Comparison of electrolyte levels, osmolality and immune function markers in blood and/or saliva, urine, and sweat, in resting and exercising males and females

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ABSTRACT

Background: The gold standard method to measure physiological markers to monitor athletes' training responses is to utilise blood samples. However, this method is invasive, requires a skilled technician and specific medical equipment for collection. Sports and exercise studies are based mostly on only male participants, and a few with males and females combined without acknowledging the sex differences. The aim of this study was to investigate differences in electrolyte and osmolality levels in blood, saliva, urine and sweat, and immunoglobulin A (IgA) levels in blood and saliva, between males and females. This study also aimed to study the changes in electrolyte and osmolality levels in blood, saliva, urine and sweat, and IgA levels in blood and saliva over time while exercising. A further aim was to explore relationships between electrolyte and osmolality levels in blood (gold standard) and saliva, urine and sweat, and IgA levels in blood and saliva, at rest and during exercise, as an alternative strategy to overcome the limitations of blood collection, particularly in a sports setting.

Methods: Blood, saliva, urine and sweat were collected to measure electrolyte, osmolality and IgA levels from 20 healthy volunteers (12 males and 8 females), who participated in a cross-over design trial that involved a resting and exercising trials. The exercise protocol involved participants continuously cycling at 70% peak power for 90 min and body fluids were collected pre, during and/or post-exercise.

Results: During exercise, significant differences ($P<0.05$) were found in serum Na^+ and Cl^-, salivary K^+, urinary Na^+ and sweat osmolality levels between males and females. Among males, there was a significant increase in serum Cl^- levels at 30 min and 60 min ($P<0.001$) of the exercising trial, and among females, a significant decrease in serum Na^+ level post-exercise ($P=0.020$) and increase in serum Cl^- at 60 min ($P<0.05$), compared to pre-exercise levels. There were no sex differences for some analytes (serum K^+ and osmolality, salivary Na^+, Cl^- and osmolality, urine K^+, Cl^- and osmolality, sweat Na^+, Cl^- and K^+) and male and female data were combined for subsequent analysis. Serum K^+ analysis (combined data) showed a significant increase serum K^+ levels at 30 min ($P<0.001$) and 60 min ($P<0.001$) of the exercising trial. In urine, there was a significant increase in K^+ levels (combined data) at 90 min and 120 min ($P=0.024$) compared to pre-exercise level. There was a strong positive correlation between serum and salivary K^+ at 60 min ($r=0.714$, $P=0.047$) among exercising females, and a positive correlation between serum and salivary IgA (combined data) pre-exercise ($r=0.445$, $P=0.49$), 60 min ($r=0.489$, $P=0.029$), and post-exercise ($r=0.513$, $P=0.016$).

Conclusion: These findings show that when studying changes in some biomarkers (i.e., serum Na^+, Cl^-, salivary K^+, urine Na^+ and sweat osmolality) during exercise, male and female

participants' results should not be pooled. The presence of positive correlations between serum and salivary IgA levels at multiple timepoints of the resting and exercising trials is promising. It opens the possibility of using saliva samples as indirect measures to predict serum IgA levels in athletes, thereby overcoming the requirement of a blood draw, which is technically demanding, invasive, stressful, and disruptive when exercising.

Keywords: Exercise, males, females, electrolytes, osmolality, IgA, body fluids

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LIST OF ABBREVIATIONS

ATP Adenosine triphosphate

AVP Arginine vasopressin

ECF Extracellular fluid

GFR Glomerular filtration rate

IgA Immunoglobulin A

ICF Intracellular fluid

IF Interstitial fluid

RPF Renal plasma flow

URTI Upper respiratory tract infections

USG Urine specific gravity

WBW Whole body wash down

CHAPTER 1: INTRODUCTION, AIM AND OBJECTIVES

1.1 Introduction

Body fluids such as blood, saliva, urine, sweat and mucus play key roles in digestion, absorption, distribution of nutrients, hormones, oxygen and heat, excretion, and maintenance of homeostasis. Body fluids contain electrolytes, hormones and immunoglobulins which play a critical role in body functioning, including conduction of nervous impulses, contraction of muscles, regulation of pH levels and protection against infection. Owing to the vital role that electrolytes and immunoglobulins play in body fluids, they are often tested as markers to indicate physiological conditions such as acute and chronic illnesses, and as an aid for diagnosis (De Bock et al., 2010). In the field of sports and exercise, markers can be used to assess the long-term effects of training as well as the acute effects of exercise. Measurement of markers will enable athletes to monitor their performance and recovery, as well as their level of fitness, hydration status, inflammation, oxidative damage and fatigue while training at different levels (Palacios et al., 2015).

The gold standard method for assessment of hormonal and immune responses to exercise and training is via a blood test. Blood testing has advantages, such as high sensitivity and the ability to test a wide range of analytes in the one body fluid (Celec et al., 2016), but it also requires a skilled technician and specific equipment. Problems with bleeding and infection can occur, and people may feel faintish during and after a blood draw. Overuse of the same area for blood collection can also cause scarring. Blood draws pose a significant risk to healthcare workers and technicians due to inadvertent exposure to infectious diseases due to the use of sharp collecting devices (Sharew et al., 2017). Alternative strategies for assessing markers by using other body fluids, such as urine, saliva and sweat are being studied to overcome the above-mentioned limitations of blood collection. Previously pairwise comparisons have only been undertaken between blood and other body fluids, such as urine, salvia or sweat in specific populations such as in diabetic patients and HIV-positive women (Labat et al., 2018; Moyer et al., 2012). However, while some studies (Ezukwa et al., 2018; Walsh et al., 2004a) showed a strong correlation between the analyte levels measured in blood and other body fluids, other studies showed no significant correlations (Francavilla et al., 2018; Wankasi et al., 2019).

For athletes, the use of other body fluids such as saliva, urine and/or sweat (instead of blood) for exercise monitoring has several potential advantages. Collection of saliva, urine and sweat are non-invasive and may induce less stress than blood sampling. These body fluids enable

the measurement of analytes without ‘‘contamination" of the results due to needle tension and also allows for self-sampling (Gatti & De Palo, 2010). In comparison to blood, saliva, urine and sweat are readily available for athletes to collect before or during athletic practice. Saliva and sweat have the added advantage that they can be collected during training without significant interruption to the training regime, thus utilisation of these fluids may provide the opportunity to monitor exercise performance with minimum disruption.

Exercise causes a significant impact to whole body homoeostasis, causing widespread changes in a variety of cells, tissues and organs, as a result of, or in response to increased metabolic activity (Hawley et al., 2014). While exercising, especially in the heat, large amounts of water are lost through sweating, leading to a water deficit. Because sweat is hypotonic, exercise-induced dehydration leads to a decrease in extracellular fluid volume (Lee et al., 2017). Blood osmolality is often used as the standard for the assessment of hydration state, especially for acute and dynamic changes of hydration (Lee et al., 2017). However, this method, requiring a blood draw, is technically demanding and invasive. Recently, increasing numbers of studies have used other body fluids such as urine, saliva and sweat, obtained non-invasively, to measure osmolality and monitor hydration status of athletes, to overcome the limitations of blood collection (Lee et al., 2017; Walsh et al., 2004a).

Exercise also affects the immune system by causing changes in hormones and cytokines (Gleeson, 2007). A strong connection has been suggested to exist between exercise, the mucosal immune system and infections, especially incidence of upper respiratory tract infections (URTI) (Rosa et al., 2014). Immunoglobulin A (IgA), one of the most abundant antimicrobial proteins, plays a critical effector role in the mucosal immune system, acting as a "first line of defence" against pathogens (Khaustova et al., 2010; Usui et al., 2011). Exercise has been shown to cause changes in salivary IgA concentrations, with the degree of change being dependent on the duration and frequency of exercise (Trochimiak & Hübner-Woźniak, 2012).

It is commonly accepted that there are physiological and morphological differences between males and females, and these differences become even more evident in the specific responses or magnitude of response to various training regimens (Lewis et al., 1986). Yet, females are significantly underrepresented in exercise research – the average percentage of female participants per article across sports and exercise journals ranges from 35% to 37% (Costello et al., 2014). Lack of female representation in exercise studies leaves them disadvantaged as there is a gap in knowledge about exercise physiology among females. Also, the validity of results from studies which pooled males and females, without

acknowledging the sex differences, is questionable, and restricts the ability to apply the findings across both sexes.

Taking into consideration the physiological and morphological differences between males and females, this study will investigate the levels of electrolytes, osmolality and/or IgA in blood, saliva, urine and sweat in males and females during exercise. To the author's knowledge, no study has investigated the level of electrolytes and osmolality in all four body fluids simultaneously during exercise. Therefore, this study will also investigate the interaction of exercise and time, in electrolytes, osmolality and IgA levels, thereby enabling a complete comparison of electrolytes, osmolality and IgA levels during exercise in the different body fluids. Finally, this study will explore the relationship between the levels of electrolytes, osmolality and IgA in blood and/or saliva, urine and sweat during exercise.

1.2 Aims

This study had 3 main aims:

1) To investigate the electrolyte (Na^+, K^+ and Cl^-) and osmolality levels in blood, saliva, urine and sweat, and IgA levels in blood and saliva, in males and females, while exercising.
2) To study the changes in electrolyte and osmolality levels in blood, saliva, urine and sweat, and IgA levels in blood and saliva over time, while exercising.
3) To explore relationships between blood levels (gold standard) of electrolytes and osmolality with those in saliva, urine and sweat, and blood and saliva IgA levels while resting and exercising.

1.3 Thesis Structure

This thesis is comprised of five chapters:

Chapter 1 presents the introduction, aim and objectives for this thesis. It provides the introduction to body fluids, explains the need for an alternative strategy for blood screening and outlines the impact of exercise on body fluids and importance of determining sex differences in exercise trials. This chapter also includes the researcher contributions.

Chapter 2 presents the literature review undertaken for this thesis. It provides a detailed background to the different types of body fluids, electrolytes and IgA using previous research data, and highlights gaps in the literature.

Chapter 3 is presented in the form of a manuscript to be submitted to the Journal of Food Nutrition and Metabolism. It has its own abstract, introduction, material and methods, discussion and conclusion sections. This chapter explores the relationships between electrolyte levels, osmolality and IgA in saliva, sweat, urine and/or blood among resting and exercising participants, and focuses on the differences in electrolyte, osmolality and IgA levels in exercising males and females.

Chapter 4 presents the conclusion to the thesis, outlining the strengths and limitations of the study, and presents recommendations for future research.

Following this are references and a series of appendices containing the pre-exercise health screening questionnaire and participant information sheet.

APA 7th referencing style will be used throughout this thesis to maintain consistency, however, the manuscripts will be updated to suit the journal's specific referencing style before submission.

In addition to the work presented here, another manuscript entitled '**Assessment of changes in physiological markers in different body fluids at rest and after exercise**' which encompasses the content of the Literature Review (Chapter 2) has been prepared during this Master's course for submission to the Journal of Sport and Health Science.

1.4 Researcher Contributions

Table 1.1 Researcher's contributions to thesis study

Contributors	Research contribution
Amalini Chithra Kandeepan	Principal researcher – thesis author Research study proposal, literature review, participant recruitment, data collection, cleaning and analysis, results formulation and subsequent discussion, thesis manuscript preparation
Associate Professor Kay Rutherfurd-Markwick	Primary academic supervisor Provided supervision for the study including the design and conduct of the study, ethics application, and advice on thesis chapter writing and manuscript preparation
Professor Ajmol Ali	Academic supervisor Provided supervision for the study including the design and conduct of the study, ethics application, and advice on thesis chapter writing and manuscript preparation

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to Body Fluids

An adult human body is made up of ~60% water by mass. Body fluids play a key role in digestion, absorption, distribution, waste excretion, and maintenance of homeostasis (Celec et al., 2016; Jain, 2015). Total body fluid can be categorised into extracellular fluid (ECF) and intracellular fluid (ICF). The system that includes all the fluid enclosed in cells by their plasma membrane is called the ICF. Most chemical reactions occur in the ICF and this accounts for 40% of total body weight. Of the remaining total body weight, 20% is due to the ECF and this can be further divided into plasma and interstitial fluid (IF). Body fluids vary in composition between body compartments, depending upon exchanges between the cells in the biological tissues and blood, for example sodium and chloride are present in high amounts in the ECF (plasma), whereas potassium and phosphate are higher in the ICF (Reddi, 2018). Changes in concentration or composition of biochemical constituents in body fluids are therefore used as indicators of physiological or pathological conditions (Colon, 1997).

A physiological marker or biological marker is a measurable indicator of the body's physiological or pathological status. Plasma, serum, urine, saliva, sweat, stool, cerebrospinal liquid, tears, and amniotic fluid are used for diagnostic purposes as well as in scientific and health research; of these, serum, plasma, urine, and saliva are the most popular due to the ease of collection, lower ethical cost and the wide range of tests that can be performed using these fluids (De Bock et al., 2010). In the field of sports and exercise, the use of physiological markers has become a popular method for monitoring an athlete's training response, as the body's physiology is significantly different at rest and after exercise (Saw et al., 2016). Certain markers may be used to assess the immediate or long-term effects of exercise. In sports and exercise, body fluids such as urine and saliva are preferred, as the collection is simple, non-invasive, cheap and does not require a specialised technician. A physiological marker's concentration during exercise depends on several variables, including, but not limited to sex, age, fitness level, the length and intensity of exercise, degree of exhaustion and hydration status of the athlete (Palacios et al., 2015). Compared to the resting state, exercise poses a substantial increase in demand on the body and disturbs the body's homeostasis, causing a shift in hemodynamic and metabolic processes which leads to both fluid and electrolyte changes in the body (Grant et al., 1996). Although the effect of exercise in various body fluids is well substantiated, to our knowledge no article has reviewed it across multiple body fluids.

This literature review will first provide an introduction to each body fluid, detail the different collection methods used for each fluid, and explain where known, the effects that exercise has on the composition and production level of body fluids, and on the osmolality of the fluids.

Next, the literature review will provide an introduction to electrolytes, and individually cover sodium, potassium and chloride, and their roles during exercise. The third section will discuss IgA as an immune function marker. Any differences reported in the existing research between males and females will be highlighted throughout the literature review where possible. Finally, this review will summarise the limitations in current research and provide future directions. This information will be relevant especially for athletes, as electrolyte levels can impact on their performance, and the osmolality level is a marker for hydration status.

2.2 Literature Review Methodology

Information for this literature review was collected from the PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) database. Comprehensive literature searches were conducted between August to March 2021 using Google Scholar, Scopus and PUBMED. Keywords, such as exercise, physical activity, serum, sweat, urine, biomarkers along with Boolean operators such as ‘and’ or ‘or’ were used.

2.3 Blood (Serum and Plasma)

Blood is a vital fluid required for the transport of nutrients, gases and waste; it also serves to protect against infection by transporting white blood cells to target pathogens and to control pH and temperature (Lindsay M. Biga et al., 2020). Blood is a complex mixture of plasma, white blood cells, red blood cells and platelets. Plasma contains about 92% water with the remainder comprising of dissolved gases, proteins such as albumin and globulin, immunoglobulins and electrolytes such as sodium, potassium, bicarbonate, chloride and calcium (Mathew et al., 2020). Both blood plasma and serum are widely used in research and many investigators consider them to be similar. However, they are not, and the inappropriate use of plasma or serum during diagnostic testing can lead to an incorrect diagnosis (Mannello, 2008). Serum is obtained from blood that has coagulated. As a result, the fibrin clot, blood cells and associated coagulation factors produced during coagulation are separated from the serum following centrifugation (Yu et al., 2011). Plasma samples are obtained when samples are collected in the presence of an anticoagulant (such as EDTA or heparin) and centrifuged to extract cellular components (Lundblad, 2003). Plasma has a slightly higher protein concentration than serum, with average values of 74.5–75.8 g/L for heparinized plasma (Ladenson et al., 1974) versus 72.1–72.9 g/L for serum (Lum & Gambino, 1974). This

difference has been attributed to the conversion of fibrinogen to fibrin during *in vitro* clot formation in serum (Ladenson et al., 1974).

During exercise, blood ensures the transport of oxygen from the lungs to tissues, and the delivery of metabolically produced carbon dioxide to the lungs for expiration (Mairbäurl, 2013). While exercising, sweat-induced hypohydration reduces plasma volume and increases plasma osmotic pressure in proportion to the amount of fluid lost (Sawka et al., 2015). Osmolality is the estimation of the osmolar concentration of plasma and is proportional to the number of particles per kilogram of solvent. During exercise, plasma volume reduces because it provides the precursor fluid for sweat, and osmolality increases because sweat is ordinarily hypotonic relative to plasma. Sodium and chloride are primarily responsible for the increased plasma osmolality. Plasma volume expansion normally occurs both with acute endurance exercise and training, with an 11.6% increase in plasma shown to be induced after 3 days of high intensity exercise (Green et al., 1984). Additionally, hemodilution caused by plasma volume expansion leads to a decrease in the hemoglobin concentration and hematocrit (Fellmann, 1992).

Blood (plasma or serum) osmolality is often regarded as the most reliable method for determining the hydration status and the gold standard method to assess body fluid balance (Hamouti et al., 2013; Sollanek et al., 2011). Blood osmolality is considered a good marker for acute or critical dehydration situations, however, it seems to lose its sensitivity when used to assess mild hydration or long-term body fluid deficit (i.e., hypohydration) (Hamouti et al., 2013). Popowski et al. (2001) demonstrated that under well-controlled conditions, with no fluid replacement, plasma osmolality increases by ~5 mOsm/kg for every ~2% loss of body mass by sweating. There are currently no studies that look at progressive changes in plasma osmolality, with fluid intake, during exercise. Also, there is a lack of studies that investigate both males and females to provide an understanding of any sex differences in plasma osmolality that may occur during exercise.

2.4 Saliva

Saliva is a clear, slightly acidic mucoserous exocrine secretion that consists of 99% water and 1% electrolytes (e.g., sodium, potassium, calcium, magnesium, bicarbonate, phosphates), proteins, immunoglobulins, metabolites, enzymes, hormones and vitamins (De Almeida Pdel et al., 2008). Whole saliva is formed primarily from the secretions of three major salivary glands (submandibular, parotid and sublingual glands), which together account for about 90% of the fluid production and electrolyte content; the minor salivary glands, which includes about

600-1000 glands, account for the remaining 10%. Each salivary gland secretes a characteristic type of saliva with different ionic and protein characteristics (Denny et al., 2008). Figure 2.1 shows the anatomical locations of the major and minor salivary glands and Table 2.1 lists the main salivary glands, composition of the saliva generated from these glands and the percentage contribution during unstimulated flow (Lee et al., 2019). Proteomic analysis has been used to catalogue the salivary proteins generated from the different salivary glands and their ductal secretions. However, variations exist between reported results, possibly due to sample collection methods, storage conditions, sample integrity, analytical methods and number of participants in the trials; this variation has hindered the use of saliva as a physiological and pathophysiological research tool and as a reliable fluid for disease diagnosis (Saitou et al., 2020).

