

**Monitoring Mucosal Immunity,
Upper Respiratory Illness, and the Influence of
Vitamin D in Athletes**

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Abstract

Introduction: Upper respiratory illness (URI) is the most frequently reported illness in athletes, resulting in lost training time and reduced success at competition. Due to the high physical demands on elite swimmers, identification of risk factors associated with URI and individuals at increased risk of illness would be paramount. Individual athletic monitoring of mucosal immunity and identifying risk factors of URI, could enable modification of training and other illness preventative strategies (Gleeson & Pyne, 2016). Vitamin D levels are known to naturally decrease in winter, further increasing risk of seasonal illnesses. However, few studies have examined the effect of vitamin D supplementation on mucosal immunity and the prevention of URI in athletes.

Methods: Study one (**Chapter 4**) investigated the effect of training load, sleep and EBV status on mucosal immunity and self-reported URI symptoms, for 8-months leading into the 2018 Commonwealth Games. The periodised training load plan for each week was classified by coaches as low, moderate, and high. When this was compared against swimmer's perceived weekly training intensity, it was significantly correlated. Weekly sIgA was examined to investigate changes in mucosal immunity over different training loads and leading into major competition. Blood samples were taken to determine EBV status and self-reported URI symptoms were quantified using an adapted illness questionnaire. In addition to monitoring self-perceived sleep, quantitative sleep parameters were measured using wrist-worn actigraphy at three different time points during the study period. **Chapter 5** presents the development and optimisation of an in-house Enzyme Linked Immunosorbent Assay (ELISA). Assay development and validation protocols were carried out in order to produce an ELISA for determination of sIgA, from the elite swimmer samples in **Chapter 4**. The final study (**Chapter 6**) provides a meta-analysis and systematic review on the effectiveness of vitamin D supplementation in prevention of URI and the effect on immune markers.

Results: Over the 8-month observation (**Chapter 4**), 70 upper respiratory symptom (URS) episodes were recorded, leading to 34 days of missed training. Significantly more URS

were reported, with increased duration and severity during high training loads. In total, 61% of swimmers had evidence of past infection with EBV. However, EBV seropositivity had no correlation with the number of episodes, severity, or duration of symptoms. When sIgA values were normalised to each individual's mean, relative sIgA concentration was significantly lower during URS (25%) than when no symptoms were present. No relationship was found for sIgA against training load, and incidence, duration, or severity of URS. Sleep duration was significantly lower during high training loads, plus sleep efficiency was poor throughout the study period. Validation and optimisation of the ELISA was conducted in **Chapter 5**, which included successful analysis of sensitivity, accuracy, precision, linearity of dilution, matrix effects, range, and intra-variation. Additionally, results from the in-house ELISA were significantly correlated with those from a commercially available assay. The meta-analysis (**Chapter 6**) found no effect on sIgA, or URS duration and severity following vitamin D supplementation.

Practical Application: The current thesis highlights the importance of individual athlete monitoring for coaches and physiologists to identify athletes at increased illness risk. Identification of risk factors associated with URS, such as increased training load, lowered sIgA and inadequate sleep, may allow for modifications in training or other illness preventative strategies. Overall, elite swimmers showed inadequate sleep; therefore promoting the use of sleep hygiene strategies and napping. There was high prevalence of EBV seropositivity for the elite swimmers; however it was not a predictor of URS. Low participant numbers could be considered for the lack of significant findings with EBV serostatus; there is ongoing debate that individual data and trends may be more useful in elite athletic research, compared to group statistical analysis. Secondly, thesis work presenting ELISA optimisation and validation was successful in offering a good basis for future work. Lastly, findings from the meta-analysis regarding the effect of vitamin D supplementation on URI contrasted with many large cohort studies from the general population. The small number of studies included, low participant numbers, and differences in study design may have influenced these findings. Thus, a clear gap in research is evident and more well-designed control trial studies are needed to establish the relationship between supplementation of vitamin D and URI in athletes.

