliva flow rate may further impair oral health by reducing the flushing of micro-organisms and their products into the gut and by limiting the availability of several other proteins (e.g. IgM, lysozyme and α-amylase) known to have important anti-microbial properties (Tenovuo, 1998; Rantonen & Meurman, 2000; West *et al.*, 2006). Future research should attempt to elucidate the mechanisms responsible for the changes in saliva

flow rate during dehydration and the decrease in s-IgA secretion rate following fluid and energy restriction.

In contrast to the proposed hypothesis, s-IgA secretion rate was not lower at any time following the TT, which suggests a limited effect of a 30 min maximal exercise bout and prior nutritional status on s-IgA availability. These results are in agreement with previous studies reporting no change in s-IgA availability after continuous exercise lasting less than 1 h in well nourished individuals (McDowell *et al.*, 1991). It remains unclear whether a similar dietary restriction affects the s-IgA response to exercise lasting longer than 30 min. The electrolyte solution provided during rehydration (0 - 2 h post-TT) returned s-IgA secretion rate to prior intervention values on ER but not on F+ER as secretion rate remained lower than 0 h at this time. Refeeding approximately two-thirds of individual daily energy requirements (8164 ± 557 kJ) normalised s-IgA availability on F+ER by 6 h post-TT. Although refeeding following fluid and energy restriction has been shown to be effective in rapidly restoring blood parameters of the immune system (Walrand *et al.*, 2001) the present investigation is the first to highlight the importance of refeeding to restore s-IgA availability.

In conclusion, a 24 to 48 h period of combined fluid and energy restriction decreased s-IgA availability but normalisation occurred rapidly with rehydration and refeeding. In addition, nutrient restriction did not alter s-IgA secretion rate responses to a 30 min maximal exercise bout.

CHAPTER SEVEN

Endurance Running Performance after 48 Hours of Restricted Fluid and/or Energy Intake

7.1 Summary: The purpose of this study was to determine the effect of a 48 h period of either fluid, energy or fluid and energy restriction on 30 min self-paced treadmill TT performance in temperate conditions. Thirteen males participated in four randomised 48 h trials. On the control trial (CON) participants received their estimated energy (12154 ± 833 $kJ \cdot d^{-1}$, mean ± SD) and water (3912 ± 500 $mL \cdot d^{-1}$) requirements. On fluid restriction (FR) participants received their energy requirements and 193 ± 50 $mL \cdot d^{-1}$ water to drink and on energy restriction (ER) participants received their water requirements and 1214 ± 84 $kJ \cdot d^{-1}$. Fluid and energy restriction (F+ER) was a combination of FR and ER. After 48 hours participants performed a 30 min self-paced treadmill TT in temperate conditions (19.7 ± 0.6°C). A separate investigation in participants with similar characteristics and running experience (n =10) showed the TT to be highly reproducible (CV 1.6%). Body mass loss (BML) at 48 h was 0.6 ± 0.4% (CON); 3.2 ± 0.5% (FR); 3.4 ± 0.3% (ER) and 3.6 ± 0.3% (F+ER). Compared with CON (6295 ± 513 m) less distance was completed on ER (10.3 ± 5.2%) and F+ER (15.0 ± 8.4%, $P < 0.01$). Although less distance was completed on FR (2.8 ± 4.2%) this was not significantly different from CON. In conclusion, these results show a detrimental effect of a 48 h period of energy restriction but no significant effect of fluid restriction alone on 30 min self-paced treadmill TT performance in temperate conditions. Therefore, these results do not support the popular contention that modest hypohydration (2-3% BML) significantly impairs endurance performance in a temperate environment.

7.2 Introduction

It is commonly believed that modest hypohydration equal to 2 to 3% BML has a detrimental effect on endurance performance (Sawka, 1992; Cheuvront *et al.*, 2003). However, the research investigating the effects of modest levels of hypohydration on endurance performance in temperate conditions remains equivocal. One widely cited paper reported significant 7% increases in time to complete 5000 and 10000 m track races when athletes were hypohydrated to approximately 2% BML using the diuretic Furosemide (Armstrong *et al.*, 1985). Another study shows that a 3% BML evoked by heat exposure decreased work completed on a cycle ergometer by 8% in a 30 min period (Cheuvront *et al.*, 2005). In contrast, similar BML evoked using a combination of exercise and fluid restriction had no significant effect on work completed on a cycle ergometer in a 15 min period (McConell *et al.*, 1999) or distance completed on a treadmill in a 30 min period (Daries *et al.*, 2000).

The different methods used to evoke hypohydration and distribution of losses from different fluid compartments may account for the equivocal findings regarding the effects of modest hypohydration on endurance performance (Cheuvront *et al.*, 2003). In line with this, endurance performance was compromised when hypohydration was evoked by diuretic administration (Armstrong *et al.*, 1985) and heat exposure (Cheuvront *et al.*, 2005) but not when similar BML was achieved using a combination of exercise and fluid restriction (McConell *et al.*, 1999; Daries *et al.*, 2000). From a practical perspective, an advantage of the latter two studies (McConell *et al.*, 1999; Daries *et al.*, 2000) is that a combination of exercise and fluid restriction represents the type of dehydration that commonly occurs in military personnel and athletes performing in temperate conditions. Unfortunately, all of the aforementioned studies induced hypohydration over a short time

period (≤5 h). Therefore, little information is available about the effects of more prolonged dehydration, similar to that encountered in many occupational and athletic settings, on endurance performance.

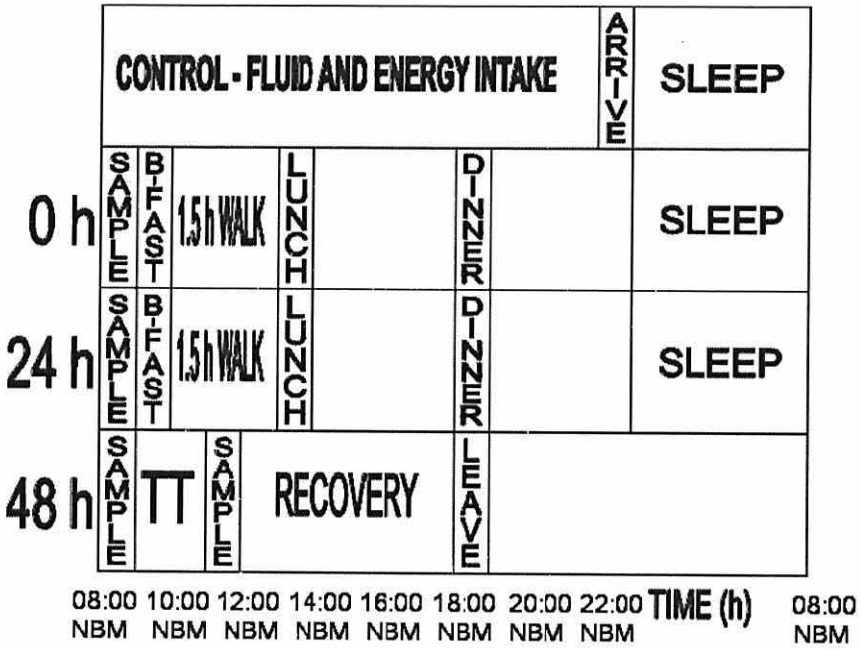
Energy restriction lasting 23 to 36 h is widely reported to decrease TTE at intensities ranging from 50 to 100% $\dot{V}O_{2\,max}$ (Nieman *et al.*, 1987; Gleeson *et al.*, 1988; Maughan & Gleeson, 1988; Zinker *et al.*, 1990). However, TTE protocols have been criticised for having poor test-retest reliability with mean CV ranging from 20 to 27% (Krebs & Powers, 1989; Jeukendrup *et al.*, 1996) compared with mean CV of less than 5% for TT protocols (Jeukendrup *et al.*, 1996; Schabort *et al.*, 1998). Variability of this magnitude causes difficulty when attempting to identify the proportion of change in individual performance attributable to the intervention and not to measurement error.

Although numerous investigations have examined the effects of energy restriction on TTE, the independent and combined effects of fluid and energy restriction on endurance performance assessed using a TT have not been examined. Therefore, the aim of the present study was to examine the independent and combined effects of prolonged (48 h) fluid and energy restriction on 30 min treadmill TT performance in temperate conditions. It was hypothesised that fluid or energy restriction would decrease TT performance and that the effects of combined fluid and energy restriction would be additive.

7.3 Methods

For details on *participants*, *preliminary measurements*, *experimental trials* and *procedures* see **Chapter 5**. A summary of the trial events is provided in **Figure 7.1**. For *analytical methods* see **Chapter 3.** In addition to previously described *preliminary measurements* all participants performed a 30 min self-paced treadmill TT familiarisation. In addition to *experimental trial* and *procedures* and for the reader's clarity the following TT description is provided. Prior to completing the TT participants performed a 5 min warm-up at 9 $km \cdot h^{-1}$. Each TT was performed under standardised conditions (19.7 ± 0.6°C, 59 ± 7% RH) in a quiet laboratory with only information about elapsed time provided. Participants were instructed to "run as far as possible in 30 minutes" and to control the speed of the treadmill (gradient set at 1%) as and when they felt appropriate. No fluids were consumed during the TT. A fan was placed in front of the treadmill during each TT with the wind speed set at 2.0 $m \cdot s^{-1}$. During the TT, T_{re} (*YSI 4000A, Daytona, USA*) and HR were measured continuously whilst ratings of perceived exertion (RPE, Borg, 1982) were obtained at 5 min intervals. Heat storage rate ($°C \cdot km^{-1}$) was estimated by dividing the change in T_{re} by distance covered. Total distance was recorded and participants were provided with this information on completion of the study. A separate investigation (n =10, **Appendix G**) showed the TT to be highly reproducible. Allowing for a familiarisation trial, the standard error of the measurement and CV between trials 1-2 and 2-3 were 106 and 114 m and 1.5 and 1.6%, respectively. A further urine sample, blood sample and NBM recording was obtained immediately post-TT. Participants then completed a 6 h rehydration and refeeding period before leaving the laboratory (for details see **Chapter 6, Section 3**).

Figure 7.1: Schematic of trial events.



Abbreviations: NBM, nude body mass; TT, time trial.

Statistical analysis. A one-way repeated measure ANOVA with *post hoc* Bonferroni corrected *t*-tests was used to determine the effects of dietary restriction on TT performance. In addition, the number of participants whose performance on FR, ER and F+ER decreased or increased by more than two standard error of measurement *vs*. CON was also recorded. Two-way fully repeated ANOVA's were performed on physiological indices, RPE, urine specific gravity, plasma volume change and metabolite concentrations.

7.4 Results

7.4.1 Body mass loss, urine volume and plasma volume change

At 48 h, prior to beginning the TT, BML was 0.6 ± 0.4% (CON); 3.2 ± 0.5% (FR); 3.4 ± 0.3% (ER) and 3.6 ± 0.3% (F+ER, **Figure 5.2**). Urine volume was lower on FR and F+ER (1597 ± 174 and 1196 ± 141 mL, respectively) and higher on ER (6457 ± 747 mL) compared with CON (4804 ± 77 mL, $P < 0.01$) over the 48 h period. At 48 h, plasma volume was unaltered on CON and FR (-1.4 ± 5.7 and -0.9 ± 4.9%, $P > 0.05$) and decreased on ER and F+ER (-5.2 ± 3.2 and -5.1 ± 4.5%, $P < 0.05$). Compared with 48 h, plasma volume decreased as a result of the TT although this only reached significance on FR (CON, -5.8 ± 3.9; FR, -6.3 ± 3.5 ($P < 0.05$); ER, -4.9 ± 4.6; F+ER, -4.8 ± 3.7%).