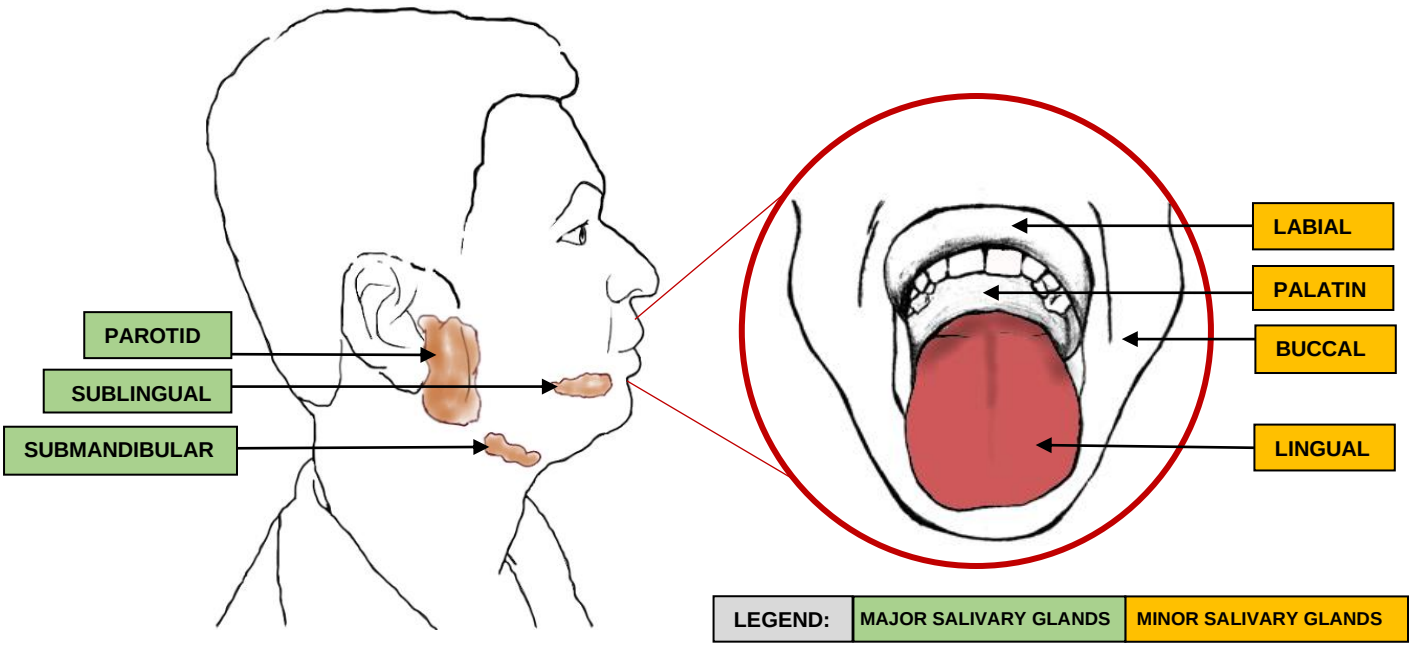

Figure 2.1 Locations of the major and minor salivary glands

Table 2.1 The different types of salivary glands, their location, composition of fluid secreted and percentage contribution to total salivary flow.

Salivary Gland	Location	Composition	Percentage contribution during unstimulated flow
Parotid	Side of the face, below and in front of each ear	Serous saliva: a watery secretion rich in enzymes e.g., amylase and proline-rich proteins (PRP)	20%
Submandibular	Near the inner side of the lower jawbone, in front of sternomastoid muscle	Mixed secretion that is both serous and mucous	65%
Sublingual	Directly under the mucous membrane covering the floor of the mouth beneath the tongue	Mucous saliva: a viscous secretion containing no enzymes and large amounts of mucus	7-8%
Minor salivary glands	Spread throughout the submucosa of the sinonasal cavity, oral cavity, pharynx, larynx, trachea, lungs, and middle ear cavity	Secretion that is dependent on its location; includes pure mucous or serous or mixed secretion	10%

The main functions of saliva are to protect the oral tissues against bacteria, maintain pH, initiate the digestion of starch, enhance taste, provide antibacterial and antiviral activity and enable speech by lubricating the moving oral tissues (De Almeida Pdel et al., 2008; Kumar, 2017). Impaired salivary secretion (hyposalivation) has been shown to increase the risk of oral diseases such as dental caries and oral candida infection (Pedersen et al., 2005).

The constituents of saliva and rate of flow differ greatly between collection methods. Saliva collection can be divided into three categories: stimulated vs. unstimulated saliva, whole saliva vs. sampling from specific salivary glands, and those collected using absorbent materials vs. techniques based on passive drooling or spitting of saliva into collection tubes (Rutherfurd-Markwick et al., 2020). At rest, there is a small continuous flow of saliva, without any exogenous or pharmacological stimulation. This unstimulated secretion is the baseline level that is present at all times in the form of a film that covers, moisturises, and lubricates the oral tissues (De Almeida Pdel et al., 2008); in healthy adults, this has a flow rate of about 0.4–0.5 mL/minute (Lorgulescu, 2009). Unstimulated whole saliva is usually collected using the `draining' or ‘drool’ method where the participant's head is tilted forward so that saliva moves towards the anterior region of the mouth and the pooled saliva is drooled into a sterile wide-mouthed container. Stimulated whole saliva is produced in response to a mechanical, gustatory, olfactory, or pharmacological stimulus and expectorated into a tube (Bellagambi et al., 2019); this accounts for 40-50% of daily salivary production (Dawes & Watanabe, 1987). The stimulated method of collection is undesirable for diagnostic applications as the foreign substances used to stimulate saliva production tend to alter the fluid pH and generally stimulate the water phase of saliva secretion, resulting in dilution of proteins. Specifically, the

contribution of the parotid gland increases from 20% to more than 50% of the total saliva (Humphrey and Williamson, 2001).

Whole salivary collection is the most common and least invasive procedure (Bellagambi et al., 2019). Sampling saliva from individual salivary glands, such as parotid, submandibular, sublingual and the minor glands is possible using unique sampling methods, and the pros and cons of saliva sampling from specific salivary glands is discussed in detail by Bellagambi et al. (2019). One study where saliva was collected in different areas of the mouth by placing absorbent cotton swabs in locations to specifically collect saliva from the three major glands yielded different values for salivary alpha-amylase from the sample of whole saliva (Harmon et al., 2008). Saliva collected using absorbent materials is performed by introducing a synthetic gauze sponge, pre-weighed swab or cotton pad into the mouth, at the orifices of major salivary glands to collect stimulated or unstimulated saliva, which can then be extracted by centrifuging the absorbent material (Rutherfurd-Markwick et al., 2020). This method is easy to perform and advantageous when the salivary flow rate is low. A disadvantage of absorbent-based collection techniques is that stimulation of salivary flow cannot be completely excluded due to the presence of the mechanical stimulus in the mouth, even if participants are instructed not to chew on the material (Navazesh, 1993).

When using saliva for measurements, it is important to note that cortisol exhibits circadian rhythm, with peak concentrations in the morning, around the commencement of diurnal activity, and reduced concentrations in the evening and overnight, and therefore caution needs to be used when interpreting results to ensure sample collection times occur at the same time of day or night (Ushiki et al., 2020).

A limited number of studies have aimed to study correlations between the composition of certain substances in saliva and blood in specific populations and Table 2.2 shows a summary of these studies. Across the three studies (Francavilla et al., 2018; Labat et al., 2018; Wankasi et al., 2019), sodium, chloride and IgA levels were lower in saliva compared to blood, whereas potassium was higher in saliva.

Table 2.2 Summary of studies comparing electrolyte and IgA levels in blood and saliva in specific populations.

Study Population	Concentration/ osmolality compared to blood	Saliva collection method	Reference
Mix of 241 healthy and hypertensive individuals	**Sodium:** 10x lower in saliva **Potassium:** 10x higher in saliva	Unstimulated - Whole buccal saliva	Labat et al. (2018)
100 Healthy and diabetic individuals	**Sodium:** 11x lower in saliva (healthy individuals) and 16x lower (diabetic patients) **Potassium:** 4.5x higher in saliva (healthy individuals) and 6x higher (diabetic patients) **Chloride:** 5x lower in saliva (healthy individuals) and 6-fold lower in saliva (diabetic patients)	Unstimulated - Spitting method	Wankasi et al. (2019)
29 Soccer players	**IgA:** 1.4x lower in saliva	Stimulated - Using absorbent material	Francavilla et al. (2018)

Electrolytes enter the saliva via osmotic gradients and are regulated by the rate of secretion, nature of the stimulus and level of mineralocorticoids in the circulation. The organic components of glandular saliva are largely from protein synthesis and are stored as granules within the acinar cells (Malamud, 1992). As serum components of saliva are derived primarily from the local vasculature that originates from the carotid arteries (Leonard, 2018), saliva has a fluid source that provides many, if not most, of the same molecules found in the systemic circulation, thus making saliva a potentially valuable fluid for the screening, diagnosis and monitoring of various systemic diseases.

Saliva provides distinct advantages over blood as it can be collected non-invasively and even by the patients themselves. It does not require any special equipment for collection and storage. In addition, because of the low concentrations of antigens in saliva, the risk of accidental HIV and hepatitis infection are much lower when handling saliva than blood (Kang & Kho, 2019). Unlike blood, saliva does not clot and can be easier to collect particularly from individuals from whom blood drawing is difficult such as in obese and/or haemophilic patients. There are other advantages for saliva collection including being inexpensive, not requiring trained medical staff and multiple samples can be collected *in situ*. Saliva can be used to detect oral and systemic illnesses at an early stage using salivary proteome (Hunggund & Rai, 2010). Significant progress has been made in cataloguing human saliva proteins and exploring their post-translational modifications for the possible use of saliva for diagnostic and

prognostic purposes. Currently, salivary diagnostics is gaining potential as a technique of choice with more flexibility, standardised techniques and consistent reference values (Sindhu & Jagannathan, 2014).

In the field of sports and exercise, recent research has focused on the possibility of testing hormonal and immunological responses to acute exercise or training using saliva rather than blood samples (Papacosta & Nassis, 2011). In comparison to blood, saliva is easily accessible for collection by athletes not only before and after sports activity but also during training. Obtaining blood or urine samples while training is difficult in terms of collection because it necessitates a break in the training. Exercise causes stress and this activates hormones such as cortisol, which can be easily measured in saliva (Gatti & De Palo, 2010). Exercise is also known to affect salivary secretion, inducing changes in concentration, secretion rate, and composition of various salivary components, such as electrolytes, immunoglobulins, hormones, lactate and proteins (Chicharro et al., 1998; Li & Gleeson, 2004). In general, during intracellular dehydration, the salivary flow rate reduces, and saliva osmolality increases significantly. Water being the predominant fluid constituent of saliva enters the saliva from plasma through acinar cells. Reduction of body fluids during dehydration leads to the salivary glands' hypofunction (Walsh et al., 2004a).

Salivary immunoglobulin A (IgA) levels have been reported to depend on the intensity and duration of exercise, hydration status of athletes, saliva collection methods, diurnal variation and the method used to express IgA (i.e., whether IgA is reported as absolute concentration, secretion rate, ratio to total saliva protein, ratio to saliva albumin or ratio to saliva osmolality) (Blannin et al., 1998; Gleeson et al., 2000; Laing et al., 2005; Nieman et al., 2002; Rutherfurd-Markwick et al., 2017). Most research shows that intensive, repetitive exercise causes a decrease in salivary IgA levels in athletes (Peters, 1997).

2.5 Urine

Urine is a clear, light-yellow fluid that is produced as a waste product by the kidneys in its process of cleaning the blood. It consists primarily of water (91%-96%), with electrolytes (e.g., sodium, potassium, chloride, magnesium, calcium etc), organic solutes (e.g., urea, creatinine, uric acid), trace amounts of enzymes, hormones, fatty acids and pigments (Rose et al., 2015). The main function of the urinary system is to filter blood, eliminate wastes from the body, regulate blood volume and pressure, control levels of electrolytes and metabolites, and regulate blood pH. A healthy adult excretes between 1000-1800 mL of urine a day. The

amount and concentration of urine excreted varies with the level of exertion, the environment, the level of hydration, and the intake of salt and protein.

Urine samples are categorised by the collection procedure used to obtain the specimen. The pooled collection sample is the most commonly used method to collect samples to measure creatinine, urea nitrogen, glucose, sodium, potassium, or analytes concentration in urine over a specified length of time, usually 8 or 24 hours. In order to determine the concentrations and ratio of the analytes, precise recording of start and stop times for the collection are important (Albert Rabinovitch et al., 2001). However, this method should not be applied in understanding post-exercise changes, as the majority of the studied parameters show changes over time during exercise, but return to normal values over the following 24 hours (Bakońska-Pacoń & Borkowski, 2003). Also, pooled urine collection is not practical for athletes during exercise. In order to examine post-exercise changes in urine biochemical parameters, single specimens are preferred (Bakońska-Pacoń, 2006).

Exercise increases the perfusion of active muscles, whereas the perfusion of body organs, such as the kidneys may decrease by as much as 25% (McAllister, 1998; Poortmans, 1984). During exercise the oxygen demand by muscle increases, and therefore blood flow is rerouted from internal organs, including the kidneys. This rerouting process leads to a decrease in glomerular filtration rate (GFR), renal plasma flow (RPF) and an increase in proteinuria and hematuria (Kachadorian & Johnson, 1970). The changes in GFR and RPF during exercise are the result of increased renal sympathetic nerve activity and a rise in catecholamine secretion (Poortmans, 1984; Tidgren & Hjemdahl, 1988; Wade & Claybaugh, 1980). In turn these changes in GFR, RPF and plasma antidiuretic hormone affect urine volume, osmolality and excretion of electrolytes (e.g., sodium, potassium, chloride and phosphate), urea nitrogen and lactic acid.

Exercise induces a state of dehydration which affects the concentration of urinary biomarkers (Lindsay & Costello, 2017). The level of dehydration depends on the type, intensity and duration of exercise, humidity and temperature of the environment, as well as the hydration status of the individual (Kachadorian & Johnson, 1970). During exercise, sweat glands have a priority over the kidneys in their demand for salt and therefore urine salt excretion is reduced due to the loss of salts in sweat (Love, 2010). Hypohydration is common during exercise when sweat loss is not replaced, and this leads to a concentration effect in the urine (Lindsay & Costello, 2017). Numerous studies have documented the deleterious effects of hypohydration on athletes and their performance during exercise (Deshayes et al., 2020; Lewis et al., 2017). Symptoms of hypohydration can range from thirst, flushed skin, apathy and discomfort to dizziness, headaches, nausea, chills and vomiting when severe (Shirreffs et al., 2004).

Two of the most common measures using urine are osmolality and urine specific gravity (USG). Urine osmolality varies between 50-1200 mOsmol/kg depending on an individual's hydration status (Ferri, 2014). Urine specific gravity is a measure of the ratio of the density of urine to the density of water, and this can vary between 1.002 to 1.030 (Stuempfle & Drury, 2003). Determination of urine osmolality is more important than USG because it gives the most accurate measurement of total solute concentration, and therefore provides the best measurement of the kidney's concentrating ability (Armstrong, 2005). Urine osmolality is also used as a tool for assessing hydration status and increases in urine osmolality have been observed after both acute and prolonged exercise (Bongers et al., 2018). Urine is an ideal clinical specimen for analysis in sports medicine as it is excreted in large quantities and collection does not require invasive methods and it can be collected in a range of field settings. A variety of urine preservatives, such as tartaric and boric acids, are now available that allow urine to be kept at room temperature while still providing results comparable to those of refrigerated urine. A greater percentage of athletes may agree to be tested regularly as urine collection, unlike blood collection does not require venepuncture or finger prick. Table 2.3 shows a summary of two studies comparing electrolyte and osmolality levels in blood and urine after exercise.

Table 2.3 Summary of studies comparing electrolyte and osmolality levels in blood and urine.

Study Population	Exercise Protocol	Concentration/ osmolality compared to blood	Urine collection method	Reference
21 males aged 21-56 years	Skiing, 70 km	**Sodium:** ~ 2X lower in urine **Potassium:** 29X higher in urine **Chloride:** 0.09X higher in urine	Single specimen urine	Refsum and Strcmme (1975)
35 males aged 18-30 years	**Acute:** Cycling, 80% HR_{max}*, 30 min **Prolonged:** Cycling, 80% HR_{max}*, 120 min or 3% hypohydration achieved	**Osmolality:** ~2x higher in urine after acute exercise 2.79x higher in urine after prolonged exercise	Single specimen urine	Bongers et al. (2018)

*HR_{max} = Maximum heart rate

Measuring sodium and potassium levels in athletes' urine is commonly done as it is a good indicator of any renal conditions involving the kidney's excretion ability (Gowda et al., 2010). A variety of hormonal and other factors affect the excretion of sodium in the urine. Any changes

that affect the kidney's ability to excrete water will lead to changes in the urinary sodium concentration. Therefore, the level of sodium in urine is measured to assess the volume status, salt intake in hypertensive athletes, to evaluate the calcium and uric acid excretion in stone formers, to calculate electrolyte-free water clearance and to diagnose hyponatremia and acute kidney injury (AKI). The level of urinary potassium in a healthy person represents the amount of daily dietary potassium intake. In a clinical setting, potassium in the urine is measured to calculate electrolyte-free water clearance and for diagnosis of dyskalemias and metabolic alkalosis, while chloride is measured for the diagnosis of metabolic alkalosis (Reddi, 2018).