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Table of Contents

Abstract.....	i
Acknowledgements	iii
Table of Contents.....	v
List of Tables.....	x
List of Figures.....	xii
Commonly Used Abbreviations	xv
CHAPTER 1: Introduction	1
CHAPTER 2: Literature Review	5
2.1 Overview of the Immune System	5
2.1.1 Mucosal Immunity.....	5
2.1.2 Immunoglobulin A.....	7
2.2 Upper Respiratory Illness.....	9
2.2.1 Monitoring URI with Self-Report Questionnaires	10
2.3. Exercise and URI Risk.....	12
2.3.1 URI Risk for Athletes	16
2.3.2 Causes of URI	18
2.4 SIgA and URI.....	22
2.4.1 Collection of sIgA.....	23
2.4.2 Variability of sIgA.....	25
2.4.3 Expression of sIgA.....	26
2.5 Exercise and sIgA.....	27
2.5.1 Acute Exercise and sIgA.....	27
2.5.2 Effect of Training Load on sIgA in Athletes.....	30
2.6 Overview of Sleep.....	33
2.6.1 Stages of Sleep and Definitions.....	33
2.6.2 Recommendations.....	34
2.6.3 Impact of Sleep Inadequacy	35
2.7 Athletes and Sleep.....	37
2.7.1 Quantity of Sleep Achieved.....	37
2.7.2 Quality of Sleep Achieved.....	38

2.7.3 Impact of Training Load and Competition on Sleep	39
2.7.4 Strategies to Improve Sleep.....	40
2.7.5 Methods of Monitoring Sleep	42
2.8 Vitamin D and URI Risk	44
2.8.1 Deficiency	46
2.9 Supplementation of Vitamin D.....	48
2.9.1 Effect on URI	49
2.9.2 Effect on Mucosal Immunity.....	50
2.10 Thesis Aims	52
CHAPTER 3: General Methods	53
3.1 Ethical Approval	53
3.2 Participant Recruitment.....	53
3.3 Saliva Collection and Storage	53
3.3.1 Saliva Flow Rate.....	54
3.4 Commercial ELISA.....	54
3.5 General Statistical Analysis.....	55
CHAPTER 4: Immune Monitoring Alongside Factors Associated with Upper Respiratory Illness in Elite Swimmers, Over an 8-month Training Period.....	56
4.1 Introduction	56
4.1.1 Study Aims.....	59
4.2 Methods	60
4.2.1 Participants	60
4.2.2 Baseline Measurements	61
4.2.3 Blood Collection and Storage	61
4.2.4 Salivary IgA Sampling and Analysis	62
4.2.5 Training Load.....	62
4.2.6 Illness Symptoms Questionnaire	63
4.2.7 Sleep Analysis	64
4.2.8 Statistical Analysis	64
4.2.8.1 Individual Analysis	65
4.3 Results.....	66
4.3.1 Training Load.....	66
4.3.2 Incidence, Duration and Severity of URS	67

4.3.3 Salivary IgA.....	70
4.3.4 Sleep	79
4.3.5 Epstein Barr Virus.....	87
4.3.6 Body Mass	88
4.4 Discussion.....	90
4.4.1 Training Load.....	90
4.4.2 Upper Respiratory Symptoms.....	91
4.4.3 Salivary IgA.....	94
4.4.4 Epstein Barr Virus.....	99
4.4.5 Sleep	101
4.4.6 Strengths, Limitations and Future Work.....	106
4.5 Conclusion	108
CHAPTER 5: Optimisation and Validation of an Enzyme Linked Immunosorbent Assay for Salivary IgA.....	109
5.1 Introduction	109
5.1.1 Types of ELISA.....	109
5.1.2 Assay Optimisation	112
5.1.3 Assay Validation	116
5.2 Methods and Materials.....	120
5.2.1 Materials	120
5.2.2 Assay Protocol.....	122
5.2.3 Assay Optimisation	122
5.2.4 Assay Validation	124
5.2.5 Participant Recruitment.....	125
5.2.6 Sample Collection and Storage	125
5.2.7 Reagent Storage.....	125
5.2.8 Analysis	125
5.3 Results.....	126
5.3.1 Assay 1	126
5.3.2 Assay 2.....	131
5.4 Discussion.....	137
5.4.1 Creation of the Standard Curve	137
5.4.2 Assessing Matrix Effects.....	139