7.4.2 Time trial performance

There was a significant difference for distance completed, where less distance was covered on ER and F+ER compared with CON and FR ($P < 0.01$, **Table 7.1**). Although less distance was covered on FR compared with CON and on F+ER compared with ER these differences were not significant. The mean percentage change in distance completed compared with CON was larger on ER (-10.3%, 95CI, -7.2 to -13.4%) and F+ER (-15.0%, 95CI, -10.0 to -20.1%) than on FR (-2.8%, 95CI, -0.3 to -5.3%). The addition of 95% confidence limits to the mean identifies the likely range of the true differences between the restrictions and CON. Individual assessment revealed all 13 participants on ER and F+ER completed less distance compared with CON. Additionally, compared with CON the differences were greater than two standard error of the measurement in 11 of 13 participants on ER and in all 13 participants on F+ER. On FR, 9 of 13 participants covered less distance compared with CON. The decrease in distance completed on FR compared

with CON was greater than two standard error of the measurement in only 7 of 13 participants.

Table 7.1: The effects of a 48 h period of fluid (FR), energy (ER) or combined fluid and energy (F+ER) restriction on 30 min treadmill time trial performance.

Participant No.	CON	FR	ER	F+ER
1	6351	5770	5366	4903
2	6302	6389	6054	5843
3	7220	6667	6233	5967
4	6245	5990	5252	5002
5	6393	6146	5101	4624
6	6299	6219	5723	4334
7	6843	6733	6661	6202
8	5489	5530	4973	4701
9	6471	5963	5888	5577
10	5968	6205	5300	5277
11	5382	5525	4906	5099
12	6029	5718	5814	5793
13	6842	6538	6124	6089
Mean	6295	6107	5646[ab]	5339[ab]
SD	513	405	541	613

Values are distance completed (m). Abbreviations: CON, control; FR, fluid restriction; ER, energy restriction; F+ER, fluid and energy restriction. a *vs.* CON, b *vs.* FR; $P < 0.01$.

7.4.3 Thermoregulatory and cardiovascular responses to the time trial

Heart rate, RPE and T_{re} increased throughout the TT ($P < 0.05$); however, HR and RPE were not different between the four trials. There was a significant interaction ($P < 0.01$) where higher peak T_{re} was found on FR compared with CON and lower peak T_{re} was found on F+ER compared with CON ($P < 0.05$, **Table 7.2**). Peak T_{re} for ER and F+ER was also significantly lower than FR ($P < 0.05$). Heat storage was significantly greater on FR ($0.37°C \cdot km^{-1}$) compared with CON, ER and F+ER ($0.32°C \cdot km^{-1}$, $P < 0.01$). NBM change (sweat loss) during the TT was greater for CON compared with FR, ER and F+ER ($P < 0.01$).

Table 7.2: Final exercising T_{re}, HR, RPE and nude body mass change (NBMΔ) after a 30 min treadmill time trial following a 48 h period of fluid (FR), energy (ER) or combined fluid and energy (F+ER) restriction.

	CON	FR	ER	F+ER
T_{re} (°C)	39.10 ± 0.26	39.33 ± 0.35[a]	38.91 ± 0.36[b]	38.84 ± 0.25[ab]
HR ($b \cdot min^{-1}$)	194 ± 9	197 ± 7	192 ± 12	191 ± 14
RPE	18.6 ± 0.8	18.8 ± 1.0	18.4 ± 1.0	18.4 ± 0.9
NBMΔ (%)	1.2 ± 0.3	0.9 ± 0.3[a]	0.9 ± 0.2[a]	0.8 ± 0.1[a]

Values are mean ± SD, a *vs.* CON, b *vs.* FR; $P < 0.05$. Abbreviations: CON, control; FR, fluid restriction; ER, energy restriction; F+ER, fluid and energy restriction; NBMΔ, nude body mass change.

7.4.4 Plasma free fatty acid, glucose and lactate responses

Plasma FFA concentration increased on energy restriction trials by 24 h (ER and F+ER, $P > 0.05$, **Table 7.3**) and was significantly greater than CON and FR from this point onwards ($P < 0.05$). Plasma FFA concentration did not alter significantly during the 48 h period on CON and FR. Plasma glucose concentration decreased on ER by 24 h and F+ER by 48 h ($P < 0.05$) and was significantly lower than CON from this point onwards on ER and F+ER ($P < 0.05$). Plasma glucose concentration did not alter significantly during the 48 h on CON and FR. Plasma lactate concentration did not alter significantly during the 48 h intervention on any trial. Plasma FFA, glucose and lactate concentration increased at post-TT compared with 0 h on all trials ($P < 0.05$). The TT evoked increases in plasma FFA and glucose concentration compared with 48 h on CON and FR ($P < 0.05$). Compared with 48 h, plasma FFA and glucose concentration did not alter significantly following the TT on ER and F+ER. The TT evoked increases in plasma lactate concentration compared with 48 h on all trials ($P < 0.05$) although plasma lactate was significantly lower on energy restriction trials (ER and F+ER) at this time ($P < 0.05$).

Table 7.3: The effects of a 48 h period of fluid (FR), energy (ER) or combined fluid and energy (F+ER) restriction and a 30 min treadmill time trial on plasma free fatty acid, glucose and lactate concentration.

		CON	FR	ER	F+ER
FFA	0 h	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.2	0.4 ± 0.2
($mmol \cdot L^{-1}$)	24 h	0.6 ± 0.2	0.5 ± 0.1	1.6 ± 0.7^{ab*}	1.7 ± 0.5^{ab*}
	48 h	0.6 ± 0.2	0.6 ± 0.2	1.8 ± 0.6^{ab*}	2.0 ± 0.7^{ab*}
	Post-TT	1.1 ± 0.3$^{*\#}$	1.1 ± 0.3$^{*\#}$	2.2 ± 0.4^{ab*}	2.4 ± 0.6^{ab*}
Glucose	0 h	5.1 ± 0.6	5.0 ± 0.6	5.3 ± 0.5	5.1 ± 0.8
($mmol \cdot L^{-1}$)	24 h	5.3 ± 0.6	5.4 ± 0.6	4.4 ± 0.7^{b*}	4.4 ± 0.8^{b}
	48 h	5.4 ± 0.6	5.5 ± 0.8	4.2 ± 0.8^{ab*}	4.1 ± 0.6^{ab*}
	Post-TT	8.2 ± 2.3$^{*\#}$	7.9 ± 1.7$^{*\#}$	4.5 ± 1.1^{ab*}	4.4 ± 0.8^{ab*}
Lactate	0 h	1.0 ± 0.4	1.1 ± 0.4	1.1 ± 0.3	1.0 ± 0.4
($mmol \cdot L^{-1}$)	24 h	1.1 ± 0.5	0.9 ± 0.4	1.2 ± 0.4	1.3 ± 0.6
	48 h	1.2 ± 0.3	1.2 ± 0.5	1.3 ± 0.3	1.4 ± 0.4
	Post-TT	10.3 ± 2.7$^{*\#}$	9.2 ± 2.8$^{*\#}$	8.4 ± 2.6$^{a*\#}$	7.4 ± 2.3$^{ab*\#}$

Values are mean ± SD. a *vs.* CON, b *vs.* FR.* *vs.* 0 h, # *vs.* 48 h; $P < 0.05$. Abbreviations: CON, control; FR, fluid restriction; ER, energy restriction; F+ER, fluid and energy restriction; FFA, free fatty acid.

7.5 Discussion

These results show a detrimental effect of a 48 h period of energy restriction (~11000 kJ deficit each day compared with CON) but no significant effect of fluid restriction alone (~2.9 L fluid deficit each day compared with CON) on 30 min self-paced treadmill TT performance in temperate conditions. In addition, TT performance was similar after a 48 h period of energy restriction and after combined energy and fluid restriction. Therefore, these results do not support the popular contention that modest hypohydration (2-3% BML) significantly decreases endurance performance in temperate conditions.

To our knowledge this is the first study to investigate the effects of a prolonged period of fluid restriction on endurance performance. The 48 h period of fluid restriction in this study evoked similar BML (3.2%) to heat exposure (3%, Cheuvront *et al.*, 2005) and larger BML than the diuretic Furosemide (~2%, Armstrong *et al.*, 1985) in studies reporting an approximate 8% decrease in endurance performance after acute dehydration (≤ 5 h). However, prolonged fluid restriction in this study did not significantly affect endurance performance (-2.8% *vs.* CON). The findings of the present study do agree with others showing no significant decrease in TT performance when approximately 2% BML was evoked by a combination of acute exercise and fluid restriction lasting less than 2 h (McConell *et al.*, 1999; Daries *et al.*, 2000). The contradictory findings maybe attributable to the different method of dehydration (e.g. diuretics, exercise and sauna, Cheuvront *et al.*, 2003) or duration of dehydration (acute *vs.* prolonged) used in previous studies; it is conceivable that these factors might alter the distribution of fluid losses from different fluid compartments. For example, compared with a control condition, peak power output was better preserved during an incremental cycle test to exhaustion (albeit lasting only 10-12 min) when approximately 4% BML was evoked by exercise with fluid and energy

restriction (-7W) than when approximately 4% BML was evoked by diuretic (-21W) or sauna exposure (-23W, Caldwell *et al.*, 1984).

Impaired endurance performance following a period of dehydration is commonly attributed to a combination of increased cardiovascular and thermoregulatory strain (Sawka, 1992; Cheuvront *et al.*, 2003). Typically, dehydration lowers blood volume (hypovolaemia) which subsequently causes a reduction in cardiac output via reduced SV (Sawka, 1992). In addition, hypovolaemia may decrease heat dissipation via reduced cutaneous blood flow (dry heat loss) and possibly also via reduced sweat rates (evaporative heat loss) which may further contribute to impaired exercise performance (Sawka, 1992). In line with this, exercise performance (5000 and 10000 m track race) decreased when sizeable reductions in plasma volume (10-12%) occurred after diuretic treatment 5 h before exercise (Armstrong *et al.*, 1985) but was better maintained when plasma volume did not alter significantly (<1%) after similar BML was evoked using a combination of exercise with fluid and food restriction over a 48 h period (Caldwell *et al.*, 1984). The present results and those previously (Caldwell *et al.*, 1984) indicate that, in contrast to acute dehydration (≤5 h), a more prolonged period of dehydration (48 h) evoked by bouts of exercise and restricted water intake does not cause hypovolaemia. However, this contention should be verified using tracer techniques (e.g. Evans Blue dye dilution) to measure blood volume as opposed to estimating changes using haemoglobin and haematocrit. Nevertheless, it might be tentatively suggested that the unaltered plasma volume (<1%) may be responsible for the non-significant effect of hypohydration on endurance performance in the present study.

Using diuretics or heat exposure to evoke similar BML to the present study impairs exercise performance (Caldwell *et al.*, 1984; Armstrong *et al.*, 1985) and possibly raises

the risk of heat illness (Claremont *et al.*, 1976; Casa *et al.*, 2005a). Although a significant decrease in 30 min TT performance was not observed on FR, the raised core temperature (+0.2°C *vs.* CON) may increase the likelihood of impaired performance and heat illness (Casa *et al.*, 2005a) when performing more prolonged exercise and/or performing in the heat; as is often the case in athletic and occupational settings. The lower sweat rate during exercise after fluid restriction (and similar work rate to control) presumably accounts for the increased final exercising core temperature compared with control (Sawka *et al.*, 1985; Buono & Wall, 2000). The mechanism(s) responsible for reduced exercising sweat rate during hypohydration include a possible role for hypovolaemia and a more likely role for hypertonicity; the latter via osmosensitive neurons in the hypothalamus or a possible effect of high interstitial osmotic pressure inhibiting fluid availability to the sweat glands (Senay, Jr., 1968; Sawka, 1992). These results, and others (Caldwell *et al.*, 1984) showing no change in plasma volume (albeit estimated) during prolonged dehydration, alongside a reported increase in serum osmolality during prolonged fluid restriction (Shirreffs *et al.*, 2004) support a more likely role for hypertonicity in the reduced exercising sweat rate after prolonged dehydration.