2.6 Sweat

The skin is the largest organ of the human body and one of the most versatile organs. One of its key functions is to maintain thermal regulation of the body by producing sweat. Up to 30% of the body's waste is secreted as perspiration from the sweat glands through approximately 2.6 million tiny pores in the human skin (Chen et al., 2020). Sweat glands can be classified into three main types: eccrine, apocrine and apoeccrine (Baker, 2019). Eccrine sweat glands, which are the most numerous, are distributed across the body and are responsible for the highest volume of sweat excretion (Baker, 2019). Apocrine and apoeccrine glands play a smaller role in overall sweat production as they are limited to specific regions of the body, such as armpits, ears and eyelids (Baker, 2019). The secretory portion of apocrine glands is larger than that of eccrine glands (making them larger overall) and rather than opening directly onto the surface of the skin, apocrine glands secrete sweat into the pilary canal of the hair follicle (Clarke, 2018). Apoeccrine consists of those sweat glands that cannot be classified as either eccrine or apocrine and share properties with both eccrine and apocrine glands (Baker, 2019).

Sweat consists mainly of water, minerals (such as sodium, potassium, calcium, magnesium), lactate, urea and some trace metals (Baker, 2019). The chemical composition of sweat and the rate of sweating varies between individuals, type of sweat glands involved, individual's food and fluid intake, reason for sweating (for example, exercise or fever), environment, their sex and exercise intensity. Sweat plays several key functions in the body including temperature balance, fluid homeostasis, electrolyte regulation, and acting as an antimicrobial and water-proofing barrier (Hussain et al., 2017). Electrolytes, particularly sodium and chloride, are important components of sweat and affect the body's fluid balance. The concentration of sodium in sweat can be highly variable (20–100 mmol/L), and some individuals can lose an estimated 4–6 g of sodium per day via sweat, especially if working in moderately hot conditions (Hussain et al., 2017).

Sweat as an alternative for blood is being explored due to a number of advantages including being able to be collected in a non-invasive manner with little-to-no pain, and without a specialised technician. Sweat collection is straightforward, well tolerated by athletes and can be achieved in an outdoor environment. Simple collection devices, using sweat as a medium, to check for drugs of abuse or alcohol are already on the market. Moreover, recent advances in wearable electronic devices that use sweat samples, have allowed at-home health monitoring of glucose and oxygen-saturation (Moonen et al., 2020). Interest in the investigation of sweat components has grown over the last century, contributing to the current value of sweat as a potential body fluid for diagnosis of tuberculosis, diabetic neuropathy and lung cancer (Adewole et al., 2016; Calderón-Santiago et al., 2015; Ponirakis et al., 2014).

The outcome of sweat analysis depends on the method of sample collection, and the accuracy and sensitivity of the analytical method. The three most common types of sweat test are the whole body wash down (WBW) technique, patch testing and non-exercise sweat testing. WBW is considered the 'gold standard' in sweat collection and analysis as it aims to collect 100% of the generated sweat (Lewis & Silkey, 2017). More details about these methods can be found in Baker et al. (2009). As the name implies, this method requires washing the subject entirely with de-ionised water and then requesting them to work out (presumably naked) in an enclosed plastic environment. Every drop of sweat generated is collected and analysed to give a true assessment of the total body sweat and electrolyte loss. However, this technique is time-consuming and complex, and is mostly used only in research studies that aim to collect 100% of the sweat loss, for assessment of total body sweat and electrolyte losses.

Patch collection methods can vary from using a simple collection absorbent sheet stuck to the body to more commercially-available products such as PharmChek® sweat patches (Hussain et al., 2017). During exercise, sweat patches are stuck on the skin and these patches absorb the sweat as it is generated. Then the patches drenched with sweat are removed and centrifuged to collect the absorbed sweat. The advantages of this system are that it can be stuck to different parts of the body, and is useful when collecting sweat from athletes in an outdoor environment, thus allowing samples to be collected in a natural setting and the results obtained are more representative of those that would occur during real world sporting scenarios. The patch method, however, does not collect 100% of the generated sweat, and some of it is lost as evaporation from patches, and contamination of patches that are not handled correctly can significantly skew the results. Baker et al. (2009) studied sodium and potassium loss during exercise, with sweat collected by both regional patch collection and WBW. The study found that the regional patch collection method generally overestimates the electrolyte excretion compared to WBW, possibly due to variations in the local sweat rate

across different regions of the body. Baker et al (2009) showed that sweat rates at the posterior mid forearm, back, upper chest and forehead were higher than the whole body sweat rate.

Large variations exist in the amount and location of sweat produced between individuals, creating major difficulties for those attempting to design a universal sweat collection device. Skin irritation, alterations of skin pH, disruptions to skin barrier properties and interactions with differing individuals' skin microbiota are just some of the difficulties encountered in designing an ideal sweat collecting apparatus (Giovanni & Fucci, 2013).

Non-exercise sweat testing is a method that has been developed to collect sweat without the subject having to exercise (Baker, 2017). This method involves applying a chemical (pilocarpine) to the skin that mimics the effect of certain neurotransmitters that stimulate the sweat glands, allowing sweat samples to be collected 'at rest'; this is especially useful for athletes who want to measure their at-rest electrolyte levels relative to exercising sweat loss. Non-exercise sweat testing is also less time consuming than manual sweat collection and does not require substantial physical effort. Moreover, it is practical in young children or cystic fibrosis patients who loose significant amounts of sodium chloride when sweating.

Multiple studies have reported that males exhibit a higher sweat rate than females, with some of the reasons being independent effects of sex and some being due to confounding physical characteristics (Andérson et al., 1995; Havenith & van Middendorp, 1990). Males have a higher body mass, metabolic heat production, greater cholinergic responsiveness and maximal sweating rate than females (Baker, 2019). On the other hand, females have a greater body fat content that allow them to better tolerate low ambient temperatures (Kaciuba-Uscilko & Grucza, 2001). However, sweat gland density is generally higher in females than males, due in part to lower body surface area leading to a reduced output per gland and therefore a reduced sweat rate in females. However, studies which looked at sex differences by matching participants for weight, surface area and metabolic heat production reported that there is a variation in whole-body sweat production only at certain environmental conditions (35–40°C, 12% rh) and metabolic heat production rate (300–500 W/m^2) (Gagnon et al., 2013; Gagnon & Kenny, 2012).

While exercising, the body temperature rises leading to sweat glands starting to secrete sweat with the subsequent evaporation of moisture from the skin helping to cool the body's temperature. The level of electrolytes such as sodium and chloride lost via sweat during exercise varies within and between athletes (Baker et al., 2016; Sawka et al., 2007). One explanation for this difference is the exercise intensity differences across training sessions, variations in environmental conditions (e.g., ambient temperature, humidity, wind, and solar

load), and individual factors (e.g., race/ethnicity, training, and heat acclimation status) (Baker et al., 2019).

Despite its advantages, sweat is still not a commonly used body fluid for routine testing. Collection of sweat in a sedentary or non-exercising state is challenging, and transport of small sample volumes requires sophisticated storage methods to enable sufficiently fast transport from a collection site to a testing site to ensure that the sample composition isn't compromised due to evaporation or condensation (Moonen et al., 2020). For a true measurement of the body's state, measures must be taken to reduce evaporation, and prevent the accumulation of dried sweat components, which can obstruct reliable and repeatable flow. Also, skin topography, sweat rate per gland, and the number of active sweat glands differ significantly between people, as well as between measurement sites on the same person (Moonen et al., 2020). All these factors restrict the use of sweat samples for routine testing of analytes in athletes on a larger scale.

2.7 Introduction to Electrolytes

Electrolytes are minerals that have an electric charge (Garrett, 2017). Electrolytes such as sodium, potassium, magnesium, calcium, phosphorus play an important role in maintaining cell membrane functions, nerve conductivity, muscle contractility, hormonal activity, bone structure, and fluid and acid-base homeostasis (Willis et al., 2015). Body fluids vary in composition between body compartments, depending upon exchanges between the cells in the biological tissues and blood, for example sodium and chloride are present in high amounts in the ECF (plasma), whereas potassium and phosphate are higher in the ICF (Reddi, 2018).

Electrolytes enter the body through the digestive tract from consumed food and fluid. While water moves passively via osmosis, sodium and potassium ions move in and out of cells via the sodium-potassium pump in the cell membrane. This pump is powered by adenosine triphosphate (ATP) to provide the energy to move the ions against their concentration gradients (i.e., sodium moves out of the cell and potassium is pumped in) and maintain the gradients inside and outside the cell (Pirahanchi et al., 2021).

2.7.1 Sodium

Sodium is the most common cation in the body (Willis et al., 2015). The normal range for blood sodium is 135-145 mEq/L and most of it is found in the ECF (Garrett, 2017). Around 40% of

the body's sodium is found in the bone, and the remainder in organs and cells. However, being a highly reactive element, it can move fairly easily between the fluid compartments and accounts for 90% of the ECF osmotic activity (Betts et al., 2013). Sodium plays three important roles, namely fluid balance, osmotic regulation and maintenance of membrane potential. As part of the sodium bicarbonate molecule, it also plays an important role in regulating acid/base balance. The body's source of sodium is from the diet. About 10% of sodium consumed occurs naturally in food, 15% is added during food preparation, and the rest comes from processed and manufactured foods (Eyles, 2011). An average person requires about 920-2300 mg of sodium per day, however daily intake usually exceeds this amount (*Food and nutrition guidelines for healthy adults: A backround paper*, 2003).

Sodium is mostly eliminated in the urine, and some is lost as sweat through the skin. The kidneys monitor arterial pressure in the renal juxtaglomerular apparatus and retain sodium when the arterial pressure is decreased and eliminate it when the arterial pressure is increased through the action of the renin–angiotensin–aldosterone system (Jain, 2015). Sodium levels are also regulated by the sympathetic nervous system though a negative feedback system stimulated by osmoreceptors in the hypothalamus and baroreceptors in the carotid sinus and thoracic veins (Garrett, 2017).

The rate and composition of sweat loss can vary considerably within and among individuals (Baker, 2017). Large sodium losses, without adequate replacement can impair performance and may cause hyponatremia and muscle cramps. After exercising, lost water and electrolytes can be replaced with normal fluid and food intake, however, if there is severe hypohydration (4-5% body mass) or a rapid recovery is required (<24 h), aggressive fluid intake and electrolyte consumption is encouraged (Shirreffs & Sawka, 2011).

Variations in sodium levels between exercising males and females have been reported in a number of studies (Eijsvogels et al., 2009; Lewis et al., 2017; Meyer et al., 1992). As discussed in Section 2.6 males exhibit a higher sweat rate than females (Andérson et al., 1995; Havenith & van Middendorp, 1990) although these results are not consistently statistically significant. Thermoregulatory function in females varies during the menstrual cycle; changes mostly attributed to the hormone progesterone, and the core body temperature increases by 0.3°C to 0.7°C in the post-ovulatory luteal phase when progesterone is high compared with the pre-ovulatory follicular phase (Baker & Wolfe, 2020). Eijsvogels et al. (2009) reported that after exercise males show an increase in plasma sodium levels and higher incidence of hypernatremia, whereas females show a decrease in plasma sodium levels. This difference in plasma sodium levels was attributed to differences in hydration status between the male and female participants. However, 70% of the female participants in this study (Eijsvogels et

al., 2009) were postmenopausal. Therefore, neither this study nor other research articles have yet explored the role of the menstrual cycle on sodium levels in females and how it may contribute to the reported differences between males' and females' sodium levels.

2.7.2 Potassium

Potassium is the major cation found in the ICF. Most of the body's potassium content is found in the intracellular space of skeletal muscle. Normal levels of potassium in the blood are between 3.6-5.0 mmol/L for adults (Garrett, 2017). Potassium plays several important roles such as nervous impulse conduction, assisting breakdown of carbohydrates for energy, building amino acids into protein and acid/base balance regulation (Kowey, 2002). Potassium is obtained from dietary sources such as fruits and vegetables. An average person requires about 2800-3800 mg of potassium per day, but the actual requirements vary depending upon an individual's physical activity, the use of diuretics and sodium intake (*Nutrient Reference Values for Australia and New Zealand* 2005). About 90% of potassium consumed is lost in the urine, with the other 10% excreted in the stool, and a very small amount lost in sweat (Stone et al., 2016).

Potassium plays a key role in homeostasis in two ways - first, by controlling the transcellular distribution of K^+ ions, and second, by regulating K^+ ion excretion by the kidney, which maintains whole body K^+ ion balance. The cell interior has a negative charge due to the presence of intracellular organic phosphates that are negatively charged, causing a gradient for K^+ ions to move across the cell membrane. Specific channels in the cell membrane permit K^+ ions to diffuse out of the cells down their concentration gradient. The movement of K^+ ions into cells requires an increase in cell interior negative voltage and can be achieved by increasing flux via the Na^+/K^+-ATPase, which exchanges two extracellular K^+ ions for three intracellular sodium (Na^+) ions, resulting in a net export of positive charges out of the cell (Kowey, 2002). Excretion of potassium is regulated in the late cortical distal nephron, namely the late distal convoluted tubule, the connecting segment, and the cortical collecting duct (Stone et al., 2016). Potassium is freely filtered by the glomerulus of the kidney, with most of it being reabsorbed (70% – 80%) in the proximal tubule and loop of Henle.

During exercise, K^+ moves from contracting muscles into the blood, leading to an increase in its levels in the plasma and a decrease in the intracellular concentration (Lindinger & Sjøgaard, 1991). This movement of K^+ is rapid during the initial stages of exercise and gradually slows until a steady-state value is reached. Following the end of exercise, K^+ concentrations quickly return to baseline levels or lower (Kowalchuk et al., 1988). The extent of the decrease in intracellular K^+ concentrations is dependent on the length and intensity of muscular contraction

during exercise. Although a large number of studies have looked at changes in plasma K^+ during exercise, there is little data looking at K^+ levels after exercise or during recovery. This may be due to some studies not finding any observable changes in intracellular K^+ levels during exercise (Paton et al., 2001), leading to a lack of interest in determining if any changes occur after exercise or during recovery.

2.7.3 Chloride

Chloride is the main anion found in the ECF. Within the body, chloride is mainly present in red blood cells and the gastric mucosal cells that secrete hydrochloric acid (Garrett, 2017). Normal levels of chloride in the blood are between 96-106 mmol/L for adults (Garrett, 2017). Chloride, together with the sodium, plays a critical role in maintaining the body's fluid balance. In addition, chloride is a signification contributor to the production of hydrochloric acid, which is an essential digestive fluid produced by the parietal cells of the stomach. Chloride is obtained from the salt (sodium chloride) added to foods when cooking or eating. It is also found in food and vegetables such as seaweed, rye, tomatoes, lettuce, celery, and olives. An average person requires about 600-750mg of chloride per day, though this varies between individuals based on age, sex, illness, and pregnancy (National Research Council Subcommittee on the Tenth Edition of the Recommended Dietary, 1989). When food is digested, chloride is absorbed by the intestines in exchange for bicarbonate, and any extra chloride is lost in the urine. Aldosterone, a hormone produced in the outer cortex of the adrenal glands acts on the kidneys which are responsible for maintaining the total body chloride balance (Morrison, 1990).

Chloride levels outside of normal reference ranges (95-110 mmol/L) generally suggest a more serious underlying metabolic disorder, such as metabolic acidosis or alkalosis. Abnormal high levels of chloride in the blood, also known as hyperchloremia, occurs when there is an excess chloride in the ECF compartment or loss of water from this compartment; and this can also indicate dehydration. Abnormal low levels of chloride in the blood (hypochloremia), occurs when chloride is lost from the ECF or there is an excess extracellular fluid levels, such as in edema or similar causes including hyponatremia (low sodium concentration in the body).

There appears to be very limited research available on chloride levels during exercise, and even fewer studies looking at sex differences. A study on salivary diagnostic makers in males and females during rest and exercise showed a trend for a decrease in salivary Cl^- levels between rest and exercise for females, but no change for males. There were also no changes in salivary Cl^- levels during the rest or exercise trials for either sex (Rutherfurd-Markwick et al., 2017)

2.8 Immune function marker - IgA

Secretory immunoglobulin A (IgA) is the main immunoglobulin in saliva. It is a glycoprotein produced by mature B-cells in the blood, and can penetrate freely through the mucous membranes before being secreted into saliva (Macpherson et al., 2008). There are two subclasses of IgA: IgA1 and IgA2, with the former the predominant subclass in saliva (Gleeson & Pyne, 2000a). Secretion of IgA into saliva is stimulated by factors such as stress or physical activity. IgA is the first line of defence against the development of oral pathogens, thereby protecting the body against infection, with decreased levels of salivary IgA being associated with URTI (Macpherson et al., 2008).

Exercise has shown to affect both the levels of serum IgA, the concentration and rate of salivary IgA secretion, as well as other elements of immune function that influence the risk of URTI or URTI symptoms (Nieman, 1997; Nieman & Nehlsen-Cannarella, 1991). Although the link between physical activity and immune system suppression is not fully understood, it is known that moderate intensity exercise can boost immunological defences while severe exertion can weaken them by increasing the risk of URTI (Bellagambi et al., 2019). Nieman and Nehlsen-Cannarella (1991) showed that intense ultra-marathon exertion may be associated with a greater and more prolonged depression of serum immunoglobulin's than is sustained at shorter distances. Optimisation of training and optimal balance of exercise and rest intervals may lower the risk of immune system compromises and URTI occurrence (Trochimiak & Hübner-Woźniak, 2012).

There appears to be very limited research available on the differences in IgA levels between sexes at rest or during/after exercising. A study by Jafarzadeh et al. (2010), which measured the levels of salivary IgA without exercise, in males and females aged 1-70 years showed no significant differences in salivary IgA levels between sexes. Studies by Rutherfurd-Markwick et al. (2017) and Martins et al. (2009) which measured the levels of salivary IgA and serum IgA after exercise in males and females also found no significant differences by sex.

2.9 Limitations of the research and future direction

The majority of studies in sports and exercise journals have used males only, or males and females combined, and there are no studies reporting female-only data (Costello et al., 2014). The results of physiological markers in different body fluids are sex-dependent (Anderson et al., 1995; Havenith & van Middendorp, 1990), therefore it would be useful to study these makers in males and females separately to gain a better understanding of how the levels of these markers change in relation to exercise and performance.

Though the effect of exercise and exercise markers have been studied extensively in different body fluids, there are currently no studies that have investigated the effect of exercise on all the four major body fluids namely, blood, saliva, urine and sweat, simultaneously. While exercising, body fluids work together to provide the required physiological functions and maintain homeostasis (Grant et al., 1996), therefore studying the analytes affected by exercise in these body fluids will provide an in-depth understanding of the changes during exercise.

There were also no studies that have looked at progressive changes in plasma osmolality, with fluid-intake, during exercise. Similarly, studies looking at the changes in potassium levels post-exercise are limited. Therefore, it is important that these areas are explored in future research.