5.4.3 Precision	140
5.4.4 Strengths, Limitations and Future work	142
5.5 Conclusion	144
CHAPTER 6: Effect of Vitamin D Supplementation on Markers of Immune Function and Upper Respiratory Illness in Athletes; a Meta-analysis and Systematic Review	145
6.1 Introduction	145
6.1.1 Aims.....	148
6.2 Methods	149
6.2.1 Search Strategy and Eligibility	149
6.2.2 Data Extraction	150
6.2.3 Risk of Bias.....	151
6.2.4 Statistical Analysis	152
6.3 Results.....	153
6.3.1 Eligibility and Selection Assessment.....	153
6.3.2 Primary Outcome - Vitamin D and markers of immune function	158
6.3.3 Secondary Outcome - Vitamin D and upper respiratory illness.....	164
6.3.4 Risk of Bias Assessment	168
6.4 Discussion.....	171
6.4.1 Vitamin D and Immune Markers	171
6.4.2 Vitamin D and Illness	175
6.4.3 Considerations.....	177
6.4.4 Strengths, Limitations and Future Work.....	180
6.5 Conclusion	182
CHAPTER 7: General Discussion.....	183
7.1 Summary of Main Findings	184
7.1.1 Chapter 4.....	184
7.1.2 Chapter 5.....	184
7.1.3 Chapter 6.....	185
7.2 Contribution to Knowledge	185
7.2.1 Risk Factors for URI in Athletes.....	185
7.2.2 Effect of Vitamin D Supplementation on sIgA and URI	188
7.2.3 Individual Athlete Monitoring vs. Group Mean Data.....	190
7.3 Strengths and Limitations of Thesis	193

7.4 Future work	195
7.5 Conclusion	197
CHAPTER 8: References	198
CHAPTER 9: Appendices	233
Appendix A	233
Appendix B	234
Appendix C	235
Appendix D	236
Appendix E	237
Appendix F	238

List of Tables

Table 2.1	<i>Definitions of Common Sleep Parameters</i>
Table 4.1	<i>Study aims</i>
Table 4.2	<i>Participant characteristics</i>
Table 4.3	<i>Average, minimum and maximum weekly training distances, plus weeks spent at each training load, for both training groups</i>
Table 4.4	<i>Illness parameters and sIgA over different training loads between training groups</i>
Table 4.5	<i>Illness parameters and sIgA over different training loads between sexes</i>
Table 4.6	<i>Average night-time sleep data</i>
Table 4.7	<i>Average nap-time sleep data</i>
Table 4.8	<i>Percentages of time the swimmers slept the national recommendation of 7-9 hours per night, for differing training loads</i>
Table 4.9	<i>Percentages of time the swimmers woke feeling fatigued each week, for differing training loads</i>
Table 4.10	<i>Average ActiGraph sleep data two weeks prior to major competition</i>
Table 4.11	<i>Comparisons of illness parameters by EBV serology status</i>
Table 5.1	<i>Materials used for in-house ELISA for detection of sIgA</i>
Table 5.2	<i>Tested dilution factors of capture antibody, concentration range and dilution factors</i>
Table 5.3	<i>Spike and recovery percentage obtained from saliva, compared to both PBS and a different 'IgA-blank' saliva.</i>
Table 5.4	<i>Precision assay, assessing intra-assay variability</i>

Table 6.1	<i>Characteristics of RCT studies included within the meta- analysis</i>
Table 6.2	<i>Characteristics of observational studies, plus one case study intervention</i>
Table 6.3	<i>RCT studies used for each random effects model</i>
Table 6.4	<i>Study results examining association between vitamin D and immune markers</i>
Table 6.5	<i>Study results examining association between vitamin D and URI</i>