Energy restriction equal to approximately 11000 $kJ \cdot d^{-1}$ for greater than 24 h caused a significant increase in plasma FFA and decrease in plasma glucose similar to those reported during a 36 h fast where TTE at 70% $\dot{V}O_{2\,max}$ decreased by 36% (Maughan & Gleeson, 1988). Indeed, TTE at intensities ranging from 50 to 100% $\dot{V}O_{2\,max}$ is widely reported to decrease after energy restriction lasting 23 to 36 h (Nieman *et al.*, 1987; Gleeson *et al.*, 1988; Maughan & Gleeson, 1988; Zinker *et al.*, 1990). Somewhat surprisingly, conflicting evidence exists about the most likely causes of decreased TTE with short-term energy restriction; these include a likely role for muscle glycogen

depletion given that muscle glycogen is the primary fuel source for the first 30 min during moderate to high intensity exercise (60-80% $\dot{V}O_{2\,max}$, Romijn *et al.*, 1993). However, decreased liver glycogen, altered acid-base balance or a role for central fatigue should not be discounted as possible causes of decreased exercise performance after energy restriction (Gleeson *et al.*, 1988; Maughan & Gleeson, 1988; Zinker *et al.*, 1990; Aragon-Vargas, 1993). This is the first investigation to assess the effects of a 48 h period of energy restriction on endurance performance using a TT. The 10% decrease in endurance performance on ER in the present study is relatively modest compared with 36% (Maughan & Gleeson, 1988) and 44% (Nieman *et al.*, 1987) decreases in TTE in studies using similar energy deficits and shorter restrictions (27-36 h). The larger decrement in endurance in previous energy restriction studies may be attributable to the lower intensity and longer duration of exercise used in their TTE protocols (mean TTE ranged 77-160 min). The relatively modest effect of energy restriction on work rate is reflected in lowered final exercising HR and T_{re} albeit these responses were not significantly different from control. Nevertheless, these results highlight the importance of adequate energy intake and the potential for impaired endurance performance in athletes and military personnel during relatively short periods of energy restriction (e.g. during an intentional weight loss regime or military operation).

These results also show that athletes who choose to ignore the ACSM's advice (Oppliger *et al.*, 1996) and practice weight loss by restricting both fluids and energy simultaneously may be mistaken to think they will evoke much larger BML compared with restricting fluids alone. Although significant, BML was only on average 0.4% greater after restricting fluids and energy simultaneously (mean BML, 3.6%) compared with restricting fluids alone (mean BML, 3.2%). This is particularly important when considering that endurance

performance was impaired significantly after the energy restriction trials but not after FR. We might have logically expected BML on combined fluid and energy restriction to be the sum of BML on fluid restriction alone and energy restriction alone. However, when fluid and energy restrictions were combined (fluid intake, 0.9 $L \cdot d^{-1}$ on F+ER *vs.* 3.9 $L \cdot d^{-1}$ on ER) we did not observe the large BML component due to urine output that was evident on ER (48 h urine volume, 1.2 L on F+ER and 6.4 L on ER). This provides an explanation for why BML on F+ER (3.6%) was not the sum of BML on FR (3.2%) plus the BML associated with the large urine output on ER (3.4%). A negative sodium balance caused by sweat sodium losses and the restricted dietary sodium intake (**Table 5.1**) most likely accounts for the increased urine output on ER *vs.* CON (Robinson *et al.*, 1956; Vokes, 1987).

In conclusion, these results show a detrimental effect of a 48 h period of energy restriction (~11000 kJ deficit each day) but no significant effect of fluid restriction alone (~2.9 L fluid deficit each day) on 30 min self-paced treadmill TT performance in temperate conditions. In addition, TT performance was similar after a 48 h period of energy restriction and after combined energy and fluid restriction. Therefore, these results do not support the popular contention that modest hypohydration alone (2-3% BML) significantly decreases endurance performance in temperate conditions.

CHAPTER EIGHT

General Discussion

8.1 Background

Fluid and/or energy deficits frequently occur in occupational and athletic settings (e.g. military recruits on field exercise, in twice daily training athletes, and athletes making weight for competition). Causes include, but are not limited to, water deprivation, restricted access to fluid and food, and exercise induced sweat loss. Modest hypohydration (2-3% BML) is associated with compromised heat dissipation (possibly raising the risk of EHI), cardiovascular function, endurance exercise performance and cognitive-motor function. Fluid and/or energy deficits may also be responsible for decreases in s-IgA availability and increased incidence of URTI reported during heavy training. Although a number of investigations have examined the effects of energy restriction on TTE, the independent and combined effects of fluid and energy restriction on endurance performance assessed using a TT is unclear. In particular, the effect of modest hypohydration evoked over a period greater than five hours on endurance performance is unknown. The broad objectives of the thesis were therefore to investigate: 1. the validity of haematological, urinary and novel salivary hydration markers during modest dehydration evoked by acute exercise in the heat and during a 48 h period of fluid and/or energy restriction; 2. the effect of a 48 h period of fluid, energy or combined fluid and energy restriction on s-IgA responses at rest and after exercise in active individuals; and 3. the independent and combined effects of fluid and energy restriction lasting 48 h on 30 min self-paced treadmill TT performance in temperate conditions.

8.2 Summary of main findings

Firstly, saliva flow rate, osmolality and total protein concentration were shown to progressively track dehydration, evoked by acute exercise and heat stress, and correlate strongly with plasma osmolality. Saliva osmolality and total protein concentration were shown to identify modest hypohydration as well as plasma and urine osmolality. These results also supported the hypothesised role of plasma sodium in the control of saliva flow rate. That is increases in plasma sodium concentration during hypertonic hypovolaemia may reduce the osmotic drive of water into the acinar lumen of the saliva gland resulting in a decrease in saliva volume (**Chapter 4**). Saliva flow rate, saliva osmolality, plasma osmolality, urine osmolality and urine colour identified modest hypohydration evoked by 48 h period of fluid restriction. Saliva and urine markers also identified modest hypohydration evoked by 48 h of combined fluid and energy restriction. In contrast, plasma osmolality remained unaltered and failed to identify hypohydration during the 48 h period of fluid and energy restriction; thereby questioning the hypothesised role of plasma sodium in the control of saliva flow rate (**Chapter 5**). Saliva-IgA secretion rate decreased following 48 h of combined fluid and energy restriction (~11000 kJ and ~2.9 L fluid deficit each day compared with control trial intake) but was not significantly decreased by fluid or energy restriction alone. In addition, s-IgA secretion rate remained unaltered following a 30 min treadmill TT irrespective of the prior 48 h period of fluid and/or energy restriction. Furthermore, following fluid and energy restriction s-IgA was shown to be normalised after the 6 h rehydration and refeeding period (**Chapter 6**). Endurance performance, measured by a 30 min treadmill TT, was significantly decreased following 48 h of restricted energy and combined fluid and energy restriction but not after 48 h of fluid restriction; questioning the negative effect of modest hypohydration (3.2% BML) on endurance performance in a temperate environment (**Chapter 7**).

8.3 Hydration markers

The development of a hydration assessment technique requires the marker to be valid to track changes in hydration evoked by common causes of water deficits including, water deprivation, restricted access to fluid and food, heat exposure and exercise induced sweat loss with inadequate fluid replacement. The marker should also be sensitive to identify water deficits equal to approximately 2 to 3% BML as it is these modest deficits that are associated with compromised heat dissipation (possibly raising the risk of EHI), cardiovascular function, endurance exercise performance (>1 h) and cognitive-motor function (Gopinathan *et al.*, 1988; Sawka, 1992; Cheuvront *et al.*, 2003; Casa *et al.*, 2005a). Moreover, to be of practical use, hydration markers should be able to reliably identify euhydration and modest dehydration in an individual with a single measure.

In **Chapter 4**, saliva flow rate, saliva osmolality and saliva total protein concentration were shown to track changes in acute dehydration (1-3 h). These findings confirmed an earlier investigation that identified saliva parameters as potential novel markers of hydration status (Walsh *et al.*, 2004b). Importantly these previous findings were developed further in **Chapter 4** by the simultaneous assessment of plasma and saliva hydration markers showing saliva flow rate, osmolality and total protein concentration to be strongly correlated ($r \geq 0.78$) with plasma osmolality, a widely accepted hydration index of acute dehydration (Sawka *et al.*, 1985; Popowski *et al.*, 2001). Furthermore, as plasma and urine osmolality, saliva osmolality and total protein concentration were shown to identify dehydration equal to approximately 2% BML. In contrast to previous findings plasma osmolality failed to identify dehydration equal to 1% BML (Popowski *et al.*, 2001). **Chapter 4** findings suggest plasma osmolality is no more sensitive to acute hypertonic hypovolaemia than urine osmolality, saliva osmolality or saliva total protein concentration.

Plasma, urine and saliva markers require basic biochemical analysis and are unable to reliably quantify the magnitude of fluid loss; therefore, in an acute setting they provide no practical advantage over measures of NBM change. Subject to validation, plasma, urine and saliva hydration markers might be more suitable to indicate changes in hydration over a more prolonged period when NBM changes may not reflect a true change in hydration status because of unaccountable changes in food intake, bowel movements and body weight changes associated with substrate metabolism or changes in body composition. However, unlike NBM change, plasma, urine and saliva hydration indices may also be able to identify an individual's hydration status with a single measure.

Chapter 5 examined the validity of plasma, urine and saliva hydration markers to chronic dehydration evoked by 48 h of fluid and/or energy restriction. Plasma, urine and saliva indices identified hypohydration during the 48 h period of fluid restriction alone suggesting the increased duration of the dehydration period had a limited effect on the validity of these hydration markers. Plasma and urine osmolality identified hypohydration equal to approximately 2% BML which is consistent with the sensitivity of these indices reported in **Chapter 4** and by previous investigations where dehydration was evoked by combined acute exercise and heat stress (Armstrong *et al.*, 1994; Armstrong *et al.*, 1998; Popowski *et al.*, 2001).

In contrast to one previous prolonged fluid restriction study (Shirreffs *et al.*, 2004), where plasma osmolality reached a plateau by 24 h, plasma osmolality increased incrementally during the 48 h period of fluid restriction and identified the additional BML (~1%) following the TT. In comparison, urine osmolality failed to identify further significant increases in BML at 48 h and post-TT which is in agreement with the findings of Shirreffs

and colleagues (2004). Saliva flow rate and osmolality identified hypohydration equal to approximately 3% BML which compares favourably with the sensitivity reported in **Chapter 4** and in one previous investigation where dehydration was evoked by a combination of acute exercise and heat exposure (Walsh *et al.*, 2004b). Saliva osmolality may be considered more sensitive than saliva flow rate as it detected further dehydration following the TT. Plasma, urine and saliva osmolality identified hypohydration evoked by fluid restriction alone with sufficient validity to fulfil the criterion of a hydration marker (Shirreffs, 2000).

Chapter 5 also demonstrates that urine and saliva osmolality identify modest hypohydration during 48 h of combined fluid and energy restriction. The decrease in whole saliva flow rate on F+ER concurs with a decrease in parotid saliva flow rate reported previously after a 24 h period of fasting (Ship & Fischer, 1997). In contrast, plasma osmolality, often referred to as a gold-standard marker of hydration (Popowski *et al.*, 2001; Casa *et al.*, 2005b), did not detect hypohydration when fluid and energy were restricted simultaneously. The inadequate sodium intake combined with sweat sodium loss most probably accounts for the unaltered plasma osmolality on both F+ER and ER (Robinson *et al.*, 1956; Consolazio *et al.*, 1968). It is widely acknowledged that much of the BML observed during the first 72 h of energy restriction is attributable to hypohydration (Consolazio *et al.*, 1967; Consolazio *et al.*, 1968), probably as a result of negative sodium balance (Robinson *et al.*, 1956). Indeed, urine output and BML were much greater after 48 h on ER compared with CON. As such, we might have expected plasma, urine and saliva osmolality to indicate hypohydration on ER alone; however, this was not the case. These results highlight the importance of adequate dietary intake when monitoring hydration using these indices, particularly when using plasma osmolality. By manipulating salt intake

and sweat loss future research should confirm the important role of adequate sodium intake for the validity of plasma osmolality as a hydration marker.