2.10 Summary of the Literature Review

In the field of sports and exercise, the gold standard method for assessing changes in hormonal and immune responses to exercise is via a blood test, owing to its high sensitivity and the ability to test a wide range of analytes. Blood collection however, requires a skilled technician and specific equipment. To measure an athlete's performance level, the use of saliva, urine and sweat, instead of blood has several advantages as they are non-invasive, and collection may induce less stress and also allow for self-sampling during a training regime.

Exercise affects salivary secretion and induces changes in concentration, secretion rate and composition of salivary components. Exercise also increases the perfusion of active muscles, whereas the perfusion of body organs, such as the kidneys, may decrease by as much as 25%. A decrease in GFR and RPF in turn affects urine volume, osmolality and excretion of electrolytes, urea nitrogen and lactic acid when exercising. Moreover, exercise also induces a state of dehydration which affects the concentration of electrolytes and osmolality levels which in turn affects the body's homeostasis. IgA is the first line of defence against the development of oral pathogens, thereby protecting the body against infection. Decreased levels of salivary

IgA are associated with upper respiratory tract illness. Although the link between physical activity and immune system suppression is not fully understood, it is known that moderate intensity exercise can boost immunological defences, while severe exertion can weaken them by increasing the risk of URTI.

Even though there are physiological and morphological differences between males and females, most research in sports and science are based on male participants only. Those few studies which have utilised both male and female participants failed to acknowledge sex differences in their findings. Therefore, the validity of these results are questionable, and restricts the ability to apply the findings across both sexes.

CHAPTER 3 : RESEARCH STUDY MANUSCRIPT

Assessing electrolyte levels, osmolality and immune function markers in blood and/or saliva, urine, and sweat, in resting and exercising males and females

The following chapter is presented as a manuscript prepared for the *Journal of Food Nutrition and Metabolism*. The participant pre-exercise health screening questionnaire and participant information sheet are presented as Appendix A and B respectively.

3.1 Abstract

Background: The gold standard method to measure physiological markers to monitor athletes' training responses is to utilise blood, however blood collection is invasive and requires a skilled technician. Sports studies using blood to investigate the impact of exercise are based mostly on only male participants and do not acknowledge sex differences. Exploring the sex differences and relationships between blood (gold standard) and saliva, urine and sweat in electrolyte, osmolality and/or IgA levels during exercise, may enable saliva, urine and sweat, which can be collected non-invasively, to be used in place of blood.

Methods: Twelve males and eight females participated in a cross-over design trial that involved a resting and a 90-min cycling trial. Blood, saliva, urine and sweat were collected pre, during and post-exercise to measure electrolyte, osmolality and IgA levels.

Results: Sex differences were found in serum Na^+ and Cl^-, salivary K^+, urinary Na^+ and sweat osmolality levels ($P<0.05$) between males and females during exercise. Among males, there was an increase in serum Cl^- levels at 30min and 60min ($P<0.001$), and among females, a decrease in serum Na^+ level post-exercise ($P=0.020$) and serum Cl^- at 60min ($P<0.05$), compared to pre-exercise. There was an increase in serum K^+ levels at 30min and 60min ($P<0.001$), and in urine K^+ levels, at 90min and 120min ($P=0.024$), when using male and female combined data. There was a positive correlation between serum and salivary IgA at pre-exercise ($r=0.445, P=0.49$), 60min ($r=0.489, P=0.029$), and post-exercise ($r=0.513, P=0.016$).

Conclusion: Male and female participants' results should not be pooled when studying changes in specific biomarkers (serum Na^+ and Cl^-, salivary K^+, urinary Na^+ and sweat osmolality) during exercise. Positive correlations between serum and salivary IgA levels

shows potential to use saliva samples as indirect measures to predict serum IgA levels in athletes, thereby overcoming the limitations of blood collection.

Keywords: Exercise, sodium, potassium, chloride, IgA, body fluids.

3.2 Introduction

In the field of sports and exercise, physiological markers are used to monitor an athlete's training response and to assess the immediate or long-term effects of exercise. Exercise causes a significant impact on homoeostasis, triggering cellular and physiological changes such as increased energy production, fat loss and/or muscle gain, as a result of, or in response to increased metabolic activity (Hawley et al., 2014). The gold standard method for measurement of physiological markers utilise blood samples, due to the high sensitivity of blood tests and the ability to test a wide range of analytes in a single sample (Celec et al., 2016; Lindsay & Costello, 2017). However, blood sampling is invasive, requires a skilled technician and specific medical equipment for collection. Alternative strategies using other body fluids, such as urine, saliva and sweat which allows for self-sampling without significant interruption to the training regime, and can be collected non-invasively are being studied to overcome some of the limitations of blood collection, particularly in a sports setting (Gatti & De Palo, 2010).

Body fluids contain electrolytes, hormones and immunoglobulins, which play a critical role in conduction of nervous impulses, contraction of muscles, regulation of the body's pH level and protection against infection. These compounds can be used as specific biomarkers and monitored in athletes to assess performance and recovery during and after exercise (Lee et al., 2017). While exercising (especially in the heat), a large amount of water is lost through sweating, leading to a water deficit. Because sweat is hypotonic, exercise-induced dehydration leads to a decrease in extracellular fluid volume. Blood osmolality is often used as the standard assessment for hydration state, especially for acute and dynamic changes of hydration state (Lee et al., 2017). Previous studies have shown that exercise-induced dehydration results in a direct increase in plasma osmolality (Brandenberger et al., 1989; Candas et al., 1986; Montain & Coyle, 1992), and this in turn leads to an imbalance in fluid homeostasis, which results in detrimental effects (such as increased core temperature, reduced sweat rate etc) as well as heat exhaustion and strokes in athletes (Wittbrodt, 2003).

Studying the levels of hormones, cytokines, and metabolites is important to monitor the immune system when exercising (Gleeson, 2007). A strong connection has been suggested to exist between the mucosal immune system and infections, especially URTI and exercise (Rosa et al., 2014). Immunoglobulin A (IgA), one of the most abundant antimicrobial proteins, plays a critical effector role in the mucosal immune system, acting as a "first line of defence" against pathogens (Khaustova et al., 2010; Usui et al., 2011). The effects of exercise on salivary IgA concentrations and secretion rate appear to be largely dependent on overall exercise intensity (Bishop & Gleeson, 2009). However, as with serum IgA, studies have reported varying changes in salivary IgA due to exercise. Most studies have reported a decrease in salivary IgA levels after exercise (Gleeson & Pyne, 2000b; Laing et al., 2005; Libicz et al., 2006; MacKinnon & Jenkins, 1993), although some have reported no change (McDowell et al., 1991) or even increases in salivary IgA concentrations following acute exercise (Blannin et al., 1998).

Numerous investigations have explored the physiological and metabolic effect of exercise on different body fluids such as blood, urine, sweat and saliva (Baker et al., 2019; Lee et al., 2017; Walsh et al., 2004a), and some research has undertaken pairwise comparison of body fluids against blood (Cadore et al., 2008). However, these results are inconsistent, mostly due to imbalance in the number of male and female participants in the study, different stimulation methods used, different collection methods, variations in hydration level, and absence or presence of supplements.

When exercising, the commonly accepted physiological and morphological differences between males and females become even more evident in the specific responses or magnitude of response to various training regimens (Lewis et al., 1986). Moreover, females are significantly underrepresented in exercise research – the average percentage of female participants per article across sports and exercise journals ranges from 35% to 37% (Costello et al., 2014). Lack of female representation in exercise studies leaves them disadvantaged as there is a gap in knowledge about exercise physiology among females. Also, the validity of results from studies which pooled data from collected from males and females, without acknowledging the sex differences, is questionable, and may restrict the ability to apply the findings across both sexes.

Taking into consideration the physiological and morphological differences between males and females, the first aim of this study was to assess the electrolyte levels, osmolality and immune function markers in blood and/or saliva, urine, and sweat, in resting and exercising males and females. As the level of these analytes are greatly influenced by the duration of physical activity, the second aim of this study was to investigate changes in electrolyte and osmolality

levels in blood, saliva, urine and sweat, and IgA levels in blood and saliva, simultaneously over time, while exercising. The final aim was to explore relationships between blood (gold standard) and saliva, urine and sweat in electrolyte and osmolality levels, and blood and saliva IgA levels, while resting and exercising. If there is a strong correlation, it will enable the electrolyte, osmolality levels in urine, saliva or sweat, and IgA level in saliva to be used to estimate the value in blood, thereby overcoming the drawbacks of blood collection in a sports setting.

3.3 Materials and Methods

3.3.1 Participants

Twenty healthy adults (12 male and 8 female) volunteered to participate in this study. All procedures had prior approval by the local institutional ethics committee (Southern A, Application 12/63). The inclusion criteria included healthy volunteers aged 18-55y, able to cycle for 90min and pass a health screening questionnaire. The exclusion criteria included smokers and taking prescription drugs. Neither the menstrual phase or oral contraceptive use of the female participants were captured in this trial. Following completion of a health screening questionnaire (Appendix A), written informed consent was obtained from all participants. Participants were asked to refrain from consuming alcohol 48 h prior to arriving at the laboratory for the experimental trials and fast for a minimum of 3 h prior. They were also required to record a food diary over the 48-h period preceding the first main trial and asked to replicate the same diet and eat at approximately the same times for the subsequent trial. Participants were reminded to arrive in a hydrated state before the main trial by drinking regular amounts of fluid on the day of the trial.

3.3.2 Familiarisation Procedure

A preliminary session was used to familiarise participants with the equipment and experimental protocol. Participants underwent a maximal heart rate (HR) test on a cycle ergometer (Ergomedic 874E, Monark Exercise AB, Vansbro, Sweden) to determine an exercise intensity relative to 70% peak power. HR was measured continuously via short-range telemetry (T31 Polar heart rate monitor, Kempele, Finland). In addition, each participant completed 10 min of continuous cycling to practice saliva sampling under exercising conditions.

3.3.3. Main Trial

Participants performed either a resting or exercising protocol in a randomised crossover design. The trials were performed at the same time of day (1500–1800 hours) in a thermoneutral (18 - 20°C) environment, approximately one week apart. Participants arrived at the laboratory dressed in appropriate exercise clothing and an initial, mid-stream urine sample was collected to assess hydration status along with subsequent anthropometric measures of height and weight. Participants' dry body mass (kg) in minimal clothing was measured using calibrated precision-weighing scales (A & D Weighing, HV-200KGL, Australia) and height was determined using a Stadiometer (Seca 240, Germany). Participants then had a cannula inserted into their arm, sweat patches placed on two sites of their body (upper chest at the mesosternale and anterior mid-thigh on the right side) and a heart rate chest strap placed just below their sternum for continuous measures of HR throughout the trial. Fifteen min prior to commencing the experimental trials, blood and saliva samples were collected and participants then given either 5 mL/kg and 2 mL/kg water to ingest prior to the exercise and resting trials, respectively.

The exercise protocol required participants to continuously cycle at 70% peak power for 90 min. Fifteen mL of blood, 1 mL of saliva and average HR were taken every 30 min. Two mL/kg of water was consumed at 15, 45 and 75 min. At 90 min, immediately following exercise completion, a second mid-stream urine sample was collected. Body mass was then measured and the HR chest strap and sweat patches were removed. The 90-min exercise protocol was used to ensure that a sufficient volume of sweat was collected for analyses. The resting protocol required participants to remain seated for the entire 90 min duration. All experimental procedures remained the same except sweat patches were not used. On completion of the 90-min protocol, participants remained in the laboratory for monitoring and further testing. They were given another 2 mL/kg of water to ingest 15 min post-exercise/rest protocol. The final blood saliva and mid-stream urine samples were collected 30 min post-exercise/rest protocol and the cannula was removed.

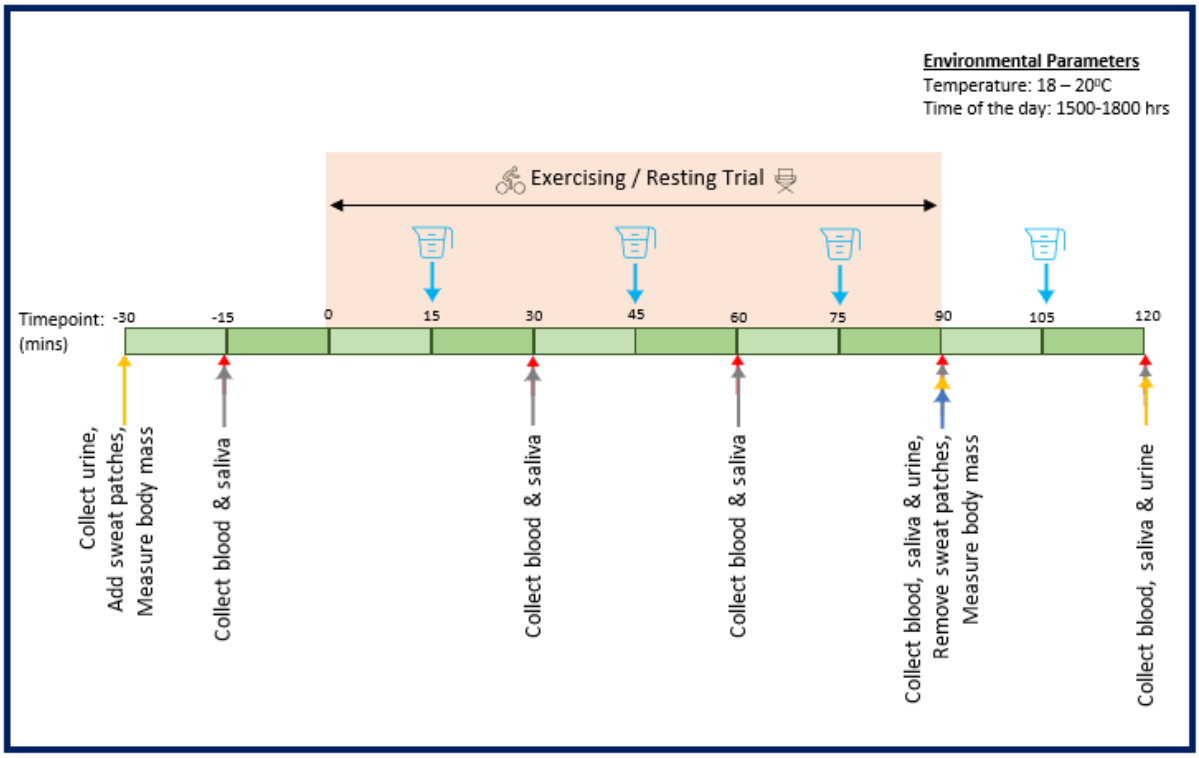

Figure 3.1 Schematic diagram of the trial procedure

3.3.4 Sample Collection and Analysis

3.3.4.1 Blood

A Becton Dickenson cannula using an 18 gauge needle was inserted into an antecubital vein of the arm in a supine position. The participant then moved to an upright position and rested in this position for 10 min before blood collection. The cannular was flushed with saline before blood draws. Fourteen mL of blood was drawn into a syringe pre-exercise/rest protocol and every 30 min thereafter; 4 mL of blood was aliquoted into an EDTA vacutainer, 4 mL into a lithium heparin (LiH) vacutainer, and 6 mL into a serum vacutainer. Vacutainers were inverted 10 times to ensure the blood was thoroughly mixed with the vacutainer additives. The LiH and EDTA vacutainers were centrifuged (MF 50, Hanil Science Industrial Co., Ltd., Korea) 10 min post-draw and the plasma and serum vacutainers were centrifuged at 3500x g for 10 min after clotting at room temperature for 30 min. Two 0.5 mL aliquots of plasma and serum were then removed from each vacutainer, aliquoted into 1.5ml microcentrifuge tubes and frozen at -80°C for later analysis.

3.3.4.2 Sweat

Prior to application of the sterile adhesive patch (10 x 12 cm; Opsite Smith and Nephew, London, UK), the participant's skin was cleaned with an alcohol swab, reverse osmosis water and a sterile gauze pad. The sweat patch was attached to the upper chest, at the mesosternale, and anterior mid-thigh on the right side of the body using disposable latex gloves and forceps before the exercising trial. Chest and thigh placement sites were selected based on Baker et al. (2009) i.e., best sites for electrolytes based on gold-standard whole body washdown method (for both sexes).

Sweat patches were removed after 90 min of exercise and individually placed into 50 mL sterile centrifuge tubes then weighed with the lid on to prevent evaporation. An accurately weighed amount of distilled water was then added into each of the tubes to achieve an approximate 2 x dilution. The patches were then refrigerated overnight and centrifuged at 1140x g at 4°C for 20 min. Representative diluted sweat samples were then pipetted into 50 mL centrifuge tubes, mixed well then aliquoted into 15 mL centrifuge tubes in duplicate and stored upright at -80°C for analysis. For sweat measurements, the mean of both sweat patches (chest and thigh) was recorded; this reflects the optimal comparison with the whole body washdown method (Baker et al., 2009).

3.3.4.3 Urine

Urine samples were collected pre-exercise/rest and at 90 and 120 min from the start of exercise/rest. Participants were required to collect their mid-stream urine into a specimen container. The urine sample was handled with disposable latex gloves and transferred into a sterile screw top tube. The samples were then frozen upright at -80°C for later analysis.

3.3.4.4 Saliva

To control for the effects of circadian variation and habitual activity known to cause alterations in salivary immunoglobulin and cortisol levels (Dimitriou et al., 2002), saliva samples were collected at the same time of day for both trials. Participants were not asked to refrain from drinking water prior to arrival to the laboratory, however, to avoid the possibility of diluting the saliva samples, they were not permitted to drink for 10-15 minutes prior to sampling. During sampling, participants were asked to perform minimal orofacial movement, except chewing when necessary. One mL saliva samples were collected using the bud method (Rutherfurd-Markwick et al., 2020). Three saliva buds were collected at each time point; pre-exercise/rest protocol and every 30 min thereafter. The buds were removed, placed into a sterile container sealed with parafilm to prevent evaporation and centrifuged at 1520x g, at room temperature

for 10 min to extract the saliva from the cotton bud. The three centrifuged samples collected at each time point were then combined, vortexed for 10 s, then aliquoted into two microtubes and stored upright at -80°C for later analysis.