With these results in mind, it might be speculated that an energy deficit could be responsible, at least in part, for the poor relationship between changes in hydration status and haematological and urinary indices previously reported in field studies lasting between 7 and 44 days in active individuals (Francesconi *et al.*, 1987; Hackney *et al.*, 1995; O'Brien *et al.*, 1996). In addition, as wrestlers frequently restrict fluid and energy intake to make weight the findings of **Chapter 5** support the validity of the current hydration testing (i.e. a urine specific gravity ≤ 1.020 g·mL^{-1}) employed by the National Collegiate Athletic Association (NCAA) and National Federation of State High School Associations (NFSHSA) to ensure wrestlers are not dehydrated prior to establishing their minimum weight for competition. However, given the findings of ER in **Chapter 5,** the NCAA and NFSHSA should be aware that large hypotonic fluid intakes during energy restriction may invalidate urine markers; that is, a urine specific gravity of less than 1.020 g·mL^{-1} despite hypohydration. It is therefore recommended that along with urine markers metabolic indices are obtained to ensure wrestlers are not energy restricted prior to minimum weight testing (e.g. blood glucose, FFA, β-hydroxybutyrate and acetoacetate).

Ultimately to be considered practically useful a hydration marker should be able to identify an individual's hydration status or at least identify euhydration and modest hypohydration in an individual. Using the upper limit of recommended ACSM euhydration ranges of 290 and 700 mOsmol·kg^{-1} for plasma and urine osmolality, respectively (Casa *et al.*, 2005b) it is possible that these hydration markers could be used to identify an individual as either

euhydrated or hypohydrated. In addition, a value of <61 mOsmol·kg^{-1} for saliva osmolality has been suggested to indicate euhydration (**Chapters 4 & 5**).

Using a combination of euhydrated baseline data from **Chapters 4 and 5** the number of individuals correctly identified as euhydrated by the currently proposed thresholds were calculated (**Table 8.1**). Likewise, using the pooled samples, indicative of modest hypohydration, obtained at approximately 3% BML on the NFI (**Chapter 4**) and FR trials (**Chapter 5**) the number of individuals correctly identified as hypohydrated by these thresholds was also calculated (**Table 8.1**). Encouragingly, the current recommended and proposed urine and saliva thresholds were able to correctly identify greater than 75% of individuals as either euhydrated or hypohydrated.

The 290 mOsmol·kg^{-1} plasma osmolality threshold correctly identified all individuals as hypohydrated following dehydration evoking fluid losses equal to approximately 3% BML. However, the threshold also incorrectly identified 61% of individuals as hypohydrated at baseline prior to the dehydration protocols in **Chapters 4 and 5**. To ensure euhydration, participants consumed 35 to 40 mL·kg^{-1} BM of water in the 24 h prior to each trial; incidentally, this is a greater amount of water than has previously been prescribed to ensure euhydration (Armstrong *et al.*, 1994; Popowski *et al.*, 2001). Furthermore, a similar daily water intake (42 mL·kg^{-1} of body mass) during the 48 h on CON in **Chapter 5** ensured mean plasma, urine and saliva osmolality remained unchanged and BML varied by less than 0.6% which compares favourably with typical daily variations in body mass in euhydrated individuals (Cheuvront *et al.*, 2004). As such, the findings of **Chapters 4 and 5** do not support using plasma osmolality of 290 mOsmol·kg^{-1} as an upper threshold for euhydration.

In fact, increasing the threshold to 295 mOsmol·kg^{-1} increases the number of individuals correctly identified as euhydrated at baseline from 39 to 83% whilst all individuals continue to be correctly identified as hypohydrated following fluid losses equal to 3% BML. In light of this it might be suggested the current ACSM recommended threshold might be amended. In line with this suggestion, the average euhydrated plasma osmolality reported by investigations is 288 mOsmol·kg^{-1} (**Table 2.2**) which is at the upper end of the recommended range for euhydration 280 to 290 mOsmol·kg^{-1} (Casa *et al.*, 2005b). Clearly, a number of individuals in these studies would have also exceeded the 290 mOsmol·kg^{-1} and been incorrectly classified as hypohydrated in spite of individuals following rigorous prior hydration protocols.

Table 8.1: Number of individuals correctly identified as euhydrated and hypohydrated using current recommended or proposed euhydration/hypohydration thresholds.

Hydration marker	Plasma osmolality (mOsmol·kg^{-1})	Urine osmolality (mOsmol·kg^{-1})	Saliva osmolality (mOsmol·kg^{-1})
Euhydration threshold	<290	<700	<61
% individuals correctly identified as euhydrated at baseline	39% (31/80)	95% (71/75)	80% (64/80)
Hypohydration threshold	≥290	≥700	≥61
% individuals correctly identified as hypohydrated at ~3% BML	100% (28/28)	88% (22/25)	75% (21/28)

Abbreviations = BML, body mass loss. Data are pooled from **Chapters 4 and 5**. For euhydration the baseline and 0 h samples were combined. For dehydration the ~3% BML samples evoked by acute exercise and heat stress (NFI trial) and 48 h of fluid restriction (FR trial) were combined.

In addition to assessing the validity of saliva flow rate and osmolality hydration markers a number of smaller investigations presented in **Appendices E and F** examined aspects of saliva markers' feasibility. Saliva flow rate and osmolality were shown to be unaffected immediately or 15 min after consuming either water or a carbohydrate-electrolyte solution (6% carbohydrate, 25 $mmol \cdot L^{-1}$ sodium) equal to 7 $mL \cdot kg^{-1}$ of BM (**Appendix E**). In addition, saliva flow rate and osmolality determined by the salivette method and a simpler dribble method were shown to be strongly correlated across a range of hydration states ($r >$ 0.79, **Appendix F**). Nevertheless, salivette and dribble methods should not be used interchangeably as differences exist in the absolute flow rate and osmolality values. This is encouraging as the dribble method of saliva collection confers both an economic (cheaper collection method) and a practical advantage (does not require a centrifuge for osmolality measurements) in comparison with the salivette method. Compared with collecting urine, saliva collection is less intrusive and in some scenarios may prove a more convenient form of sampling (e.g. when individuals cannot produce a urine sample or are attired in protective clothing). However, compared with urine indices, saliva flow rate and osmolality possess large interindividual variability as illustrated by the large standard deviation in **Figures 4.2, 5.3 and Table 5.2**. This individual variation is more likely to cause incorrect identification of an individual's hydration status using proposed euhydration/dehydration threshold value. It is therefore recommended that a euhydrated reading is determined for saliva markers for each individual.

Given the validity and practicality it is recommended that urine indices (osmolality or specific gravity) are used to identify individual hydration status with a single measure. Urine indices are valid indicators of changes in hydration status evoked by acute and chronic exercise and fluid restriction (Armstrong *et al.*, 1994; Shirreffs *et al.*, 2004),

Chapters 4 & 5) and combined fluid and energy restriction (**Chapter 5**). Moreover, using the current recommended threshold of 700 mOsmol·kg^{-1} (which is interchangeable with 1.020 g·mL^{-1} for urine specific gravity), urine osmolality was able to correctly identify hydration status in more than 88% of individuals. In addition, urine indices can be considered truly practical hydration markers on account of non-invasive sampling and the recent development of simple, portable handheld devices. Practitioners should consider the limitation of urine indices to detect the magnitude of hypohydration as they tend to plateau in response to modest dehydration irrespective of further dehydration (**Chapter 4 & 5**). In addition, during periods of large fluid intake urine indices have been shown to incorrectly indicate euhydration, as suggested by hypotonic urine production, irrespective of hypohydration (Robinson *et al.*, 1956; Kovacs *et al.*, 1999, **Chapter 5**). Caution should therefore be used, especially following a known period of rapid water turnover, when using a single urine measurement to determine an individual's hydration status. At present no single marker provides enough evidence to definitively identify an individual's hydration status and therefore in line with recent recommendations a combination of hydration markers should be used to increase the likelihood of detecting the correct hydration status (Cheuvront & Sawka, 2005).

8.4 Mucosal immunity and saliva control

A decrease in s-IgA has been implicated as a possible causal factor in the increased susceptibility to URTI during heavy training in athletes and military personnel (Gleeson *et al.*, 1999; Carins & Booth, 2002). As previously reported in a number of reviews nutrient restrictions have the potential to weaken many aspects of the immune system (Chandra, 1997; Bishop *et al.*, 1999; Gleeson *et al.*, 2004a). Nevertheless, until recently, the effect of

fluid and/or energy deficits on s-IgA at rest and after exercise remained unclear (Gleeson *et al.*, 2004b).

Increases in saliva protein concentration (i.e. saliva total protein and s-IgA) and simultaneous decreases in saliva flow rate were observed following modest hypohydration (~3% BML) evoked by acute exercise and heat stress (**Chapter 4**) and 48 h of fluid restriction (**Chapter 6**). These findings are consistent with previous studies that report increases in salivary proteins and reductions in saliva flow rate after dehydration evoked by prolonged water deprivation (48 - 72 h, Gregersen & Bullock, 1933) and acute exercise and heat stress (Ford *et al.*, 1997; Bishop *et al.*, 2000; Walsh *et al.*, 2004b). The concentration of salivary proteins and saliva flow rate were also shown to be unaltered when water was provided to offset fluid losses (i.e. total protein in **Chapter 4** and IgA in **Chapter 6**). In comparison with previous studies, these changes in saliva flow rate were accompanied by a number of other hydration markers that were able to qualify hydration status. Removal of concentrating and dilution effects of saliva flow rate ensures a true indication of salivary proteins availability at the oral mucosa. For instance, s-IgA concentration was significantly increased compared with all other trials following 48 h of fluid restriction which might be considered representative of an enhanced mucosal response (**Chapter 6**). However, expressing s-IgA as a secretion rate indicated a 19% reduction in s-IgA availability, albeit non significant. This decrease in s-IgA secretion rate is most likely a consequence of the previously mentioned concurrent decrease in saliva flow rate. Accounting for changes in saliva flow rate is not a new idea (Blannin *et al.*, 1998). However, **Chapters 4 to 6** underline the importance of correcting for changes in hydration status by expressing saliva parameters as a secretion rate.

Chapter 6 also identified that 48 h of energy restriction equal to approximately 22000 kJ caused a similar (20%) non significant reduction in s-IgA secretion rate compared with hypohydration equal to approximately 3% BML evoked by 48 h of fluid restriction. In contrast a 48 h period of fluid and energy restriction was shown to significantly decrease s-IgA secretion rate by 39%. The reduced s-IgA availability on F+ER at 48 h is likely to be attributable to a combination of decreased saliva flow rate and impaired local synthesis and/or secretion of s-IgA proteins. In fact the decrease in saliva flow rate was of a similar magnitude to that reported after modest hypohydration evoked by acute exercise and heat stress (**Chapter 4**) and 48 h of fluid restriction alone (**Chapter 5 & 6**). In previous athletic and occupational studies the stressor(s) responsible for the reported decrease in s-IgA availability and increased URTI remain unclear because any one or a combination of stressors (e.g. arduous exercise, psychological stress, sleep deprivation, fluid and energy deficits) may impact upon s-IgA availability (Gleeson *et al.*, 1999; Carins & Booth, 2002). The results of **Chapter 6** are the first to demonstrate, under controlled laboratory conditions, a role for fluid and energy restriction in the reduction of s-IgA availability in active healthy individuals. Furthermore, these findings suggest the decreases in s-IgA availability and increased URTI previously reported after intense military training (Carins & Booth, 2002; Gomez-Merino *et al.*, 2003) may at least in part be caused by fluid and energy deficits.

Chapter 6 also highlighted a limited effect of a 30 min maximal exercise bout on s-IgA secretion rate, irrespective of prior nutritional status. These results are in agreement with previous studies reporting no change in s-IgA availability after continuous exercise lasting less than 1 h in well nourished individuals (McDowell *et al.*, 1991). Future studies should examine whether a similar dietary restriction would affect the s-IgA responses to more

prolonged exercise (>30 min). Encouragingly, rehydration and refeeding was shown to return saliva flow rate, s-IgA concentration and s-IgA secretion rate to at least baseline values following the 6 h rehydration and refeeding period on all trials. Although refeeding following fluid and energy deficits has been shown to be effective in rapidly restoring blood parameters of the immune system (Walrand *et al.*, 2001) this study was the first to highlight the importance of refeeding to restore s-IgA availability. Specifically, refeeding approximately two-thirds of individual daily energy requirements (8164 ± 557 kJ) normalised s-IgA availability on F+ER by 6 h post-TT.

The exact mechanism(s) by which water deficits decrease saliva flow rate (**Chapters 4 - 6**) or a combination of fluid and energy restriction impair local synthesis and/or secretion of s-IgA (**Chapter 6**) remain unclear. Given the independent effects of fluid and energy restriction on s-IgA secretion rate it is likely the significantly reduced s-IgA availability on F+ER is attributable to a combination of decreased saliva flow rate and impaired synthesis and/or secretion of s-IgA. It has previously been hypothesised that an increase in plasma sodium (osmolality) during dehydration may account for the production of smaller amounts of more concentrated saliva (Ship & Fischer, 1997; Walsh *et al.*, 2004b). This hypothesis is supported where increases in plasma osmolality simultaneously occur with decreases in saliva flow rate (**Chapters 4 & 5**). However, the decrease in saliva flow rate on F+ER with no concomitant change in plasma osmolality questions a direct effect of plasma sodium in decreasing saliva flow rate (**Chapter 5**).

The observed decrease in saliva flow rate following dehydration may also result from an up-regulated renin-angiotensin-aldosterone system. That is, decreases in blood volume or blood pressure, common after dehydration, cause the release of angiotensin II and

consequently an increase in SNS activity and circulating vasopressin and aldosterone concentration. Increases in SNS acitivity, vasopressin and aldosterone may decrease saliva flow rate by vasoconstriction of the blood vessels to the salivary glands or by increasing water and electrolyte reabsorption in the saliva gland (Lavelle, 1988; Talke *et al.*, 2003). For example, in sheep the infusion of angiotensin II into the carotid artery ipsilateral to the parotid gland caused a reduction in saliva secretion (McKinley *et al.*, 1979). Future investigations should attempt to examine the relationship between saliva flow rate and the renin-angiotensin-aldosterone system during dehydration by measuring changes in vasopressin, aldosterone and SNS activity. These investigations may provide further insight into the mechanism responsible for the decrease in saliva flow rate following water deficits.

The relative modest reduction in plasma glucose ($\leq$1.1 mmol·L^{-1}) and unchanged plasma cortisol after 48 h of energy restriction (**Chapter 6**) do not support a role for decreased plasma glucose or increased plasma cortisol in the impairment of s-IgA synthesis and/or secretion observed following 48 h of fluid and energy restriction (Saxon *et al.*, 1978; Sabbadini & Berczi, 1995; Gleeson *et al.*, 2004a). The restricted protein intake at least in part may be responsible for the impaired local synthesis and/or secretion of s-IgA as protein deficiency is associated with impaired cell replication and the production of proteins (i.e. Ig and cytokines, Chandra, 1997; Gleeson *et al.*, 2004a).

A limitation of **Chapter 6** was the measurement of the monomeric form of IgA which accounts for a relatively small (<20%) proportion of total saliva IgA compared with polymeric secretory IgA (>80%, Brandtzaeg, 2007). As IgA enters the oral cavity by different transport mechanisms (**Section 2.5.1**) the effects observed in the monomeric

proportion of s-IgA may not be representative of the larger secretory IgA proportion. Nonetheless, nutrient restrictions have also been shown to decrease the availability of secretory IgA at the mucosal surface via pIgR regulated translocation. Specifically, intestinal IgA was decreased following energy restriction in mice (Ha & Woodward, 1997; Ha & Woodward, 1998) and pIgR expression *in vitro* has been shown to be dependent on the adequate presence of vitamin A (Sarkar *et al.*, 1998).

Other potent modulators of the immune system including the sympathetic nervous system, other hormones (e.g. catecholamines) and cytokines which are altered during energy restriction (Pequignot *et al.*, 1980; Savendahl & Underwood, 1997; Gleeson *et al.*, 2004a) may also influence the availability of secretory and monomeric s-IgA (Norderhaug *et al.*, 1999; Proctor & Carpenter, 2002; Kaetzel, 2005). Indeed, sympathetic and parasympathetic nerve stimulation has been shown to increase translocation of secretory IgA in rats (Carpenter *et al.*, 1998) whilst a recent review identified numerous cytokines (e.g. interferon-gamma, tumour necrosis factor, interleukin 1 and 4) and hormones (e.g. estradiol, progesterone, androgens) that may affect pIgR expression (Kaetzel, 2005). However, many of these studies were conducted *in vitro* or with rodents and as aspects of the mucosal system differ considerably between rodents and humans, it is difficult to determine the significance of autonomic nervous system, cytokines or hormones on s-IgA production in humans (Bosch *et al.*, 2002). Therefore it might be hypothesised that a prolonged period of fluid and energy restriction may also decrease the availability of saliva secretory IgA in a similar manner to monomeric IgA as described in **Chapter 6**. Given the recent development of a specific saliva secretory IgA ELISA (*Immundiagnostik AG, Bensheim, Germany*), future investigations should examine the effect of fluid and/or energy restriction on secretory s-IgA.

Adding to existing literature which suggests nutrient deficiencies lead to compromised immune function in otherwise healthy recreationally active individuals (Bishop *et al.*, 1999; Gleeson *et al.*, 2004a) the findings of **Chapter 6** highlight the importance of adequate dietary intake for mucosal immunity. Although, dehydration alone was not shown to significantly reduce s-IgA availability fluid intake has been shown to be beneficial in preventing or attenuating reductions in s-IgA secretion rate in acute exercise studies and following energy restriction (Bishop *et al.*, 2000, **Chapter 6**). Athletes and other active individuals are therefore advised to consume adequate fluid during training and competition to ensure the effects of dehydration on mucosal immunity are minimised.

8.5 Endurance performance

The results of **Chapter 7** demonstrated a detrimental effect of a 48 h period of energy restriction but no significant effect of fluid restriction on 30 min TT performance in temperate conditions. Additionally, TT performance was similar following fluid and energy restriction and after energy restriction alone. The reduction in TT performance following energy restriction is in agreement with previous studies that identified significantly reduced TTE at exercise intensities ranging from 50 to 100% (Nieman *et al.*, 1987; Gleeson *et al.*, 1988; Maughan & Gleeson, 1988; Zinker *et al.*, 1990). However, the 10 and 15% reduction in TT performance on ER and F+ER is modest compared with 36% (Maughan & Gleeson, 1988) and 44% (Nieman *et al.*, 1987) reduction in TTE after similar energy deficits. The larger decrement in performance in TTE studies may be attributable to the lower intensity and longer duration of exercise protocols used. In addition, the "open" ended nature of TTE versus "known end point" TT protocols may affect an individual's motivation to continue following energy restriction (Jeukendrup *et al.*, 1996).

By identifying a limited effect of modest hypohydration on endurance performance in a temperate environment the findings of **Chapter 7** are the first to highlight the effect of prolonged dehydration on endurance performance. The 48 h period of fluid restriction evoked similar BML (3.2%) to heat exposure (3%, Cheuvront *et al.*, 2005) and larger BML than the diuretic Furosemide (~2%, Armstrong *et al.*, 1985) where studies reported an approximate 8% decrease in endurance performance measured by TT. However, prolonged fluid restriction did not significantly decrease endurance performance (-2.8% *vs.* CON). These findings are in agreement with others showing no significant decrease in TT performance when approximately 2% BML was evoked by a combination of acute exercise and fluid restriction lasting less than 2 h (McConell *et al.*, 1999; Daries *et al.*, 2000).

Nevertheless, it is important to appreciate that **Chapter 7** findings are only valid for endurance performance lasting approximately 30 min in recreationally active individuals in temperate conditions. The completion of longer distances in the same time (e.g. elite athletes ~10000 *vs.* 6500 m from participants in **Chapter 7**) or more prolonged exercise and/or performing in the heat may increase the likelihood of modest hypohydration significantly decreasing endurance performance. The completion of greater distances and/or performing exercise in the heat will increase heat production and sweating subsequently raising core temperature and further decreasing blood volume which in turn may lead to the attainment of critical core temperatures sooner and/or earlier compromised cardiac output which consequently decrease exercise performance. Although not significant, the raised core temperature on FR (+0.2 °C) versus CON supports this concept. Therefore, the completion of greater distances and/or performing in the heat after prolonged dehydration may also increase the incidence of heat illnesses. Future studies

should examine the effect of prolonged dehydration on more prolonged exercise and/or performing in the heat.

The contradictory findings of modest hypohydration on endurance performance may be attributable to the different methods of dehydration (e.g. diuretics, sauna, fluid restriction and exercise, Caldwell *et al.*, 1984) or the duration of dehydration (i.e. acute *vs.* prolonged). In support of this concept, one investigation reported better preserved peak power output on an incremental cycle test to exhaustion, after hypohydration was evoked by 48 h of diet restriction and exercise compared with hypohydration evoked by diuretics or sauna exposure (Caldwell *et al.*, 1984). Furthermore, the diet restriction and exercise dehydration method was associated with better preserved plasma volume compared with dehydration by diuretics or sauna exposure. Endurance performance following a period of dehydration is commonly attributed to a combination of increased cardiovascular and thermoregulatory strain (Sawka, 1992; Cheuvront *et al.*, 2003). For example, amongst the contradictory findings of modest hypohydration in temperate environment, endurance performance (5000 and 10000 m track race) was shown to be significantly decreased when sizeable reductions in plasma volume (10-12%) occurred after diuretic treatment 5 h before exercise (Armstrong *et al.*, 1985), whereas, endurance performance was not significantly decreased when plasma volume was shown to be unaltered after hypohydration was evoked using a combination of exercise and fluid restriction over a 48 h period (**Chapter 7**).

The exact mechanism for the differences in plasma volume loss between methods of dehydration is unclear. However, it might be speculated to be related to the redistribution of remaining water between fluid compartments (Sawka, 1992; Cheuvront *et al.*, 2003). As

increased ECF sodium content has been shown to cause fluid to be mobilised from the intracellular to ECF compartment by osmotic pressure and therefore water redistribution has been suggested to be dependent on the solute concentration of fluid lost (Nose *et al.*, 1988). Specifically, Nose and colleagues (1988) suggested the greater the sodium concentration for a given fluid loss the larger the plasma (blood) volume decrease (Nose *et al.*, 1988). For example, diuretics are associated with a more equal loss of electrolytes and fluids and have been shown to cause a much greater ratio of plasma fluid loss to TBW loss (Sawka, 1992). In contrast it may be suggested plasma volume was better maintained after 48 h of fluid restriction because the sodium losses were replaced by dietary intake and therefore the relatively larger plasma sodium (osmolality) may cause fluid to be mobilised from extravascular sources to intravascular sources thereby preserving plasma and blood volume.

A limitation of the investigations to have currently assessed different methods of dehydration on endurance performance is plasma and blood volume has been estimated using haemoglobin and haematocrit (Dill & Costill, 1974). Further research is therefore required using tracer techniques (e.g. Evans Blue dye dilution) to confirm whether different methods of dehydration influence the magnitude of plasma and blood volume loss which may subsequently have an effect on endurance performance. It might be tentatively suggested that different methods of dehydration are responsible for the equivocal findings of modest hypohydration on endurance performance in a temperate environment. Future studies should compare endurance performance following equivalent dehydration (i.e. 3% BML) evoked by diuretics, prolonged fluid restriction, exercise and heat stress. It may be hypothesised that methods evoking the largest decreases in plasma volume and subsequent greater cardiovascular strain will result in the largest deterioration in endurance

performance. Similarly, individuals consuming fluids with no or low electrolyte concentrations compared with high concentrations to replace sweat losses during exercise might be expected to have greater reductions in plasma and blood volume. Subsequently, it might be hypothesised that exercise performance would be improved when individuals consume fluids containing higher concentrations of electrolytes as a result of enhanced conservation of blood volume and cardiovascular function. This future research will further elucidate the importance of electrolyte (sodium) balance for exercise performance.

8.6 Conclusions

The major conclusions from this thesis are:

1. Saliva flow rate, saliva osmolality and saliva total protein concentration track progressive acute dehydration evoked by a combination of exercise and heat stress (hypertonic hypovolaemia) and fulfil the criterion of a hydration marker by identifying modest hypohydration (2-3% BML).