3.3.2.5 Electrolyte, Osmolality and IgA Measurements

Electrolyte concentrations were measured using an ion selective electrode analyser according to the manufacturer's instructions (EasyLyte Medica Corporation, Bedford, MA, USA). Osmolality was measured using a freezing point depression osmometer according to the manufacturer's instruction (Osmomat 030, Gonotec, Berlin, Germany). Serum IgA, and salivary IgA concentrations were determined by a two-step sandwich ELISA as described elsewhere (Crooks et al., 2006). The coefficients of variation for all assays were <5%.

3.3.2.6 Statistical Analysis

Statistical analysis was performed using SPSS (v27, IBM SPSS Statistics). Results are presented as means ± SD (Section 3.4).

Two-way ANOVA was used to investigate the effect of sex, and the change over time while exercising for electrolytes, osmolality and IgA levels. If Mauchly's test of sphericity was statistically significant ($P<0.05$), the Greenhouse-Geisser correction was applied. Within the exercising trial, the change in the electrolyte, osmolality and IgA levels over time in the different body fluids was determined using one-way ANOVA, followed by post-hoc tests (Bonferroni corrected).

Pearson's correlation was used to determine the association between the urine, sweat, saliva against blood (as the criterion measure). Based on the r value, the strength of the relationship was defined as follows: $r=0$ (none), $0<r<0.3$ (very weak), $0.3<r<0.5$ (weak), $0.5<r<0.7$ (moderate) and $r>0.7$ (strong) (Moore et al., 2013). $P<0.05$ was used for statistical significance.

3.4 Results

3.4.1 Descriptive Data: Baseline characteristics of the study population

A total of 20 participants (12 males and 8 females) with a mean age of 27.2 (± 7.6) years, body weight of 71.4 (± 12.4) kg and height of 176 (± 10) cm completed this study. Mean body weight change of the participants after exercising (after correction for fluid intake) was 0.18 ± 0.23 kg.

Analysis was possible from all 20 participants' blood (serum and plasma) samples, but not from all the urine, saliva and sweat samples. For some samples the concentration of the analyte being measured was too low for the ion selective electrode analyser (EasyLyte) to report a numerical value.

3.4.2 Differences in blood electrolyte, osmolality and IgA levels between males and females during exercise

Figure 3.2 shows the mean (± SD) levels of electrolytes, osmolality and IgA in blood during the exercising trial for males and females. There was a significant effect of sex on serum Na^+ (P=0.019) levels, with males displaying a higher mean value than females in the exercising trial. Serum Na^+ was statistically higher in males than females at pre-exercise, 60 min and 90 min (P<0.005). For serum Na^+ levels, there was a trend for a change over time (P=0.053), but there was no interaction of time*sex (P=0.446). For serum K^+ levels, there was a change over time (P<0.001), but there was no significant effect of sex (P=0.949) and no interaction of time*sex (P=0.197). There was a significant effect of sex (P=0.021) for serum Cl^-, but there was no change over time (P=0.376) and no interaction of time*sex (P=0.636).

There was no significant effect of sex (P=0.739), or change over time (P=0.755), or interaction of time*sex (P=0.173) for plasma osmolality. Similarly, for serum IgA, there was no significant effect of sex (P=0.074), or change over time (P=0.754), or interaction of time*sex (P=0.302).

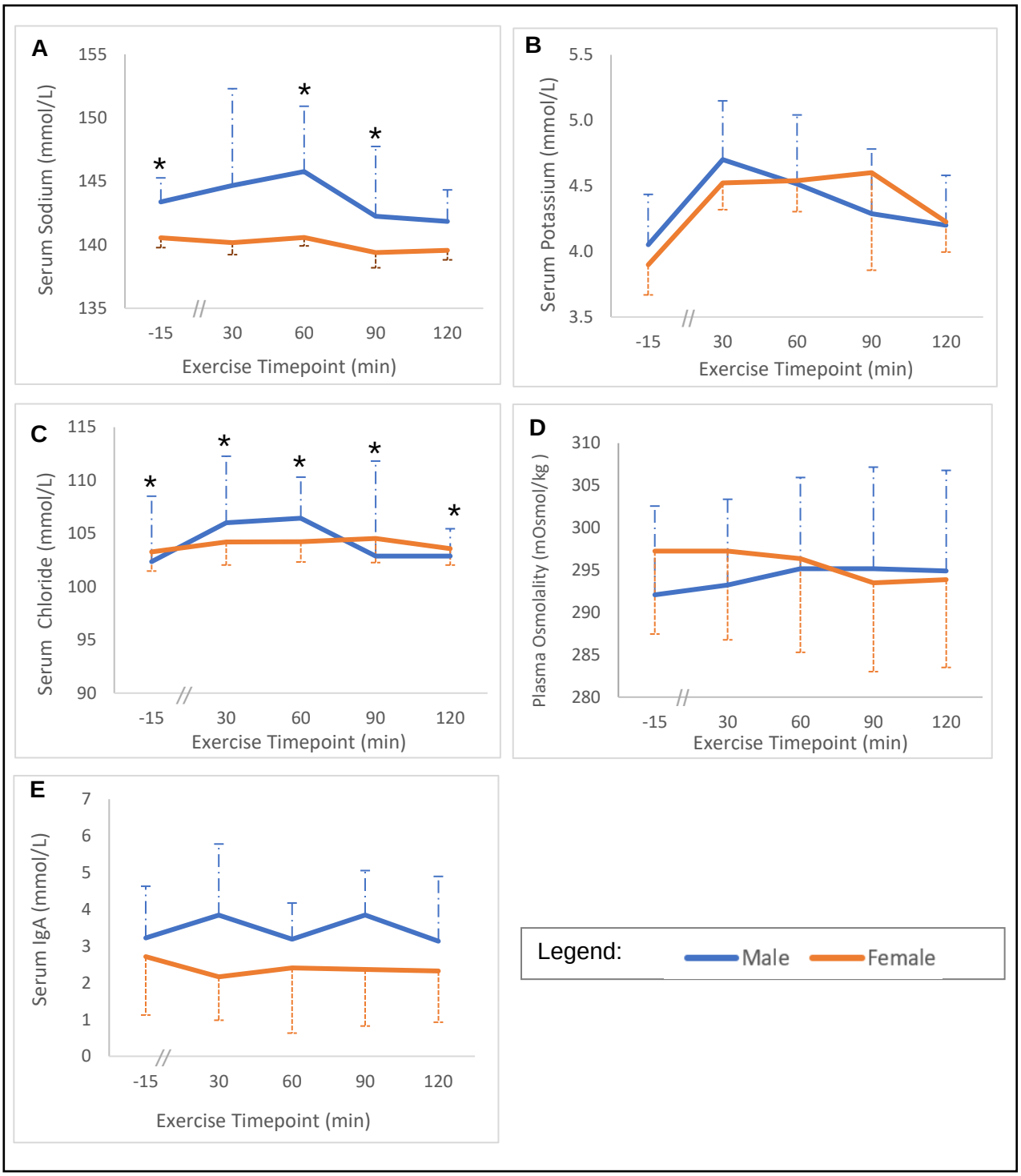

Figure 3.2 Mean levels (± SD) of blood sodium (A), potassium (B), chloride (C), osmolality (D) and IgA (E) in males (12M) and females (8F) at -15, 30, 60, 90 and 120 min of the exercising trial. Timepoints with a significant difference in serum electrolyte levels between males and females are indicated with an asterisk (*, P<0.05).

3.4.3 Differences in salivary electrolyte, osmolality and IgA levels between males and females during exercise

Figure 3.3 shows the mean (± SD) salivary levels of electrolytes, osmolality and IgA for males and females during the exercising trial. There was no significant effect of sex on salivary Na^+ (P=0.392) levels, or change over time (P=0.073), or interaction of time*sex (P=0.994). For salivary K^+ levels, there was a significant effect of sex (P=0.024), however there was no significant change over time (P=0.087) or interaction of time*sex (P=0.249). For salivary Cl^-, there was no significant effect of sex (P=0.111), or change over time (P=0.164), or interaction of time*sex (P=0.669). For saliva osmolality, there was no significant effect of sex (P=0.428), or interaction of time*sex (P=0.811). Similarly, for salivary IgA, there was no significant effect of sex (P=0.446) and no interaction of time*sex (P=0.858).

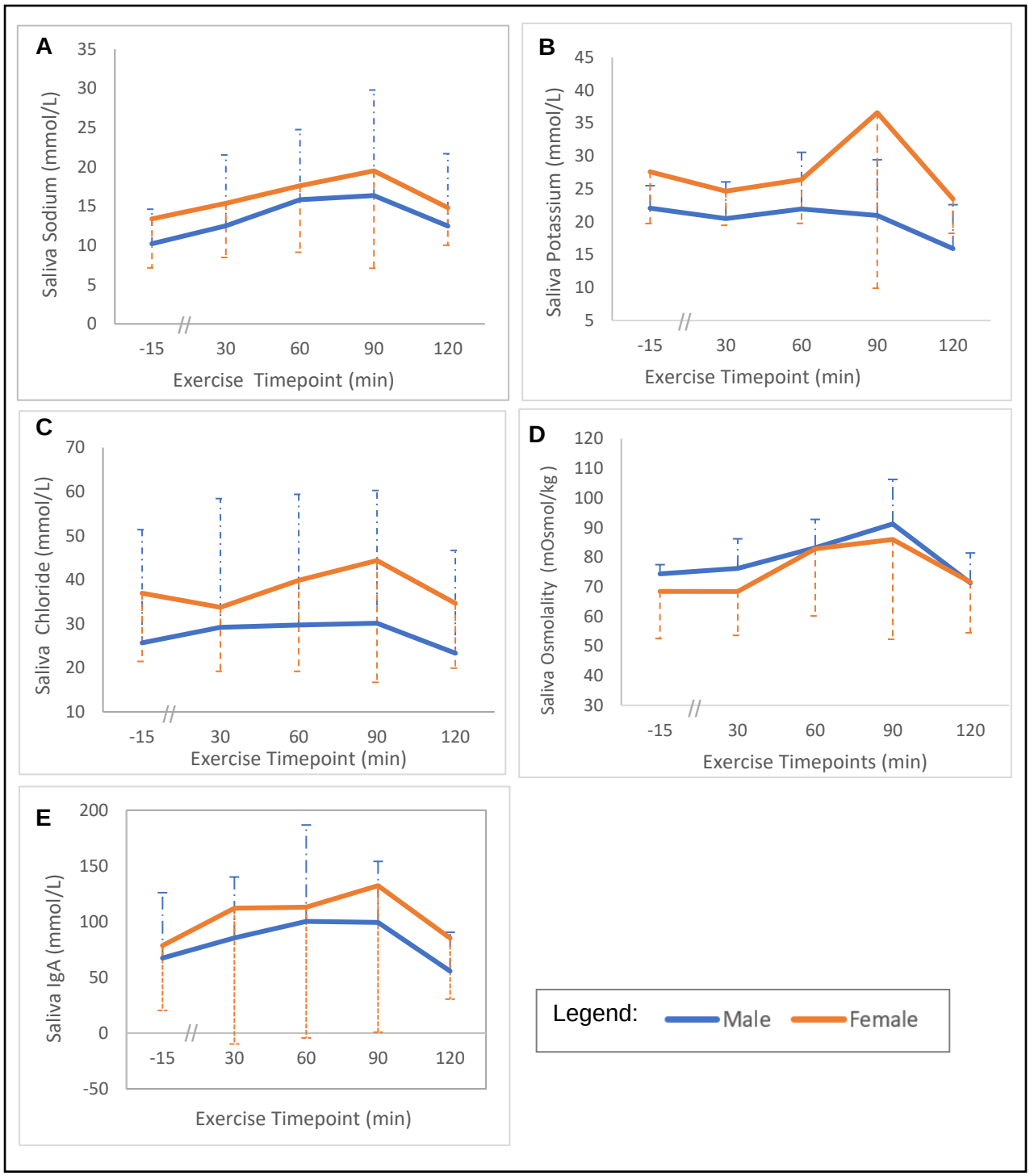

Figure 3.3 Mean levels (± SD) of salivary sodium (A), potassium (B), chloride (C), osmolality (D) and IgA (E) in males (11M) and females (8F) at -15, 30, 60, 90 and 120 min of the exercising trial.

3.4.4 Differences in urinary electrolyte, osmolality and IgA levels between males and females during exercise

Figure 3.4 shows the mean (± SD) levels of urinary electrolytes and osmolality for males and females during the exercising trial. There was a significant effect of sex in urinary Na^+ (P=0.006) levels, with males displaying higher mean Na^+ levels than females. There was no interaction of time*sex (P=0.122). For urinary K^+ levels, there was no significant effect of sex (P=0.180) or interaction of time*sex (P=0.573). For urinary Cl^-, there was no significant effect of sex (P=0.05), or change over time (P=0.166), or interaction of time*sex (P=0.356). Likewise for urine osmolality, there was no significant effect of sex (P=0.363) or change over time (P=0.153) or interaction of time*sex (P=0.917).

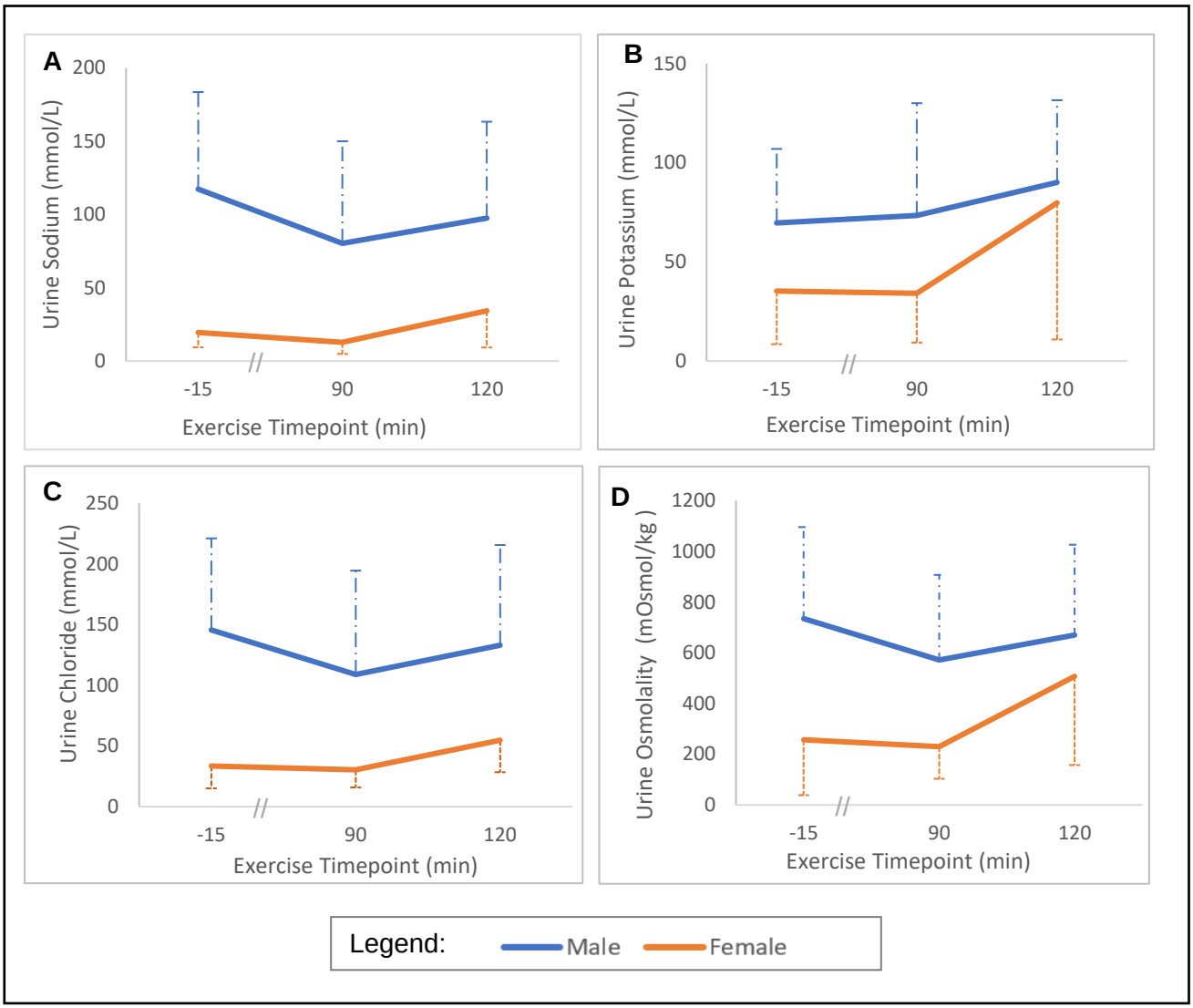

Figure 3.4 Mean levels (± SD) of urine sodium (A), potassium (B), chloride (C) and osmolality (D) in males (12M) and females (7F) at -15, 90 and 120 min of the exercising trial.

3.4.5 Differences in sweat electrolyte, osmolality and IgA levels between males and females during exercise

Figure 3.5 shows the mean (+SD) levels of sweat electrolytes and osmolality for males and females at 90 min of the exercising trial. There were no differences between males and females for sweat Na^+, Cl^- or K^+ levels, but these results should be viewed with caution as only 45% of the sweat samples were able to be analysed due to their low electrolyte content. However, all of the sweat samples were able to be analysed for osmolality levels and it was noted that males had significantly higher (P=0.017) sweat osmolality levels, than females.

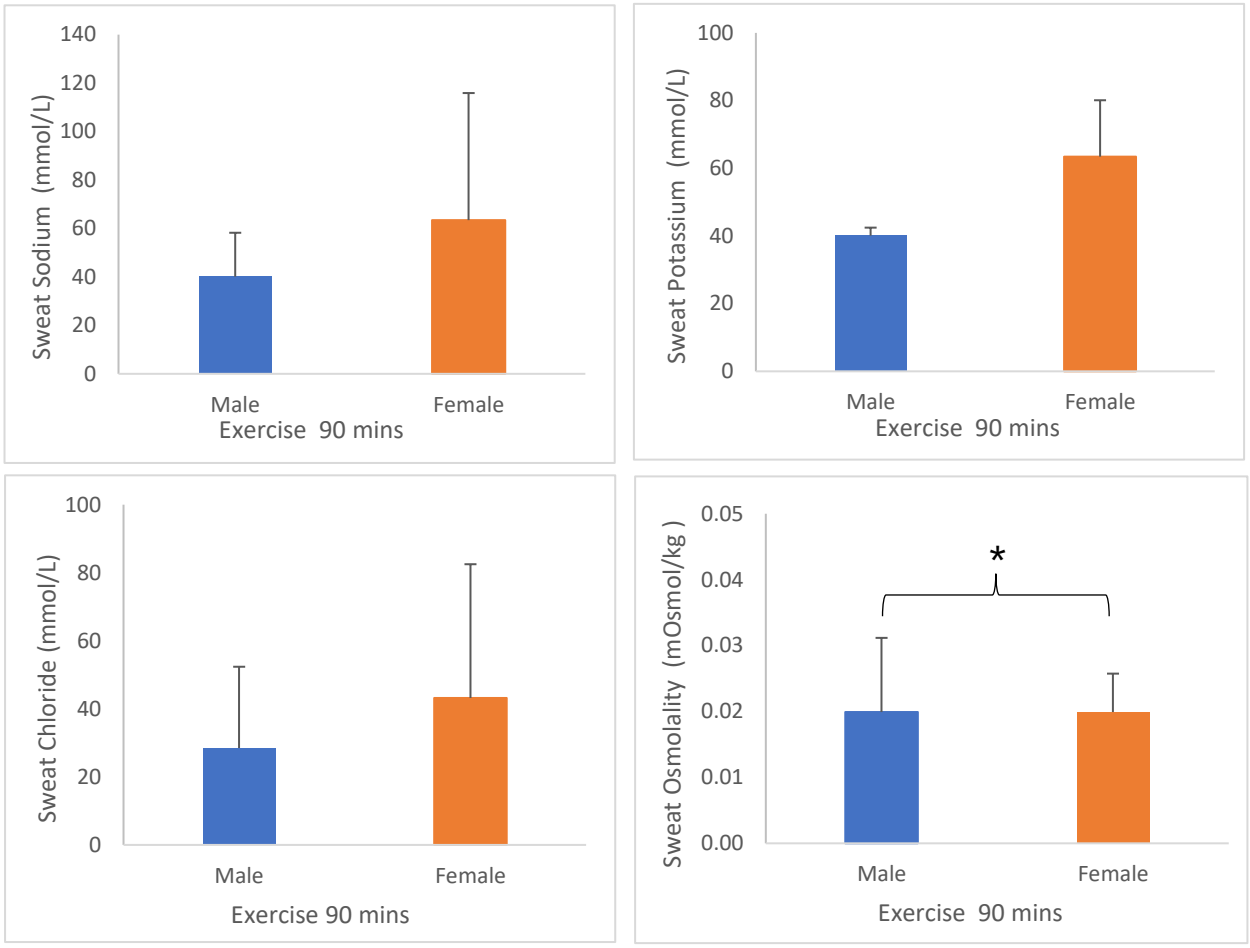

Figure 3.5 Mean levels (+SD) of sweat sodium (A), potassium (B), chloride (C) and osmolality (D) in males and females measured from the sweat patch removed at 90 min of the exercise trial. N = 13 (sweat sodium and chloride; 9 males and 4 females), 7 (potassium; 3 males and 4 females) and 20 (osmolality; 12 males and 8 females). Mean of both sweat patches (chest and thigh) was used. Significant difference in sweat osmolality level between males and females at 90 min is indicated with an asterisk (*, P<0.05).

3.4.6 Implications of differences in analyte levels between males and females

The above results indicate there were significant differences in serum Na^+, Cl^-, salivary K^+, urinary Na^+ and sweat osmolality levels, between males and females during the exercising trial. However, no sex differences were seen for the other measures. This information indicates that male and female participants' results should not be pooled when studying changes in serum Na^+, Cl^-, salivary K^+, urinary Na^+ and sweat osmolality levels during exercise, however, the data for the other measures which did not differ by sex, can be pooled.

For the following sections, male and female data will be analysed separately for serum Na^+, Cl^-, salivary K^+, urine Na^+ and sweat osmolality levels, and combined for the other measures.

3.4.7 Interaction of exercise and time on electrolytes levels in blood, saliva, urine and sweat

The mean (+SD) data for Na^+, K^+ and Cl^- levels in blood, saliva, urine and sweat during the exercising trial are shown in Fig 3.6. Among males, there was a significant increase in serum Cl^- levels at 30 min ($P<0.001$) and 60 min ($P<0.001$), compared to the pre-exercise level (-15 min). Among females, compared to the pre-exercise level, there was a significant decrease in serum Na^+ levels at 120 min ($P=0.020$) and serum Cl^- at 60 min ($P=0.014$). Serum K^+, which used combined (male and female) data exhibited significant increases at 30 min ($P<0.001$) and 60 min ($P<0.001$) when compared to the pre-exercise level. No significant changes in salivary Na^+, K^+ or Cl^- levels using combined male and female data were observed during exercise. There was a significant increase in urinary K^+ levels ($P=0.024$; combined data) between 90 min and 120 min.

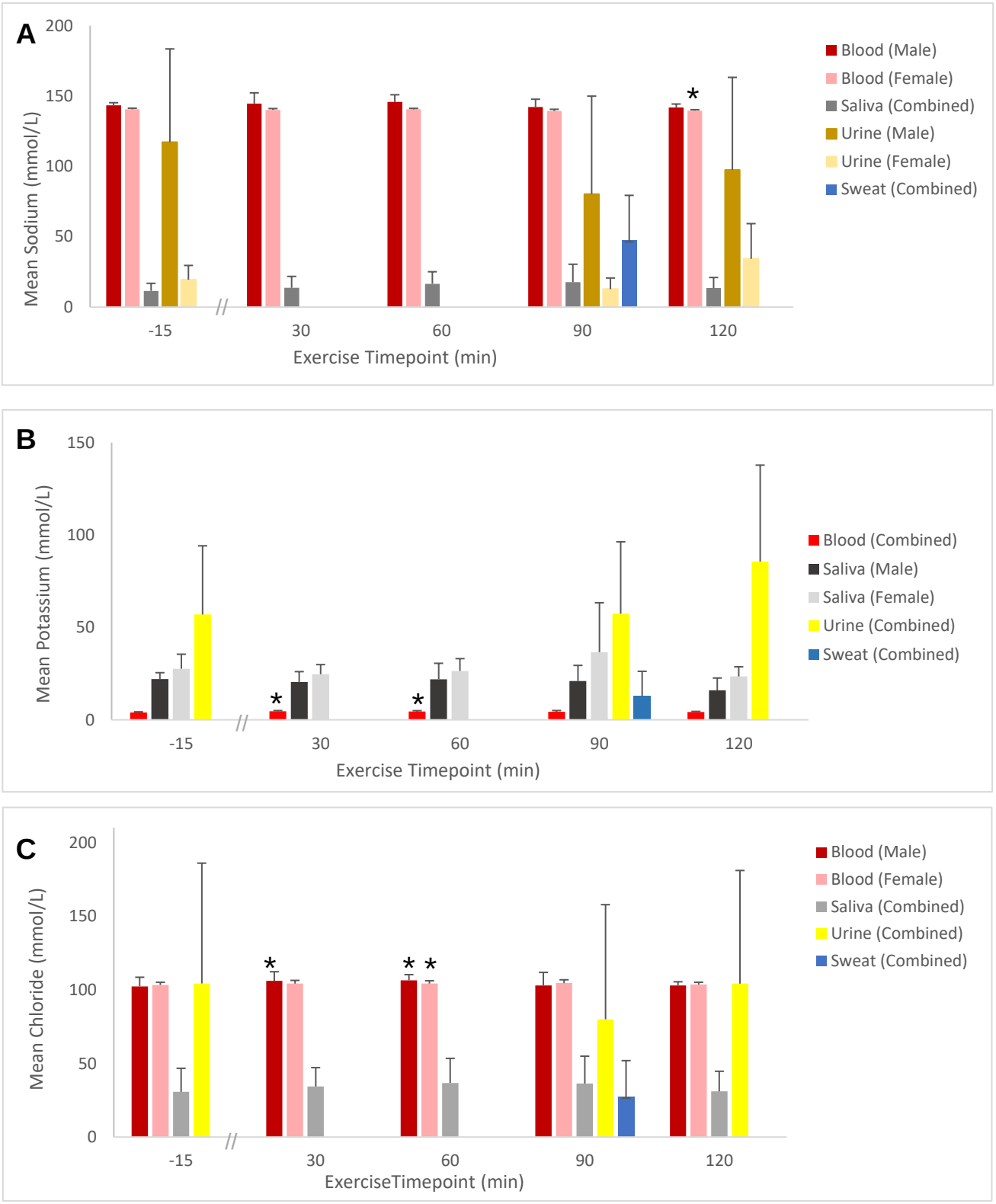

Figure 3.6 Mean blood, saliva, urine and sweat levels (+ SD) for sodium (A), potassium (B) and chloride (C) while exercising. Blood sodium and chloride, saliva potassium and urine sodium levels are presented separately for males and females. Blood and saliva were measured at -15, 30, 60, 90 and 120 min, urine was measured at -15, 90 and 120 min, and the sweat patch was removed at 90 min of the exercising trial. Significant differences in electrolyte levels compared to pre-exercise levels are indicated with an asterisk (*, $P<0.05$).

3.4.8 Interaction of exercise and time on osmolality levels in blood, saliva, urine and sweat

The mean (+SD) data for osmolality levels in plasma, saliva, urine and sweat during exercise are shown in Fig 3.7. During the exercising trial, there were no significant changes in osmolality levels in blood, saliva or urine using the combined male and female data.

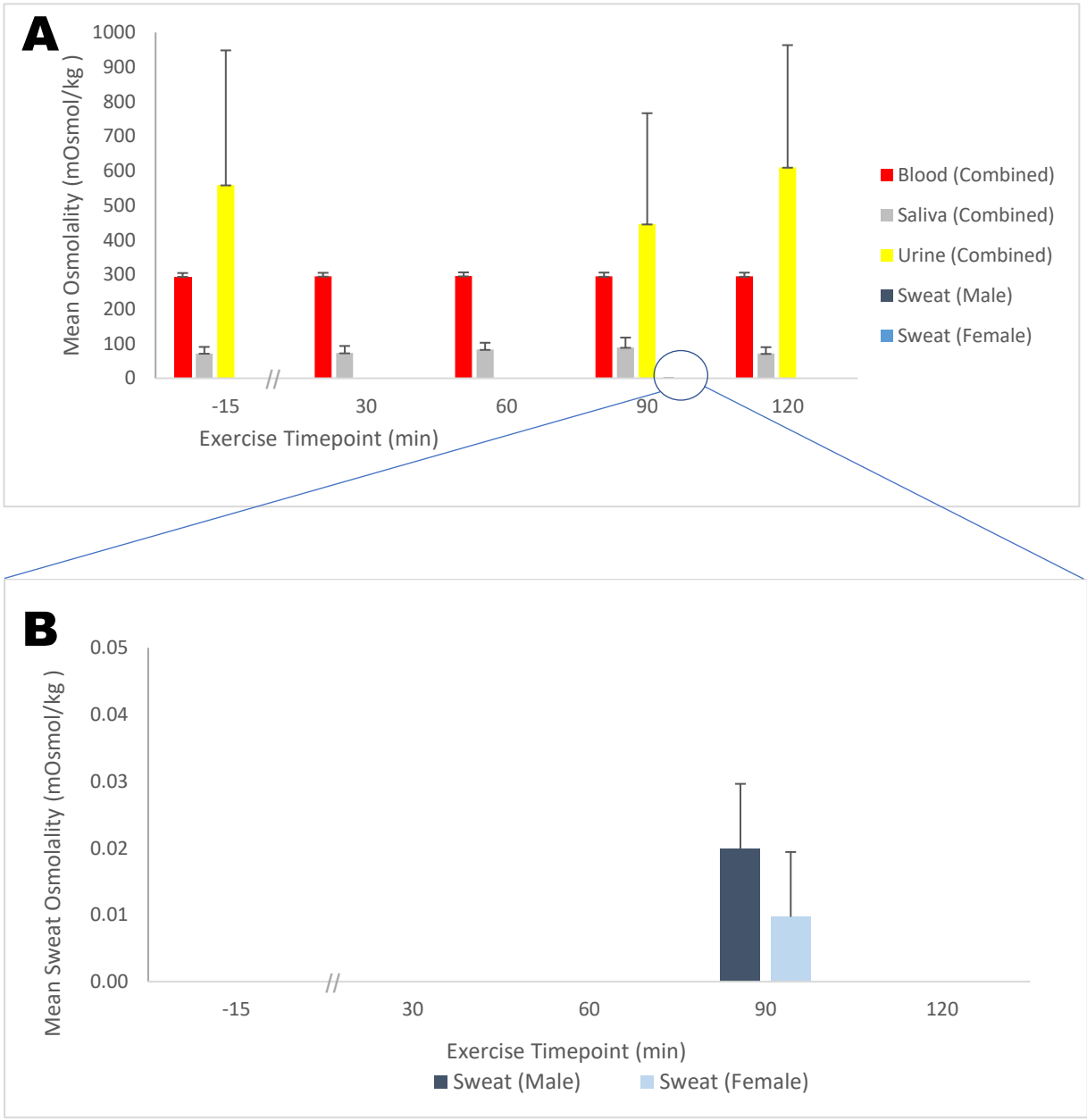

Figure 3.7 Mean blood, saliva, urine and sweat osmolality levels (+SD) while exercising (A). Sweat osmolality has been plotted separately (B) to enable a better comparison. Blood and saliva were measured at -15, 30, 60, 90 and 120 min, urine was measured at -15, 90 and 120 min, and sweat patch was removed at 90 min of the exercising trial.

3.4.9 Interaction of exercise and time on IgA levels in blood and saliva

The mean (+ SD) data for IgA levels in serum and saliva during exercise are shown in Fig 3.8. Serum and salivary IgA levels did not change significantly during exercise (combined male and female data).

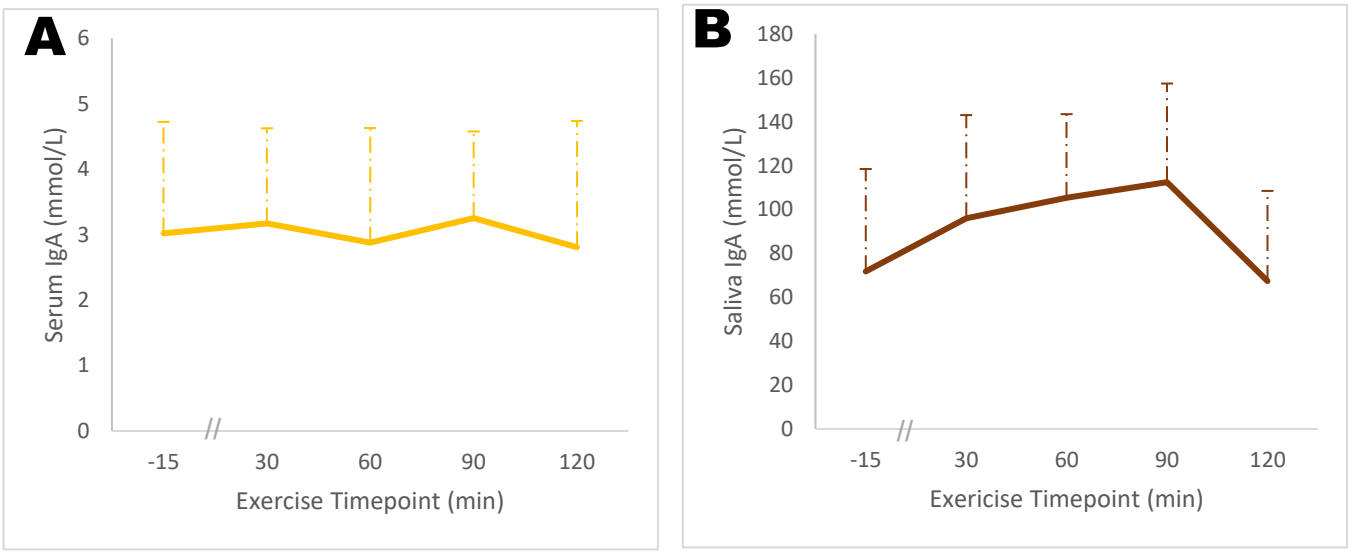

Figure 3.8 Mean serum (A) and saliva (B) IgA levels (+ SD) while exercising. Serum and saliva IgA were measured at -15, 30, 60, 90 and 120 min of the trials.

3.4.10 Correlational analysis comparing electrolyte levels between blood (gold standard) and saliva, urine and sweat, while resting and exercising

The correlation coefficients of electrolyte levels in saliva, urine and sweat compared to blood are shown in Table 3.1. Based on Section 3.4.6, where sex differences were noted for serum Na^+ and Cl^-, salivary K^+ and urinary Na^+ levels in the exercising trial, the correlation coefficients for Na^+, Cl^- and K^+ were investigated separately for males and females during the exercising trial and combined for other analytes and body fluids.

In the resting trial, there was a moderate positive correlation (r=0.522, P=0.022) between serum and urinary Na^+, at pre-exercise, and a strong positive correlation (r=0.702, P=0.001) at 90 min. There was also a strong positive correlation between serum and salivary Cl^- (r=0.712, P=0.001) levels at 90 min of the resting trial.

In the exercising trial there was a moderate negative correlation between serum and salivary Na^+ levels (r=-0.664, P=0.026) at pre-exercise, in males only. There was also a strong positive correlation between serum and salivary K^+ (r=0.714, P=0.047) levels at 60 min of the exercising trial in females only.

Table 3.1: Correlation coefficients comparing sodium, potassium and chloride levels in blood (gold standard) and saliva, urine and sweat at -15, 30, 60, 90 and 120 min. *denotes that the correlation is significant at the 0.05 level (2-tailed).