2. Saliva flow rate and saliva osmolality are able to identify modest hypohydration evoked by 48 h of fluid restriction alone (hypertonic hypovolaemia) and a combination of fluid and energy restriction (isotonic hypovolaemia).

3. Using the proposed euhydration/hypohydration threshold of 61 $mOsmol \cdot kg^{-1}$ saliva osmolality was able to correctly identify 80% of individuals as euhydrated following rigorous hydration strategies the day prior and 75% of individuals as hypohydrated following acute and chronic exercise and fluid restriction protocols.

4. Plasma osmolality, often referred to as a gold-standard hydration marker, identifies modest hypohydration evoked by a 48 h period of fluid restriction alone but is unable to detect hypohydration when fluid and energy restriction are combined.

5. Urine osmolality and colour identify modest hypohydration evoked by 48 h of fluid restriction alone or a combination of fluid and energy restriction. Using the current ACSM recommended upper euhydrated range value of 700 mOsmol·kg^{-1} as a euhydration/hypohydration threshold urine osmolality was able to correctly identify 95% of individuals as euhydrated following rigorous hydration strategies the day prior and 88% of individuals as hypohydrated following acute and chronic exercise and fluid restriction protocols.

6. Saliva flow rate, saliva osmolality, plasma osmolality, urine osmolality and urine colour are unable to identify hypohydration associated with energy restriction alone.

7. A strong relationship between plasma osmolality and saliva flow rate and saliva osmolality exists during hypertonic hypovolaemia but not isotonic hypovolaemia; consequently these results do not support the hypothesised role for plasma sodium in the control of saliva flow rate during dehydration.

8. A 48 h period of fluid or energy restriction alone, evoking hypohydration equal to 3.2% or an energy deficit equal to approximately 22000 kJ did not significantly decrease s-IgA availability.

9. Saliva IgA availability is decreased following a 48 h period of combined fluid and energy restriction evoking hypohydration and an energy deficit equal to approximately 3% BML and 22000 kJ, respectively. However, s-IgA availability was normalised following 6 h of rehydration and refeeding.

10. Saliva-IgA availability is unaffected by a 30 min maximal exercise bout irrespective of prior fluid and energy deficit equal to approximately 3% BML and 22000 kJ, respectively.

11. Modest hypohydration (3.2% BML) evoked by 48 h of fluid restriction does not significantly impair 30 min self-paced treadmill TT performance. Furthermore, the combination of 48 h fluid and energy restriction had no additive effect on 30 min TT performance compared with 48 h energy restriction alone. These results do not support the popular contention that modest hypohydration significantly impairs endurance performance in temperate conditions.

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Appendix A:

The University of Wales, Bangor
School of Sports, Health and Exercise Sciences

Subject Information Sheet

Project Title: Are changes in saliva - flow rate, osmolality and protein concentration, associated with changes in body mass during acute progressive dehydration and rehydration in humans?

Project investigators:
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Invitation to take part
You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being conducted and what it will involve. Please take time to read the following information carefully and discuss it with the investigators. Ask us if there is anything that is not clear or if you would like more information.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time without giving a reason.

Objectives
To assess whether changes in saliva flow rate, osmolality and protein concentration are associated with changes in body mass during acute progressive dehydration, induced by exercising in a hot environment, and subsequent rehydration. Also, we wish to compare the changes in these saliva measures with the more commonly used blood and urine markers of whole body hydration status.

Visit One (approximately 50 minutes):
You will be asked to refrain from exercise and alcohol consumption for 24 hours prior to each visit. At this visit you will be familiarised with the procedures for collection of saliva and venous blood. You will also perform an incremental cycle test to volitional exhaustion in order to determine maximal oxygen uptake ($\dot{V}O_2$ max) and peak power output (PPO). Non-invasive measures of breathing and heart rate will be taken. After the physiological tests have been conducted, you will be familiarised with the cognitive functioning tests and the rates of perceived exertion scale.

Visit Two (approximately 3 hours):
At this visit you will perform an exercise dehydration protocol. You will be instructed to drink 30 $ml.kg^{-1}$ body mass of water the day before the trial. You will arrive at the Physiology laboratory in the appropriate clothing (shorts, trainers and T shirt) following an overnight fast. You will not be allowed to eat for the duration of this visit. Prior to the exercise protocol you will be scanned on the DEXA machine to estimate your body composition. On arrival at the laboratory you will be given a disinfected rectal probe to fit

and a universal container to collect a urine sample. After completely emptying your bladder and bowels you will be weighed. No blood samples will be obtained on this visit. The rectal probe will be used to monitor core temperature and a polar HRM to monitor heart rate at rest and throughout exercise. You will be cycling on a Monark 824e cycle ergometer at 60% $\dot{V}O_2$ max in an environmental chamber maintained at 30°C and RH 70%, after cycling for 10 minutes, dismount, towel dry, and you will be weighed nude on digital scales. You will repeat this 10 min cycling and weighing procedure until reaching 1, 2, and 3% body mass loss. This trial will be used to estimate your individual sweating rate and exercise duration for the two subsequent trials. Following the dehydration protocol you will be rehydrated and then be free to leave the laboratory.

Visit Three and Four (approximately 4 hours):
Visit three and four will be completed in a random order. You will be asked to follow the same prior exercise procedures as described in visit two. Except after providing a urine sample and being weighed nude you will be asked to sit for 10-15 minutes prior to a resting blood and saliva collection. At rest a blood sample will be collected along with a 2 min saliva collection. You will not be permitted to consume any fluid 15 min prior to each saliva collection. You will then complete either a trial in which you will receive no fluid or a trial in which you will receive fluid sufficient to prevent dehydration. On both trials you will be cycling on a Monark 824e cycle ergometer at 60% $\dot{V}O_2$ max in an environmental chamber maintained at 30°C and RH 70%, after cycling for 10 minutes, dismount, towel dry, and you will be weighed nude on digital scales. You will repeat this 10 min cycling and weighing procedure until reaching 1%, 2%, and 3% body mass loss on the no fluid trial or until the corresponding times on the fluid intake trial. At each of these stages you will be removed from the environmental chamber and seated for 5 minutes. On the no fluid intake trial blood, saliva and urine samples will be collected before returning to the chamber to perform further 10 min periods of cycling and weighing until the next 1% body mass loss is achieved. Blood and saliva samples will only be obtained after completing the exercise bout on the fluid intake trial. On the no fluid intake trial, after the final samples are obtained, you will be given a volume of carbohydrate-electrolyte solution to drink within the first 45 minutes of the rehydration period. During this rehydration period, nude body mass, blood, saliva and urine will be collected. You will then be free to leave the laboratory.

Dr Neil Walsh, and any of the above contacts will happily answer any further questions regarding this research.

The University of Wales, Bangor
School of Sport, Health and Exercise Sciences

Subject Information Sheet

Research Title: The effects of restricted food and/or fluid intake on markers of hydration status, immune function and incidence of upper respiratory tract infections

Research Co-ordinator: Dr Neil Walsh
Tel: 01248 383480
Email: n.walsh@bangor.ac.uk

Additional Investigators: Sally Wilson, Sam Oliver and Stewart Laing.

Invitation to take part

You are being invited as an athletic male to take part in a research investigation. Before you decide to take part it is important for you to understand why the research is being conducted and what will be required of you should you agree to be involved. Please take time to read the following information carefully and discuss it with the investigators. Ask us if there is anything that is not clear or if you would like more information.

Do I have to take part?

This is entirely your decision. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. **If you decide to take part you are still free to withdraw at any time without giving a reason.** If you decide not to participate or withdraw during the study, your decision will not affect your relationship with the School of Sport, Health, and Exercise Sciences, or any of the investigators involved in the study. All information collected during the study will be treated confidentially.

Background

An athlete's health and physical performance are negatively affected by body fluid losses (dehydration). Therefore, it is important to be able to identify body fluid losses before severe dehydration disrupts health and performance. Recent investigations by our group have found that simple measurements performed on saliva samples are able to track fluid losses and therefore determine the hydration status of athletes over short periods of time (2-3 hours). Dehydration often results from a combination of improper fluid intake and sweat losses over a number of days. It is not clear if these simple salivary measurements can track hydration status over longer periods of time (e.g. over a number of days). One of the aims of the study is to compare these new saliva markers of hydration with more traditionally used blood and urine hydration markers over a period lasting 3 days. This will enable us to conclude whether saliva markers can be used outside of the laboratory setting to determine an athlete's hydration status.

This study will also examine if a 2 day period of food and/or fluid restriction will decrease immunity and in turn increase the susceptibility to infections (e.g. colds). Military personnel often have to carry out demanding exercises, lasting for many days, on restricted rations and fluid intake. The effects of restricted rations and fluid intake on immunity and infection incidence are relatively unknown. In summary, the aims of this study are to examine the effects of restricted food and/or fluid intake on markers of hydration status, immunity and the incidence of infection.

What will be expected of you?

If you decide to take part in this study there will be a number of constraints placed upon your normal everyday life and activities.

The day prior to completing a trial you will be expected;

- to avoid drinking alcohol, caffeinated drinks (i.e. coffee, tea, coke and diet coke) and avoid participating in any exercise for 24 hours before arriving for each visit.

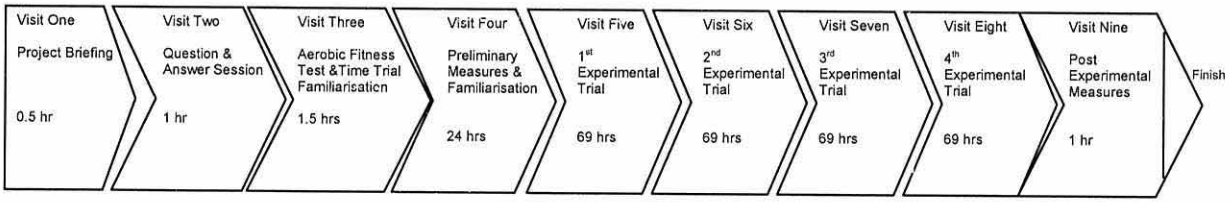
Whilst completing the experimental trials in the laboratory you will be expected;

- to spend 3.5 consecutive days in the laboratory on 4 separate occasions.
- to live and sleep in the laboratory during each 3.5 day trial (Soft furnishings, bedding, computers, TV, video, DVD will be provided and living temperature will be between 17-21°C)
- to eat and drink only what is given to you.
- to follow the study's daily timetable, including going to bed and waking from sleep when asked.
- to perform prescribed physical activity (130 minutes walking per day).
- to be weighed nude (behind screens to maintain privacy).
- to have 6 blood samples taken during each 3.5 day trial (~84 ml during each trial) totalling 25 blood samples in all during the 6- week study period (~340 ml blood)
- to perform a maximal aerobic test and four 30 minute time trials.

You will be excluded if you are a smoker or you are on specific types of medication or supplements (i.e. creatine).

IN TOTAL, THE STUDY WILL REQUIRE YOU TO GIVE UP ~300 HOURS OF YOUR TIME.

Summary of visits



Visit one: Project briefing (~30 minutes)

At this meeting, you will be fully briefed about the requirements of the project. You will be talked through the subject information sheet by an investigator and given the opportunity to ask any questions. You will then leave with the information sheet so as to allow you time to discuss your possible involvement in the study with significant others. This will also allow you additional time to think of questions.

Visit two: Project briefing (Question and answer session ~ 1 hour)

Visit two is provided as an additional opportunity for you to ask questions. Once you are fully satisfied with the information and on agreement to take part in the study you will be asked to complete an informed consent form, a medical questionnaire and make arrangements for the following visits.

Visit three: Aerobic fitness test and time trial familiarisation. (~ 1.5 hours)

On an agreed day between 09:00-10:00 hours you will be required to perform a 10-15 minute treadmill test that will be used to measure your aerobic fitness (maximal oxygen uptake). This test will require you to run at your maximal aerobic capacity for approximately one minute. After a recovery period, this test will be followed by a familiarisation of the 30 minute treadmill time trial that will be used in the main experimental trials. This time trial will require you to run as far as possible in 30 minutes on a treadmill. You will control the speed of the treadmill throughout the

time trial. Completing a practice time trial will help you to pace yourself during the time trial in the main experimental trials.