Electrolyte	Timepoint Trial	Correlation Coefficient								
		Males and females combined			Males only			Females only		
		Saliva	Urine	Sweat	Saliva	Urine	Sweat	Saliva	Urine	Sweat
Sodium	-15 Rest	0.362	0.522*							
	30 Rest	-0.141								
	60 Rest	-0.190								
	90 Rest	-0.255	0.702*							
	120 Rest	0.126	-0.279							
	-15 Exercise				-0.664*	0.195		-0.649	-0.076	
	30 Exercise				-0.005			-0.275		
	60 Exercise				-0.042			-0.534		
	90 Exercise				0.012	-0.113	-0.141	-0.051	-0.063	0.010
	120 Exercise				-0.178	-0.135		-0.126	-0.117	
Potassium	-15 Rest	-0.099	0.218							
	30 Rest	-0.218								
	60 Rest	0.138								
	90 Rest	0.245	-0.136							
	120 Rest	0.333	0.114							
	-15 Exercise				-0.329	-0.160		0.383	-0.009	
	30 Exercise				0.101			0.323		
	60 Exercise				-0.246			0.714*		
	90 Exercise				0.292	-0.260	-0.362	-0.018	0.300	0.599
	120 Exercise				0.051	-0.332		-0.034	-0.471	
Chloride	-15 Rest	0.006	0.392							
	30 Rest	0.316								
	60 Rest	-0.215								
	90 Rest	0.712*	-0.136							
	120 Rest	0.047	0.092							
	-15 Exercise				-0.143	0.482		0.026	0.595	
	30 Exercise				0.215			-0.086		
	60 Exercise				0.026			0.359		
	90 Exercise				-0.105	0.022	0.419	0.059	0.617	0.478
	120 Exercise				0.269	-0.154		-0.037	0.301	

3.4.11 Correlational analysis comparing osmolality levels in saliva, urine and sweat to blood

The correlation coefficients comparing osmolality levels in saliva, urine and sweat to blood are shown in Table 3.2. Based on Section 3.4.6, as sex differences were noted for sweat osmolality in the exercising trial, correlations coefficients for osmolality levels were investigated separately for males and females.

In the resting trial at pre-exercise, there was a weak negative correlation between plasma and salivary osmolality levels (r=-0.481, P=0.037). There were no other significant correlations for osmolality levels between body fluids at other timepoints. A consistent but non-significant negative correlation was noted between plasma and saliva osmolality levels across all timepoints, in both the resting and exercising trials.

Table 3.2: Correlation coefficients comparing osmolality levels in saliva, urine and sweat to blood at -15, 30, 60, 90 and 120 mins. ***** denotes that the correlation is significant at the 0.05 level (2-tailed).

		Correlation Coefficient								
	Timepoint/Trial	Males and females combined			Males only			Females only		
		Saliva	Urine	Sweat	Saliva	Urine	Sweat	Saliva	Urine	Sweat
Osmolality	-15 Rest	-.481*	0.045							
	30 Rest	-.141								
	60 Rest	-.123								
	90 Rest	-.090	-0.402							
	120 Rest	-.045	0.028							
	-15 Exercise				-0.130	0.242		-0.484	-0.009	
	30 Exercise				-0.130			-0.070		
	60 Exercise				-0.356			-0.054		
	90 Exercise				-0.407	-0.305	0.16	-0.112	0.273	0.130
	120 Exercise				-0.548	-0.348		-0.466	-0.208	

3.4.12 Correlation analysis comparing IgA levels in saliva to blood

The correlation coefficients comparing IgA levels in saliva to blood are shown in Table 3.3. Salivary IgA showed a moderate positive correlation with serum IgA in the resting trial at 30 min (r=0.560, P=0.010), 60 min (r=0.613, P=0.004) and 90 min (r=0.643, P=0.02). In the exercising trial, salivary and serum IgA showed a weak positive correlation at pre-exercise

(r=0.445, P=0.49) and 60 min (r=0.489, P=0.029), and a moderate positive correlation at 120 min (r=0.513, P=0.016). Although not all time points were significant, a consistent positive correlation was observed between serum and salivary IgA levels at all timepoints, in both the resting and exercising trials.

Table 3.3: Correlation coefficients comparing IgA levels in saliva to blood at -15, 30, 60, 90 and 120 mins. *****denotes that the correlation is significant at the 0.05 level (2-tailed).

	Timepoint/Trial	Correlation Coefficient
		Saliva
IgA	-15 Rest	0.185
	30 Rest	0.560*
	60 Rest	0.613*
	90 Rest	0.643*
	120 Rest	0.296
	-15 Exercise	0.445*
	30 Exercise	0.042
	60 Exercise	0.489*
	90 Exercise	0.060
	120 Exercise	0.513*

3.4.13 Summary of Correlation Analysis

In the resting trial, with males and females combined, there was a moderate positive correlation pre-exercise, and a strong positive correlation at 90 min between serum and urinary Na^+ levels. There was also a strong positive correlation at 90 min between serum and salivary Cl^- using combined male and female data in the resting trial. There was a weak negative correlation between plasma and salivary osmolality level pre-exercise, and a moderate positive correlation between serum and salivary IgA at 30 min, 60 min and 90 min of the resting trial, using the combined male and female data.

In the exercising trial, there was a moderate negative correlation pre-exercise between serum and salivary Na^+ among males only. There was a strong positive correlation between serum and salivary K^+ at 60 min of the exercising trial among females only. There was also a weak positive correlation between serum and salivary IgA (combined data) at pre-exercise and 60 min, and a moderate positive correlation at 120 min.

3.5 Discussion

This study investigated electrolyte (Na^+, K^+ and Cl^-) and osmolality levels in blood, saliva, urine and sweat, and IgA levels in blood and saliva, in males and females. This study also examined the changes in electrolyte and osmolality levels in blood, saliva, urine and sweat, and IgA levels in blood and saliva over time while exercising, and explored relationships between blood (gold standard) and saliva, urine and sweat in electrolyte, osmolality and/or IgA levels while resting and exercising.

3.5.1 Differences in electrolyte, osmolality and IgA levels between males and females during exercise

The present study found that in serum, the mean Na^+ level was significantly higher in males than females at pre-exercise, 60 min and 120 min, however there was no main interaction effect. The higher mean Na^+ level in males is similar to previous studies that showed that Na^+ concentrations in the red blood cells of healthy females are lower than in males (M'Buyamba-Kabangu et al., 1985; M'Buyamba-Kabangu et al., 1986). These differences between males and females are most likely due to sex-related differences in the function of the Na^+ transport systems (Na^+/H^+ exchanger, $Na^+/K^+/2Cl^-$ cotransport, Na^+, K^+ -pump) (Grikiniene et al., 2004).

The present study found that in saliva, there were no significant differences in Na^+, Cl^- and K^+ levels between males and females except for K^+ at 120 min. These results are similar to a previously reported results which found no sex differences in salivary Na^+, Cl^- and K^+ levels (Rutherfurd-Markwick et al., 2017).

In urine, although males had a significantly higher Na^+ level than females at pre-exercise, 90 min and 120 min, and higher Cl^- and K^+ levels at pre-exercise in the exercising trial there was no main interaction effect. During exercise, fluid balance is regulated by arginine vasopressin (AVP) by increasing renal water reabsorption and reducing urinary output (Eijsvogels et al., 2013). Sex hormones modulate the synthesis and osmotic regulation of AVP. At rest, males have a higher baseline of plasma AVP level and a greater sensitivity to changes in plasma osmolality than females (Sar & Stumpf, 1980; Stachenfeld & Taylor, 2009). Therefore, exercise-induced increases in AVP in males may contribute to their urine being more concentrated, which is reflected in the higher levels of Na^+, Cl^- and K^+ seen in the males of this study.

In sweat, there were no significant differences in Na^+, K^+ and Cl^- levels between exercising males and females, in contrast to another study which showed that males have higher sweat

Na^+ and Cl^- levels than females (Meyer et al., 1992). This apparent difference in study outcomes could be due to the significant number of sweat samples that could not be analysed (55% non-analysable) in the current study, as the value was too low for the analytical machine.

Sweat osmolality was significantly higher in males than females at 90 min, however there were no sex differences in plasma, urine or saliva osmolality levels. The higher sweat osmolality reported in the males of this study agree with the findings of Meyer et al. (1992), who reported that males have higher sweat Na^+ and Cl^- levels than females, hence males have a higher sweat osmolalty level. A study by Gagnon and Kenny (2012) that involved 8 males and 8 females cycling for 90 min, and looked at differences in plasma osmolality between the sexes found no significant differences at either baseline or at the end of the exercise period, similar to the current study which found no sex differences in plasma osmolality.

The mean salivary IgA levels were consistently higher in females than males, and serum IgA levels were higher in males than females at all timepoints of this study, though these differences were not all statistically significant. These results are similar to the findings of Rutherfurd-Markwick et al. (2017) and Martins et al. (2009) who studied the levels of salivary IgA and serum IgA after exercise in males and females and found no significant differences by sex.

This study has shown that there are sex differences in a number of biomarkers (serum Na^+, serum Cl^-, saliva K^+, urine Na^+, sweat osmolality) when exercising, and data from males and females should be treated separately when trying to understand the true effect of exercise on these markers. This finding brings into question the validity of other published studies which have not considered sex differences when measuring electrolyte levels during exercise and have pooled the results for analysis.

It must be noted that the present study did not record the menstrual cycle of the females which makes it challenging to understand if sex hormones may have influenced the results. The female hormones oestrogen and progesterone fluctuate regularly during the menstrual cycle, and these hormones regulate the physiological system that affect strength, power and cardiorespiratory endurance (Wikström, 2016). Oestrogen has been suggested to have an anabolic effect on muscle, whereas progesterone may have a catabolic effect, however the detailed mechanisms behind this remain unclear (Wikström, 2016). Fluid balance and electrolytes are known to play a key role in performance and recovery, so indirectly, the menstrual cycle and female sex hormones may have influenced the differences seen in the electrolyte levels between sexs (Więcek, 2018).

3.5.2 Electrolyte levels in serum, urine, saliva and sweat during exercise

In the exercising trial, males exhibited a gradual increase in serum Na^+ levels up to 60 min (non-significant), and then a trend towards a decrease at 120 min of exercise to below the pre-exercise level. In females, there was a decrease in serum Na^+ level from pre-exercise to 120 min of exercise. This decrease in serum Na^+ levels at 120 min among both males and females may be related to exercise-associated hyponatremia (EAH), which is defined as an acute fall in the serum or plasma Na^+ level to below 135 mmol/L that occurs during or up to 24 h after prolonged physical activity (Rosner, 2019). In this study, the mean serum Na^+ level at 120 min among exercising males was 141.8 mmol/L and females 139.6 mmol/L and thus did not reach the EAH-defined level. However, we do not know if the serum Na^+ level continued to decline during the subsequent 24 h after exercise, as this was not measured. Another reason why the serum Na^+ level may not have reached 135 mmol/L (the EAH-defined level), is that the intensity and length of the cycling exercise may not have been sufficient to induce this state.

In the exercising trial, in both males and females, there was a significant increase in serum K^+ levels at 30 min and 60 min and it decreased once exercise stopped. The rise and fall in blood K^+ concentrations is consistent with other studies (Poortmans, 1975; Vøllestad et al., 1994), with the increase in K^+ likely caused by the efflux of this ion from the intercellular stores into the circulation, due to either a change in the permeability of the muscle cell membranes or a decrease in plasma volume or a possible release of K^+ from erythrocytes (Lindinger & Sjøgaard, 1991). To regulate the increasing K^+ in the blood, the body stimulates the uptake of K^+ ions from the blood by noncontracting tissues. Once exercise is stopped, the efflux of K^+ into the blood is stopped (Medbø & Sejersted, 1990), and this would explain the decrease in serum K^+ concentrations noted at 120 mins of this study (30 mins post-exercise).

Among males, serum Cl^- increased significantly at 30 min and 60 min, and among females at 60 min, compared to the pre-exercise level. The significant increase in Cl^- levels in both males and females at 60 min mirrors the trend of increases in serum Na^+ levels when exercising. Due to osmoregulation, the movement of cation (Na^+) in and out of the extracellular space are always accompanied by an anion (Cl^-) (Garrett, 2017). Therefore, as expected, when the serum Na^+ level increased, serum Cl^- level also increased. The effect of exercise on Cl^- levels across other studies have shown varied results, with some showing a decrease (Kratz et al., 2003) and others no change (Adner et al., 2002). This variation in observed changes in Cl^- levels may be due to differences in type, duration and intensity of exercise and possibly sample numbers in some studies.

In urine, Na^+ levels showed a trend for a decrease at 90 min and then a significant increase at 120 min for both males and females. Decreases in urinary Na^+ levels during exercise have been noted in previous studies (McDowell et al., 1991; Refsum & Strcmme, 1975). Within the urinary system aldosterone (a mineralocorticoid hormone) plays an important role in the regulation of electrolytes by acting on the renal distal tubes (Patlar & Unsal, 2019). It promotes the absorption of Na^+ from the renal tubules, and together with the reduction in glomerular filtration rate (to balance the rise in sodium loss through sweat), leads to a decrease in urinary Na^+ level (as seen at 90 min) (Nelson et al., 1989). Aldosterone also increases the urine excretion of K^+, as was evidenced by the significant increase in K^+ at 90 min in the exercising trial.

3.5.3 Osmolality levels in plasma, saliva, urine and sweat during exercise

There were no significant changes in plasma, urine or sweat osmolality during the exercising trial of this study. However, there was a significant change over time with salivary osmolality levels increasing at 60 min and 90 min, before decreasing at 120 min. This increase in salivary osmolality is likely due to the vasoconstriction of blood arteries to the salivary glands, leading to an increase in sympathetic nervous system activity, which in turn caused a decrease in salivary flow rate after continuous exercise (Chicharro et al., 1998; Dawes & Watanabe, 1987). This decrease in salivary flow rate would have led to smaller amounts of more concentrated saliva with increased osmolality levels as noted in this study.

3.5.4 Serum and salivary IgA levels during exercise

In the exercising trial, salivary IgA levels showed a pattern of increasing at 60 min and 90 min. Results from this study mirror that of Laing et al. (2005), who reported that salivary IgA levels increased during training and then reduced below the pre-exercise level 2 h after training. Other studies (Steerenberg et al. (1997), Libicz et al. (2006), Farzanaki et al. (2008)) have also shown increases in salivary IgA levels during training and then decreases at the end of training. The observed increase in IgA levels could be due to reduction in the amount of saliva secreted during exercise which would lead to a more concentrated saliva.

It is well known that IgA plays an important role in the immune system's defence against infections (Papacosta & Nassis, 2011), and decreased levels of salivary IgA are linked to an

increased risk of URTI. Previous studies have shown that salivary IgA levels depend on the intensity and duration of training as well as the type of physical activity (Klentrou et al., 2002; Nieman, 2001; Rahimi et al., 2010). This study showed an increase in salivary IgA levels at 60 min and 90 min, therefore this result suggests that the physiological stress produced by continuous cycling at 70% peak power for 90 min does not reduce immune function.

There were no significant changes in serum IgA levels observed during the exercising trial. Unlike salivary IgA, very few studies have investigated the effect of exercise on serum IgA levels, and the mechanisms behind variations in serum immunoglobulin levels in athletes in response to exercise are yet to be fully understood (Hejazi & Hosseini, 2012).

3.5.5 Correlation coefficients comparing electrolyte levels in saliva, urine and sweat to blood, while resting and exercising

During the exercising trial, there were no significant correlations in electrolyte levels, except between serum and salivary Na^+ levels at pre-exercise in males, and serum and salivary K^+ at 60 min in females. The pre-exercise timepoints in both the resting and exercising trials were the same (did not involve any exercise), so if the correlation between serum and salivary Na^+ levels among males at pre-exercise was true, then a correlation should also exist at the same time point in the resting trial. However, there were no correlations in the resting trial between serum and salivary Na^+ levels among males at pre-exercise, therefore the correlation noted in the exercising trial may not be reliable. In females, there was only one significant correlation between serum and salivary K^+ at 60 min. The fact that there was no correlation at any other timepoints or between any other body fluids indicates that this sporadic correlation between serum and salivary K^+ at 60 min may also not be reliable. The lack of correlations between blood and salivary electrolyte levels in this study is similar to the Wankasi et al. (2019) study, who reported that there were no significant correlations in potassium, sodium and chloride levels between blood and saliva for healthy participants.

There were no significant correlations for electrolyte levels at any timepoints, between serum and urine, except for Na^+ levels at pre-exercise and 90 min of the resting trial. One challenge with the urine measurements was the large standard deviations in Na^+, K^+, Cl^- levels seen in both the resting and exercising trials. This is similar to what has been observed in previous studies which reported a drawback of using urinary biomarkers due to considerable intra-individual variation (i.e., variation in ion concentrations from urine samples collected from the same individual at different time points) and an even higher levels of inter-individual variation

(Kang & Kho, 2019; Sun et al., 2009). Also, unlike blood, urine is a pooled body fluid that is stored in the bladder, that therefore does not immediately reflect the changes in the body occurring due to exercise.

There were no significant correlations between serum and sweat electrolyte levels, and these results are similar to other studies which reported no correlations between blood and sweat for sodium and chloride (Johnson et al., 1944; McCubbin et al., 2019; Mickelsen & Keys, 1943). Due to the fact that extracellular fluid is the precursor for sweat in the coil of eccrine sweat glands (Kuno, 1957), direct relationships between blood and sweat composition are sometimes assumed. However, before eccrine sweat is released onto the skin surface, modifications to the primary solution are made during its passage along the sweat glands duct. As a result of these modifications, serum and sweat composition do not always correlate (Baker & Wolfe, 2020) and may explain the lack of correlation in electrolyte levels noted between serum and sweat in this study.

3.5.6 Correlation coefficients comparing osmolality levels in saliva, urine and sweat to blood, while resting and exercising

There was no significant correlation between plasma and urine osmolality levels at any timepoints during the resting and exercising trials. Though urine osmolality has been used to assess water balance, it has been reported to be a poor standard to reliably track changes in body mass corresponding to acute dehydration and rehydration (Kovacs et al., 1999; Popowski et al., 2001). In this study, there were large standard deviations (202 – 389 mOsmol/kg) for the urine osmolality levels, indicating high inter-individual variability for urine osmolality levels between participants. Urine being a pooled body fluid, collected and stored over a period of time, is slow in detecting changes in hydration status during periods of rapid body fluid turnover (Popowski et al., 2001). Urine osmolality is less sensitive and shows a delayed reaction, lagging behind plasma osmolality changes, which increases and drops in an essentially linear pattern (Shirreffs, 2000) and this difference in sensitivity and response time may explain the lack of correlation noted between plasma and urine osmolality levels.

Even though a weak negative correlation was noted between plasma and salivary osmolality levels at pre-exercise in the resting trial, there was no correlation at the same time point in the exercising trial. Since neither of these time points involved exercise, the weak negative correlation noted between plasma and salivary osmolality levels at pre-exercise in the resting trial may not be reliable. The lack of correlation noted between plasma and salivary osmolality

levels could be due to the large variability in salivary flow rate noted in previous studies during low levels of hydration (Walsh et al., 2004b). Moreover, although participants in this study fasted for 3 h before the trial started, the interference of food and beverage consumption before the 3 h fast, as well as oral hygiene routines and dental erosions between participants may have contributed to the variations in salivary osmolality (Mulic et al., 2012), and consequently on the lack of significant correlation between plasma and salivary osmolality levels.