Visit four: Preliminary measurements and familiarisation (~24 hours)
As you will be required to spend approximately 69 hours residing in the physiology laboratory for each of the four trials we feel it is necessary to familiarise you with the surroundings and measures we will be taking from you. This will help to reduce any anxiety you may have about any of the visits. This visit will also provide us with the time to perform body composition measurements and procedures to estimate your daily energy expenditure. You will also have blood pressure, urine, saliva and blood taken at this visit.

You will arrive at the Physiology laboratory at 07:45 hours after an over night fast (**no breakfast**). You will also have refrained from exercising or consuming alcohol and caffeine (tea, coffee, coke, and diet coke) the day before your visit. Measurements performed on your blood and urine will be used to ensure you are properly hydrated on arrival at the laboratory.

On arrival at the laboratory, measures of height and weight will be obtained; these will be followed by **a whole body scan using an x-ray** machine to determine your body composition. The x-ray is painless and involves you lying down on a flat bed for 20 minutes. The x-ray does emit a very small dose of ionising radiation. The dose of radiation you will receive is the same as an additional day of natural background radiation. This is substantially less than the radiation associated with a normal X-ray (approximately one fortieth of a chest x-ray).

Your energy expenditure will be assessed using breath by breath gas analysis which involves you wearing a face mask at rest and during normal activities moving around the laboratory. Familiarisation of blood, urine and saliva techniques will also be performed at this visit. All blood samples will be collected by a qualified member of staff from a forearm vein using a small needle (~14 ml of blood at each collection). A saliva sample will be obtained by placing a cotton swab in your mouth for 2 minutes and urine will be collected in privacy by passing urine into a plastic container. In order to complete the investigation these samples are a necessity. Breakfast will be provided (~10:00 hours) followed by lunch (~13:30 hours) and an evening meal (~18:00 hours). After the overnight stay you will also receive breakfast and be free to leave the laboratory.

Experimental trials
You will be required to complete four experimental trials in a random order each separated by eight days. Each experimental trial will consist of a 69 hour period where you will reside in the laboratory under treatment conditions (Visit Five). **Including the briefing meeting, familiarisation visit and all four experimental trials you will be required to visit the laboratory on nine occasions for a total of ~300 hours.**

Visit five (~69 hours)
You will arrive at the laboratory at 22:00 hours. We will provide you with water before you go to bed and when you wake up in the morning to make sure you are well hydrated at the start of the trial. The next morning, after confirmation that you are well-hydrated you will begin one of the four 48 hour experimental conditions at random. The trials are as follows: control trial, fluid restriction trial, calorie restriction trial and a fluid and calorie restriction trial. The control trial will involve you consuming a range of foods (approx. 3000 kcal per day), and sufficient water (approx. 3.0 litres per day), to cover your normal living requirements. The fluid restriction trial will involve you consuming the same foods as the control trial (approx. 3000 kcal per day) but with restricted fluid intake (approx. 0.2 litres per day). The calorie restriction trial will involve you consuming a range of foods providing 1/10 of your estimated energy requirements (approx. 400 kcal per day), and sufficient water (approx. 3.0 litres per day), to maintain hydration. The fluid and calorie restriction trial will involve you consuming a range of foods providing 1/10 of your estimated energy requirements (approx. 400 kcal per day) and restricted fluid intake (approx. 0.2 litres per

day). In addition and on both days of the trial you will be asked to perform a 1.5 hour bout of moderate ('brisk') paced walking (4mph/6.4kph).

After 48 hours (day 3) you will be required to perform a 30 minute treadmill time trial. You will then begin the rehydration and refeeding process. During the first 2 hours after the time trial, you will be provided with an electrolyte drink that will help replace any losses that may have occurred during the trial. After 2 hours recovery you will be provided with the first of two meals with the second being provided 2 hours later (4 hours post exercise). After 7 hours of recovery you will be free to leave the laboratory. On leaving the laboratory you will be provided with additional meals to replace any calorie deficit that has occurred during the trial for you to consume over the proceeding week. You will also be given a simple 8 day health and sickness log where for each day you are asked to record the occurrence of any health problems (e.g. cold symptoms). We will ask that you bring the completed health and sickness log to the laboratory the next time you visit.

During each trial you will have blood, urine and saliva samples taken every 24 hours and immediately after and 2 and 6 hours after the treadmill time trial (**6 blood samples in each trial**). In addition to the previously mentioned measures, we will also ask you to drink a small volume of water containing a safe dose of deuterium oxide. After an overnight period, for the deuterium oxide to mix with body fluids, an additional urine sample will be collected. This is a well established and safe method to allow us to estimate the amount of water in your body at the start of this trial.

Visit Six, Seven and Eight (~69 hours each)
Six days after visit four you will be required to return to complete one of the three remaining experimental trials**.** Once again, you will be asked to complete a 48 hour experimental trial (see visit 5). You will be required to repeat this cycle until you have completed all four experimental trials.

Visit Nine (1 hour)
After completing all experimental trials you will be asked to return to the laboratory after 8 days for a final visit. During this visit you will have your weight assessed and also undergo a whole body x-ray scan as in the familiarisation visit.

Advantages and disadvantages of taking part

A benefit of taking part in this study is that you will receive comprehensive feedback, with full explanations, for your fitness level, body composition (e.g. body fat %) and blood measures (immune function). This feedback should help you with planning and monitoring your athletic training programme. The feedback you will receive regarding your fitness level is similar to that which many testing facilities provide as a fee-paying service.

The disadvantages of taking part in this study, which you will probably be most concerned about, are giving blood samples, the food and fluid restriction treatments and the amount of time you will be asked to commit to the study. Firstly, regarding the blood samples, we will only be collecting a single blood sample every 24 hours with the addition of the three samples after the 30 min treadmill time trial (6 samples in each trial). Only qualified Phlebotomists with experience at performing this procedure will collect blood samples. The blood samples will be taken with a very small butterfly needle without a tourniquet. In addition, the blood sample volume will only be approximately 14 ml at each collection. We will collect ~84 ml of blood during each 3 day trial giving a total blood volume of ~340 ml over the 4 trials spread across 6 weeks. The second of your concerns will probably be the 2 days of restricted food and fluid intake. Military recruits, of similar age and fitness to you, consume on average 330 kcal per day, with fluid restriction, for periods lasting 2-3 weeks on escape and evasion training. On top of the energy and fluid restriction the recruits cover 10-15 km per day on foot in difficult desert conditions with severe sleep deprivation. Clearly, the food and fluid restriction in this study is modest in comparison with that experienced during escape and evasion training. In case of emergency there will be an experimenter qualified in

First Aid present through-out the study. The third disadvantage is that to complete all aspects of the study we will require you to visit the laboratory on eight occasions for a total of approximately 300 hours.

We understand that this study will ask a lot of you as a participant and so we are offering a financial incentive. As a participant you will be eligible for a £80 reward on completion of each of the first three experimental trials and a further £160 bonus on successful completion of the final experimental trial (**£400 total**). This incentive does not in anyway affect your freedom to withdraw without reason from the study at anytime. Any further questions will be happily answered by Dr Neil Walsh or any of the additional investigators.

Appendix B:

INFORMED CONSENT TO PARTICIPATE IN A RESEARCH PROJECT OR EXPERIMENT

The researcher conducting this project subscribes to the ethics conduct of research and to the protection at all times of the interests, comfort, and safety of participants. This form and the information it contains are given to you for your own protection and full understanding of the procedures. Your signature on this form will signify that you have received information that describes the procedures, possible risks, and benefits of this research project, that you have received an adequate opportunity to consider the information, and that you voluntarily agree to participate in the project.

Having been asked by **Dr Neil Walsh** of the School of Sport, Health and Exercise Sciences at the University of Wales, Bangor to participate in the research project titled:

The effects of restricted food and/or fluid intake on markers of hydration status, immune function and incidence of upper respiratory tract infections.

I have received information regarding the procedures of the experiment.

I understand the procedures to be used in this experiment and any possible personal risks to me in taking part.

I understand that I may withdraw my participation in this experiment at any time.

I also understand that I may register any complaint I might have about this experiment to Professor Roger Eston Head of the School of Sport Health and Exercise Sciences, and that I will be offered the opportunity of providing feedback on the experiment using standard report forms.

I may obtain copies of the results of this study, upon its completion, by contacting:
Dr Neil Walsh (Tel: 01248 382756 or Email: n.walsh@bangor.ac.uk)

I confirm that I have been given adequate opportunity to ask any questions and that these have been answered to my satisfaction.

I have been informed that the research material will be held confidential by the researcher.

I agree to participate in the study

_____

NAME (please type or print legibly): _____

ADDRESS: (Optional)_____

_____

SIGNATURE: _____

DATE: _____

Appendix C:

PHYSIOLOGY INFORMED CONSENT & MEDICAL QUESTIONNAIRE

Name: .....

Age:.....

Are you in good health? Yes/No
If no, please explain:

How would you describe your present level of activity? Tick intensity level and indicate approx. duration.

vigorous moderate low intensity

Duration (min):

How often:
< once per month
once per month
2-3 times per week
4-5 times per week
> 5 times per week

Have you suffered from a serious illness or accident? Yes/No
If yes, please give particulars:

Do you suffer, or have you ever suffered from:
Asthma Yes/No
Diabetes Yes/No
Bronchitis Yes/No
Epilepsy Yes/No
High blood pressure Yes/No

Are you currently taking medication? Yes/No
If yes, please give particulars:

Are you currently attending your GP for any condition or have you consulted your doctor in the last three months? Yes/No

If yes, please give particulars:

Have you, or are you presently taking part in any other laboratory experiment? Yes/No

PLEASE READ THE FOLLOWING CAREFULLY

Persons will be considered unfit to do the experimental exercise task if they:

have a fever, suffer from fainting spells or dizziness;
have suspended training due to a joint or muscle injury;
have a known history of medical disorders, i.e. high blood pressure, heart or lung disease;
have had hyper/hypothermia, heat exhaustion, or any other heat or cold disorder;
have anaphylactic shock symptoms to needles, probes or other medical-type equipment.
have chronic or acute symptoms of gastrointestinal bacterial infections (e.g. Dysentery, Salmonella)
have a history of infectious diseases (e.g. HIV, Hepatitis B); and if appropriate to the study design, have a known history of rectal bleeding, anal fissures, haemorrhoids, or any other condition of the rectum;

DECLARATION
I agree that I have none of he above conditions and I hereby volunteer to be a participant in experiments/investigations during the period of
.....20___.

My replies to the above questions are correct to the best of my belief and I understand that they will be treated with the strictest confidence. The experimenter has explained to my satisfaction the purpose of the experiment and possible risks involved.

I understand that I may withdraw from the experiment at any time and that I am under no obligation to give reasons for withdrawal or to attend again for experimentation.

Furthermore, if I am a student, I am aware that taking part or not taking part in this experiment, will neither be detrimental to, nor further my position as a student.

I undertake to obey the laboratory/study regulations and the instructions of the experimenter regarding safety, subject only to my right to withdraw declared above.

Signature of Participant.....

Date:.....

Signature of Experimenter.....

Date: .....

Appendix D: *The effect of time prior to blood centrifugation on serum osmolality and anticoagulants on plasma osmolality*

Significant increases in serum osmolality have previously been shown when blood samples were left for 3.5 h at room temperature (24°C) before centrifugation. Additionally, serum osmolality was greater than heparinised plasma osmolality when determined between 1 and 4 h post blood collection. These increases in serum osmolality were shown to be related to lactic acid produced by glycolytic activity of erythrocytes and leukocytes (Redetzki *et al.*, 1972). In contrast, recently no difference was reported between osmolality determined on serum or heparinised plasma although EDTA was shown to increase plasma osmolality by approximately 10 $mOsmol \cdot kg^{-1}$ (Seifarth *et al.*, 2004). Blood samples are often and have been recommended to be left for greater than 1 h prior to centrifugation to collect serum (Maughan *et al.*, 2001) and few previous hydration studies explicitly state if an anticoagulant is used. *Purpose*. To assess the validity of serum osmolality measurements determined from blood samples left at room temperature for up to 2 h and also compare plasma osmolality determined from blood samples containing lithium heparin and EDTA anticoagulants with serum osmolality *Methods*. Blood samples were obtained in vacutainers from a single venepuncture with a standardised draw order to prevent contamination: 4 serum, 1 lithium heparin (HEP, 17 $U \cdot mL^{-1}$) and 1 EDTA (EDTA, 1.8 $mg \cdot mL^{-1}$). Serum blood vacutainers were left to clot at room temperature (19°C) whilst HEP and EDTA vacutainers were centrifuged immediately. Serum vacutainers were centrifuged upon clotting which occurred between 10-14 min and after 30, 60 and 120 min. Following centrifugation plasma and serum osmolality measurements were made in triplicate within 20 min. *Results*. The duration prior to centrifugation did not influence serum osmolality (**Table A.1**). Osmolality was not different between serum and heparinised plasma. However, the plasma osmolality determined from EDTA blood

resulted in a significant increase (mean 12 mOsmol·kg^{-1}, $P < 0.01$) compared with serum osmolality and heparinised plasma osmolality.

Table A.1: *The effect of time prior to blood centrifugation on serum osmolality and anticoagulants on plasma osmolality*

Subject	S (10-14)	S (30)	S (60)	S (120)	HEP	EDTA
1	299	297	296	296	296	309
2	289	293	285	288	287	300
3	292	292	296	296	294	306
4	292	291	291	292	292	303
5	291	289	291	288	293	302
Mean	292	292	292	292	292	304[a]
SD	4	3	5	4	4	3

$n = 5$. Abbreviations: S, serum (min prior to centrifugation); HEP, lithium-heparinised plasma; EDTA, ethylenediaminetetraacetic acid plasma. a vs. serum (10-14), serum (30), serum (60) and serum (120) and heparinised plasma; $P < 0.01$.

Conclusion. Serum osmolality may be determined from clotted blood samples centrifuged within 2 h of blood collection. Serum and HEP blood may be considered comparable when assessing osmolality. However, EDTA blood should not be used to determine osmolality because elevated osmolality compared with serum and HEP plasma may lead to misinterpretation of an individual's hydration status (e.g. using ACSM recommended 280-290 mOsmol·kg^{-1} euhydrated range). The increase in plasma osmolality is most likely attributable to the addition of potassium by the anticoagulant.

Appendix E: *Saliva flow rate and osmolality responses to water alone and a carbohydrate solution at rest*

Fluids are commonly consumed *ad libitum* in populations (e.g. athletes) where saliva hydration indices could have significant utility. Although parotid saliva flow rate has been shown to be unaltered immediately after consuming 1 L of water (Shannon & Chauncey, 1967) the effect of water ingestion on whole saliva flow rate and osmolality remains unclear. Furthermore, the effect of a carbohydrate-electrolyte solution on saliva parameters is unknown. *Purpose*. To assess the validity of saliva hydration indices following the ingestion of water or a carbohydrate-electrolyte solution on saliva flow rate and osmolality at rest. *Methods*. Six non-smoking individuals with no significant oral, dental or systemic disease volunteered to participate (mean ± SD: BM, 67.0 ± 7.5 kg). Participants were required to complete three trials in a randomised order on three successive days (08:00-10:00 h). Participants received no fluids on the control trial (CON) whilst receiving water or a carbohydrate-electrolyte solution (CHO, 6% carbohydrate and 25 $mmol \cdot L^{-1}$ sodium) equal to 7 $mL \cdot kg^{-1}$ of BM on the water and CHO trials, respectively. Participants were instructed to ingest the fluid evenly over a 5 min period. Salivette saliva samples were obtained as described in **Chapter 3** prior to the ingestion of fluid (pre), immediately following the 5 min fluid ingestion period (post) and 15 min post fluid ingestion (15 min post). On CON participants provided saliva samples at the same times as the fluid ingestion trials. A two-way fully repeated measures ANOVA was performed (3 trials X 3 sample collections) on the saliva parameters. *Results*. Compared with CON saliva flow rate and osmolality were not significantly different at any time point (pre, post or 15 min post) following the consumption of water or a carbohydrate-electrolyte solution (**Table A.2**).

Table A.2: The effects of 7 $mL \cdot kg^{-1}$ body mass of water or a carbohydrate-electrolyte solution (6% carbohydrate and 25 $mmol \cdot L^{-1}$ sodium) on saliva flow rate and osmolality at rest.

		Pre	Post	15 min post
Saliva Flow Rate	CON	380 ± 258	280 ± 274	410 ± 273
($\mu L \cdot min^{-1}$)	Water	436 ± 330	433 ± 280	570 ± 320
	CHO	502 ± 284	581 ± 170	575 ± 235
Saliva Osmolality	CON	63 ± 17	69 ± 34	58 ± 19
($mOsmol \cdot kg^{-1}$)	Water	71 ± 30	51 ± 16	54 ± 17
	CHO	51 ± 7	51 ± 10	45 ± 9

Values are mean ± SD. $n = 6$. Abbreviations: CON, control trial; CHO, carbohydrate-electrolyte solution.

Conclusion. These results suggest a limited effect on unstimulated whole saliva flow rate or osmolality of consuming water alone or a 6% carbohydrate-electrolyte solution equal 7 $mL \cdot kg^{-1}$ of BM either immediately post or 15 min post drink consumption.

Appendix F: *The relationship between salivette and dribble saliva collection methods for saliva flow rate and osmolality determination*

Purpose. As the salivette collection method requires a centrifuge, which may limit its practical application, the relationship between the salivette and dribble saliva collection methods was examined for both saliva flow rate and osmolality. *Methods*. Saliva samples ($n = 69$) representative of a range from euhydrated to approximately 4% BML were collected and analysed as described in **Chapter 3**. The relationship between the two saliva collection methods was examined by Pearson's correlation analysis and paired *t*-tests. *Results*. Pearson's correlation analyses revealed a significant relationship between the salivette and dribble methods of collection for both saliva flow rate ($r = 0.79$, $P < 0.01$, **Figure A.1**) and saliva osmolality ($r = 0.83$, $P < 0.01$, **Figure A.2**). However, the salivette method was signifcantly greater for both saliva flow rate (mean ± SD: salivette, 318 ±193 $\mu L \cdot min^{-1}$ vs. dribble, 229 ± 136 $\mu L \cdot min^{-1}$, $P < 0.01$) and saliva osmolality (salivette, 64 ± 26 $mOsmol \cdot kg^{-1}$ vs. dribble, 53 ± 21 $mOsmol \cdot kg^{-1}$, $P < 0.01$). Comparing the line of identity (dotted line) to the trendline (solid line) further highlights the differences between methods for determining absolute values. *Conclusion*. As the dribble and salivette collection methods are significantly related it may be suggested the dribble method could replace the salivette method to indicate possible changes in hydration status. This is encouraging as the dribble method confers both economic (cheaper collection method) and practical advantages (does not require a centrifuge) in comparison with the salivette method. Nevertheless, results obtained by salivette and dribble methods should not be used interchangeably as significant differences exist between absolute saliva flow rate and osmolality values for the same sample.

Figure A.1: The relationship between saliva flow rate measurements collected by salivette and dribble methods

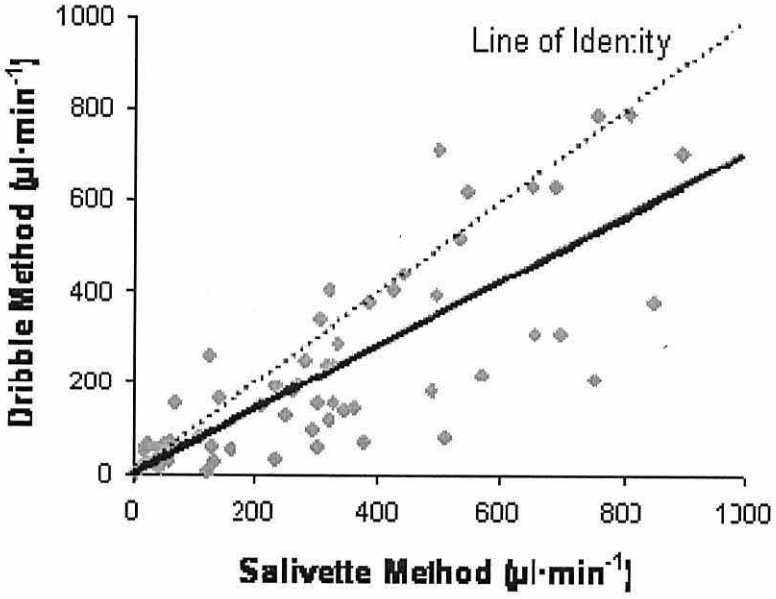
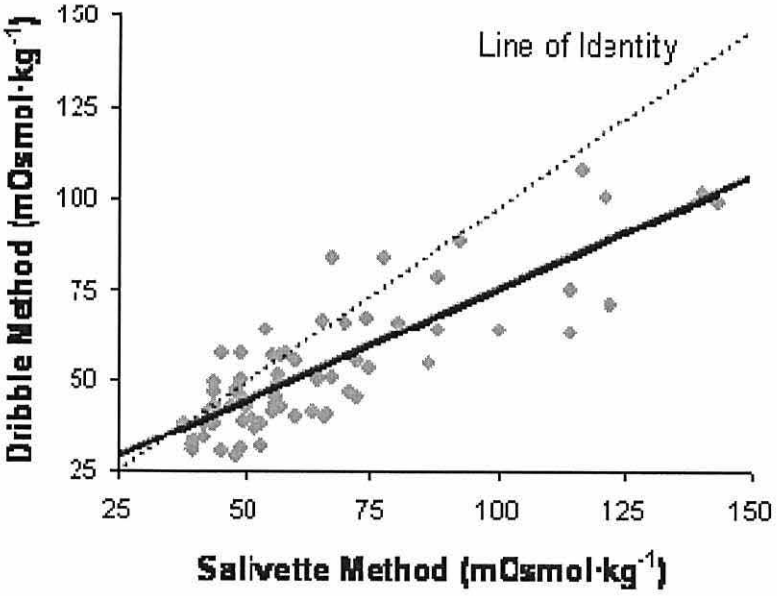


Figure A.2: The relationship between saliva osmolality measurements collected by salivette and dribble methods



Appendix G: *The reproducibility of a 30 minute self-paced treadmill time trial*

Purpose. To assess the reproducibility of a 30 min self-paced TT. *Methods*. Ten males (mean ± SD: age, 22 ± 3 years; height, 179 ± 9 cm; BM, 74.2 ± 9.1 kg; $\dot{V}O_{2max}$ 54.8 ± 7.5 $mL \cdot kg^{-1} \cdot min^{-1}$) performed four TT comprising of a familiarisation and three further TT evenly spaced over a two week period. Within-subject variation was minimised by testing at a similar time of day. In addition, on the day prior to each TT, participants refrained from exercise and consumed a similar diet and water equal to 40 $mL \cdot kg^{-1}$ of BM. All TT were performed as previously described in **Chapter 7**. To minimise heteroscedasticity, reproducibility was derived from log-transformed final distances (Hopkins, 2000). Typical error of the measurement, expressed as an absolute value (standard error of the measurement) and CV, were obtained as described elsewhere (Hopkins, 2000). *Results*. Participants completed 6832 ± 249, 6782 ± 208 and 6801 ± 196 m on trials 1, 2 and 3 respectively. Allowing for a familiarisation trial, the standard error of the measurement and CV between trials 1-2 and 2-3 were 106 and 114 m and 1.5 and 1.6%, respectively. A CV of 3.8% was noted between the familiarisation (6630 ± 186 m) and trial 1, highlighting the importance of allowing participants a single familiarisation session. In addition, no order or learning effect was identified as ANOVA revealed no significant differences between trials 1, 2 and 3 ($P > 0.05$). *Conclusion*. The CV of 1.6% is much smaller than the CV reported for the more traditional TTE protocols and compares favourably with that reported previously in endurance trained runners performing a 1 h TT (CV = 2.7%, Schabort *et al.*, 1998).