Plasma osmolality has been recognised as the gold standard for hydration status in previous studies (Lee et al., 2017). For a hydration status marker to be useful, it must be able to detect body water losses of 2–3% of body mass (Shirreffs, 2000), as a 2-3% reduction in body mass due to fluid loss has been linked to reduced heat dissipation, cardiovascular function, and exercise performance (Armstrong et al., 1985; Buskirk et al., 1958). However, in the current study, the body mass change after fluid correction was <1%. Moreover, this change was recorded over a single exercise bout, rather than over longer periods of time when body weight changes may be influenced by food intake, fluid intake and faecal losses. A study with 15 males who experienced a mean weight loss up to 3% by cycling at 60% $\dot{V}O_{2\,max}$, with no fluid intake, found a strong correlation between plasma and saliva osmolality (r=0.87) (Walsh et al., 2004a). Therefore, it is challenging to assess the relationship between blood osmolality and urine, saliva or sweat osmolality as markers of hydration when there is no significant body mass loss as noted in this study.

3.5.7 Correlation coefficients comparing IgA levels in saliva to blood, while resting and exercising

The present study found significant positive correlations between serum and salivary IgA levels at pre-exercise, 60 min and 120 min of the exercising trial, and 30 min, 60 min and 90 min of the resting trial. All the other timepoints, even though not significant, also showed a positive correlation between serum and salivary IgA levels. This trend is promising as it opens the possibility of using saliva samples as indirect measures to predict serum IgA levels (e.g., to evaluate antibody immunodeficiencies) among athletes, overcoming the requirement of a blood collection, which is technically demanding, invasive, stressful, and disruptive when exercising. Outside the world of exercise, serum IgA has been used as a diagnostic biomarker in patients with oral cancer and seropositive rheumatoid arthritis, so a correlation between salivary IgA and serum IgA may enable these patients to assess their serum IgA level, based

on their salivary IgA level, without the need of a hospital or clinical setting (Inamo et al., 2021; Madki et al., 2021).

3.6 Conclusion

This study showed that serum Na^+ and Cl^-, salivary K^+, urinary Na^+ and sweat osmolality levels are different in males and females, and results from male and female participants should not be pooled when studying changes in these biomarkers during exercise. However, it is acceptable for other biomarkers (serum K^+ and plasma osmolality, salivary Na^+, Cl^- and osmolality, urine K^+, Cl^- and osmolality, sweat Na^+, Cl^- and K^+) which did not differ by sex, to be pooled. During exercise, there were significant changes in electrolyte levels in blood, urine and sweat over time. The presence of a positive correlation between serum and salivary IgA levels during exercise is promising, as it opens the possibility of using saliva samples as indirect measures to predict serum IgA levels in athletes, thus overcoming the requirement of blood collection, which is technically demanding, invasive, stressful, and disruptive when exercising.

CHAPTER 4: FINAL CONCLUSION AND RECOMMENDATIONS

4.1 Summary of results and main findings

In the exercising trial, serum Na^+, Cl^-, salivary K^+, urine Na^+, and sweat osmolality levels were significantly different between males and females. These findings demonstrate that males and females react differently while exercising and indicate that male and female participants' results should not be pooled when studying changes in these specific biomarkers (i.e., serum Na^+, Cl^-, salivary K^+, urine Na^+ and sweat osmolality) during exercise. However, it is acceptable for male and female data to be pooled for the other biomarkers (serum K^+ and plasma osmolality, salivary Na^+, Cl^- and osmolality, urine K^+, Cl^- and osmolality, sweat Na^+, Cl^- and K^+) analysed in this study. While exercising, there were significant changes in electrolyte levels in blood, urine and sweat over time, and these changes can be explained by the physiological alterations that occur in the body when exercising. There were strong positive correlations at multiple timepoints between blood and saliva IgA levels in the resting and exercising trials. This is promising as salivary IgA levels can be used as an alternative and non-invasive method to assess blood IgA levels. For athletes, it provides the added advantage of estimating their serum IgA level without disrupting their exercising regime. However, for electrolyte and osmolality levels, there were no reliable correlations between blood and saliva, urine or sweat. This indicates that blood biomarkers are still the best tool to measure athletes' electrolyte and osmolality levels when monitoring their performance and recovery, as blood biomarkers reflect the body changes immediately and provide an accurate representation of the body's status when compared to the other body fluids.

4.2 Strengths

As indicated earlier, when compared to males, studies investigating the effects of exercise in females are significantly underrepresented in sports and exercise journals. One of the major strengths of the present study is the inclusion of 8 females in the pool of 20 individuals recruited for this study and its aim to understand if there were differences in electrolyte, osmolality and IgA levels between the sexes. Secondly, the advantage of cross-over designs means that the same individuals can participate in both arms of the trial to avoid inter-individual variability between the resting and exercising trials. Lastly, several studies involving collection of saliva samples have failed to take into account the circadian variation of cortisol secretion.

However in this study, saliva samples were collected at the same time of the day for both trials to control for the effects of circadian variation and habitual activity known to cause alterations in salivary immunoglobulin and cortisol levels (Dimitriou et al., 2002).

4.3 Limitations and recommendations for future research

There were a number of limitations in this study. Participants were required to record a food diary over the 48-h period preceding the first main trial and asked to replicate the same diet and timing for the subsequent trial. As this was self-reported, there is no assurance that the information provided was accurate. Food and fluid can have a big impact on the levels of electrolyte, IgA and on osmolality (Laing et al., 2005; Lee et al., 2017). A better approach would have been to provide a standard meal at set times to all participants before both trials.

A number of sweat samples were unable to be analysed using the ion selective electrode analyser as it was not sensitive enough to detect the low concentrations in these particular samples. For some analysis, this led to relatively small sample size and some samples did not have a baseline measurement for comparison. Another model of an electrode analyser with a better sensitivity, or an alternative technique such as flame photometry is recommended for future studies.

The pre-exercise blood samples were collected just 15 min before the trial. This may not be representative of a true resting state due to anxiety and preparations for the 90 min cycling trial. Bringing in the participants earlier, for example 2-4 h before the start of the trial and providing all participants with the same controlled meal, then allowing a 3-hr fasting period before collecting the pre-exercise blood samples would be advantageous. This would not only provide blood samples with a baseline level that is close to a true resting state, but would also help to achieve the recommendation (stated above) of providing a standardised meal to all participants at a set time before both trials.

The menstrual phase and use of oral contraceptives of the female participants were not captured in this trial. As the female hormones oestrogen and progesterone fluctuate regularly during the menstrual cycle, and these hormones regulate the physiological systems of the body, having this information may have helped to address the electrolyte and osmolality differences seen in this study better.

The results of this study are applicable to indoor cycling exercise performed on a cycle ergometer. It would be beneficial to understand if other exercise modalities such as running, swimming, or rowing also produce the same results, indoors and in the field.

Lastly, the sample size consisted of only 20 participants, and most were young healthy individuals (mean age ~27 years). This restricts the ability to generalize the findings of this study across a wide population. In addition, measurements of body composition to characterize fat mass and fat free mass, which were not collected in this study, may have helped to explain some of the sex differences of the current results. Therefore, a larger sample size, including people of various ages and fitness levels, would offer a more accurate representation of the population.

4.4 Use of Findings

This study has shown that there are sex differences in certain electrolyte and osmolality levels between males and females when exercising; to the author's knowledge this is a novel finding. Future researchers need to take these sex differences into account when designing and carrying out their trials.

This study was also the first to demonstrate the changes in electrolytes and osmolality levels in all four body fluids (namely, blood, saliva, urine and sweat) simultaneously during exercise. This provided an understanding of how electrolyte and osmolality levels change during exercise in each of the four body fluids, as well as a comparison of how they change in comparison to the other body fluids.

This study also showed significant correlations between blood and salivary IgA levels at multiple timepoints of the resting and exercising trials. Moreover, there were consistent negative correlations (not all statistically significant) in osmolality levels between blood and saliva osmolality levels. Further research using a larger sample size will help to validate the findings of this study. This information, if further validated, will be relevant to athletes and exercisers who can opt for a convenient, non-invasive and cost-effective sample collection method that can be performed by themselves with minimal skills in order to assess their hydration status (osmolality) and immune status (salivary IgA). Information about their hydration and immunity status will help athletes to change their nutritional intake or alter their training regime to improve their performance and recovery. The ability to track osmolality and levels of an immune function marker using saliva will enable them to track it outdoors, rather than requiring a clinical or laboratory setting. Monitoring of the mucosal immune status through

salivary IgA will be relevant to practitioners, academics and researchers, who can inform guidelines for nutritional support and management of training levels, and thereby minimise the risk of URTI.

The finding of correlation between blood and salivary IgA levels is promising and highlights the opportunity to explore correlations of other biomarker levels (HIV and SARS-CoV-2 antigen levels) in non-invasive body fluids such as saliva, urine and sweat, instead of blood. If correlations exist, the use of non-invasive body fluids can be extended to a hospital setting where clinicians would benefit from the use of other body fluids such as saliva due to its ease of collection, affordability, simplicity and less chance of accidental viral transmission to medical staff (Ezukwa et al., 2018). Babies and aged patients with thin veins would also benefit from the use of less invasive and pain-free sample collection techniques.

This study indicated that electrolyte levels changed significantly in both males and females during exercise. This data will be relevant to the food and nutrition industry (e.g., supplement manufacturers) who can advocate for new dietary options, such as using supplements or energy drinks, that benefit athletes to better complement changes in electrolyte levels during exercise.

CHAPTER 5: REFERENCES

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APPENDIX

Appendix A: Pre-exercise health screening questionnaire

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Pre-Exercise Health Screening Questionnaire

Name:_____

Address: _____

Phone: _____

Age: _____

Please read the following questions carefully. If you have any difficulty, please advise the medical practitioner, nurse or exercise specialist who is conducting the exercise test.

Please answer all of the following questions by ticking only one box for each question:

This questionnaire has been designed to identify the small number of persons (15-69 years of age) for whom physical activity might be inappropriate. The questions are based upon the Physical Activity Readiness Questionnaire (PAR-Q), originally devised by the British Columbia Dept of Health (Canada), as revised by [1]Thomas *et al.* (1992) and [2]Cardinal *et al.* (1996), and with added requirements of the Massey University Human Ethics Committee. The information provided by you on this form will be treated with the strictest confidentiality.

Qu 1. Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?

Yes ☐ No ☐

Qu 2. Do you feel a pain in your chest when you do physical activity?

Yes ☐ No ☐

Qu 3. In the past month have you had chest pain when you were not doing physical activity?

Yes ☐ No ☐

Qu 4. Do you lose your balance because of dizziness or do you ever lose consciousness?

Yes ☐ No ☐

Qu 5. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?

Yes ☐ No ☐

Qu 6. Do you have a bone or joint problem that could be made worse by vigorous exercise?

Yes ☐ No ☐

Qu 7. Do you know of any other reason why you should not do physical activity?

Yes ☐ No ☐

Qu 8. Have any immediate family had heart problems prior to the age of 60?

Yes ☐ No ☐

Qu 9. Have you been hospitalised recently?

Yes ☐ No ☐

Qu 10. Do you have any infectious disease that may be transmitted in saliva?

Yes ☐ No ☐

Qu 11. Do you have any infectious disease that may be transmitted in blood?

Yes ☐ No ☐

Qu 12. Do you have any allergies to skin preparations (e.g., alcohol swabs), adhesives (e.g., plasters) or dressings (e.g., bandages)

Yes ☐ No ☐

You should be aware that even amongst healthy persons who undertake regular physical activity there is a risk of sudden death during exercise. Though extremely rare, such cases can occur in people with an undiagnosed heart condition. If you have any reason to suspect that you may have a heart condition that will put you at risk during exercise, you should seek advice from a medical practitioner before undertaking an exercise test.

I have read, understood and completed this questionnaire.

Signature: _____ Date: _____

Signature of Parent: _____
or Guardian (for participants under the age of consent)

References

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2. Cardinal BJ, Esters J and Cardinal MK. Evaluation of the revised physical activity readiness questionnaire in older adults. *Med Sci Sports Exerc* 28(4): 468-472

Appendix B: Participant information sheet

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Comparison of electrolyte levels and immune function markers in saliva, sweat, urine and/or plasma in resting and exercising humans

PARTICIPANT INFORMATION SHEET

Invitation to Participate in Research Study

We, Mr Simon Bennett, Dr Ali, Dr Rutherfurd-Markwick, Ms Sherina Holland, Ms Stevie Robinson, Ms Saskia Stachyshyn and Ms Micaela Makker (Massey University) and Mr John Taulu, Mr Olivier Morin and Dr Fletcher (AUT University) would like to invite you to participate in a study investigating measurement of electrolytes and immune function markers from various bodily fluids.

Various markers can be used to assess how the body responds to exercise and to examine the effects of training programmes. Some of the best ways to do this require invasive procedures such as muscle biopsies and blood sampling. However, it may be easier to analyse urine, saliva and/or sweat samples – especially for certain electrolytes (salts), hormones and immune markers – and this may increase the likelihood of getting more participants for exercise studies. Therefore, the aim of this study is to compare electrolyte, hormones and immune markers in various bodily fluids; we will examine responses in males vs. females and rest vs. exercise. Following familiarisation, participants will come to the lab on two different occasions for resting and exercise trials. Body fluid samples will be taken at 0, 30, 60 and 90 minutes for each trial. Blood samples will be taken via indwelling catheter; saliva samples using a passive drool method; sweat samples via the use of sweat patches taped to the skin and mid-stream urine will also be collected (all standard procedures). Samples will be stored in the freezer for later analysis. The results from this research will be applicable for exercise scientists, food and nutrition industry as well as epidemiologists.

Participant Recruitment

Approximately 26 participants (13 male and 13 female) will be recruited to provide sufficient statistical power to the study. All participants will be reimbursed for travel expenses with $20 of MTA vouchers upon completion of the second experimental trial.

To be included for this study you must be:
- Aged 18-55 years
- Willing and able to undergo 90 min of continuous cycling exercise at an intensity corresponding to 60-70% of your maximum heart rate.
- A non-smoker
- Not taking any prescription or recreational drugs, including inhalers or corticosteroid creams
- Not menstruating during the actual trials (females only)
- Willing to provide blood, saliva, sweat and urine samples for measurement of electrolytes and immune function markers

Risks/Discomforts of the study could include:
• Feeling tired or fatigued after the exercise sessions.
• Mild soreness from blood sampling

You will be informed verbally and in writing, of the aims, procedures, demands and any potential risks and discomfort that the study will entail. You will then have the opportunity to give written consent. Please note that you will still retain the right to withdraw from the study, without reason, at any stage; your relationship with Massey University will not be affected whether or not you choose to participate in this study.

Project Procedures and Participant Involvement

Before taking part in this study you will be asked to complete a Health Screening Questionnaire. This screening questionnaire is used to ascertain any information that may conflict with the study, (e.g., if you are taking any medications that may interfere with the outcome of the study) and may ultimately prohibit you from participating. If you have any medical condition listed in the Health Screening Questionnaire then we will have to exclude you from taking part. The information obtained on all study questionnaires is strictly confidential and will be used for the purposes of the present study only.

Preliminary trial

You will be asked to attend an initial session for a maximal heart rate test to determine an exercise intensity relative to 60-70% of your maximum heart rate. Heart rate will be measured throughout the trials using a heart rate monitor. This test should take no more than 12 min. After this test is completed you will be provided with a paper copy of your heart rate training zones. After a brief rest the four collection methods will be explained to you and you will have the opportunity to ask any questions as required.

You will then be asked to cycle continuously for 10 min so that you can practice the saliva sampling under exercise conditions. Upon successful completion of this initial trial we will book a time for your first experimental session. We will provide you with water to drink prior to your first experimental trial.

Main trials

Prior to the main trials we will remind you of your appointment time, and to consume the water which was provided to you. When you arrive for your main trial we will ask you to provide a mid-stream urine sample to assess your hydration status, measure your height and weight (using electronic scales in private room) and then allocate you to either the resting or exercise trial. We will ask you to provide details of your dietary intake for 48 h (2 days) prior to the main trial so that we can quantify your intake of electrolytes. We would like you to replicate the same 2-day diet prior to the second main trial. As the trial order is randomised we will ask you to arrive to your first experimental session with clothing suitable for exercise. You will then have a cannula placed in your arm. You will also have sweat patches placed on 2 sites of your body (chest and mid-thigh). Please note that these areas may need to be shaved to allow for better placement of the sweat patches. You will have a heart rate strap wrapped around your chest to continuously measure heart rate throughout the trial.

After completing these preliminary procedures, a 90 min resting or exercise trial will begin. The exercising condition requires you to continuously exercise on a stationary cycle at an intensity relating to 60-70% of your maximal heart rate. The resting condition requires you to remain seated for the entire 90 min duration. Ten mL blood samples and 1 mL saliva samples will be taken at 0, 30, 60 and 90 minutes of each trial. A second mid-stream urine sample will be collected at 90 minutes. The sweat patches will be removed after the 90-minute trial.

Individuals trained in resuscitation (NZ Red Cross First Aid, Level 2) and use of a defibrillator will be present for all exercise sessions. In addition, the researchers will be constantly monitoring physiological and perceptual variables that will aid in identifying any major issues.

Participant's Rights

You are under no obligation to accept this invitation. Should you choose to participate, you have the right to:

- decline to answer any particular question
- withdraw from the study at any time, even after signing a consent form (if you choose to withdraw you cannot withdraw your data from the analysis after the data collection has been completed)
- ask any questions about the study at any time during participation
- provide information on the understanding that your name will not be used unless you give permission to the researcher
- be given access to a summary of the project findings when it is concluded (in the form of a summary sheet containing mean data for the whole group)

Confidentiality

All data collected will be used solely for research purposes and has the possibility of being presented in a professional journal. All personal information will be kept confidential by assigning numbers to each participant. No names will be visible on any papers on which you provide information. All data/information will be dealt with in confidentiality and will be stored in a secure location for five years on the Massey University Albany Campus. After this time it will be disposed of by an appropriate staff member from the School of Sport and Exercise.

Project Contacts

If you have any questions regarding this study, please do not hesitate to contact any of the following people for assistance:

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Committee Approval Statement

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 12/63. If you have any concerns about the conduct of this research, please contact Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A telephone 06 350 5799 x 8717, email humanethicsoutha@massey.ac.nz

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.