List of Figures

- Figure 2.1 *Transportation of IgA to the mucosal surface*
- Figure 2.2 *Secretory IgA in dimeric form*
- Figure 2.3 *The “J-shaped” curve*
- Figure 2.4 *The “S-shaped” curve*
- Figure 2.5 *Factors affecting measurement of sIgA*
- Figure 2.6 *Decision tree for athletic supplementation of vitamin D*
- Figure 4.1 *Study design diagram to shown what measurements were taken and when*
- Figure 4.2 *Total number of URS episodes over different training loads*
- Figure 4.3 *Mean duration of URS episodes over different training loads*
- Figure 4.4 *Total number of missed training days over different training loads*
- Figure 4.5 *Mean symptom severity score over different training loads*
- Figure 4.6 *Seven weeks of salivary IgA (%), leading in and out of URS episodes*
- Figure 4.7 *Four weeks of salivary IgA (%), leading in and out of URS episodes*
- Figure 4.8 *Variation from the average IgA concentration, plotted over the 8-month season for one swimmer*
- Figure 4.9 *Illness measures presented alongside sIgA concentration for the sprint group*
- Figure 4.10 *Illness measures presented alongside sIgA concentration for the middle-distance group*
- Figure 4.11 *IgA concentration ($\mu\text{g}\cdot\text{mL}^{-1}$), sleep score and overall number of URS episodes for the sprint group*

- Figure 4.12 *IgA concentration ($\mu\text{g}\cdot\text{mL}^{-1}$), sleep score and overall number of URS episodes for the distance group*
- Figure 4.13 *Percentage of time the national recommendation of 7-9 hours of sleep was met (%), during two weeks leading into competition compared to two weeks mid-season training*
- Figure 4.14 *Percentage of time swimmers woke feeling fatigued (%), during two weeks leading into competition compared to two weeks mid-season training*
- Figure 4.15 *Reported number of URS episodes for EBV positive (+) and negative (-), over all three training loads*
- Figure 4.16 *Change in body mass from baseline of female swimmers*
- Figure 4.17 *Change in body mass from baseline of male swimmers*
- Figure 5.1 *Chosen method; Sandwich (direct) ELISA*
- Figure 5.2 *Assay template layout*
- Figure 5.3 *Standard curve ($25\text{-}50\ \mu\text{g}\cdot\text{mL}^{-1}$) for sIgA using four different dilutions of coating antibody*
- Figure 5.4 *Low range standard curve ($6.25\text{-}50\ \mu\text{g}\cdot\text{mL}^{-1}$) for sIgA using four different dilutions of coating antibody*
- Figure 5.5 *Image of completed assay, examining five coating antibody dilutions*
- Figure 5.6 *Standard curve ($12.5\text{-}400\ \mu\text{g}\cdot\text{mL}^{-1}$) for sIgA following checkerboard titration assay*
- Figure 5.7 *Standard curve for sIgA in PBS and IgA-blank saliva*
- Figure 5.8 *Interpolated standard curves following the checkerboard titration assay*

- Figure 5.9 *Standard curves for sIgA shown with two different substrate incubation times*
- Figure 5.10 *Image of completed precision assay*
- Figure 5.11 *Comparison of commercial and in-house ELISA*
- Figure 5.12 *Variation from the average IgA concentration plotted over the 8-month season, with comparison of commercial and in-house ELISA*
- Figure 6.1 *PRISMA flowchart*
- Figure 6.2 *IgA Concentration, intervention vs. placebo (n = 100)*
- Figure 6.3 *IgA secretion rate, intervention vs. placebo (n = 75)*
- Figure 6.4 *TNF- α , supplementation vs. placebo (n = 62)*
- Figure 6.5 *URI Duration, intervention vs. placebo (n = 91)*
- Figure 6.6 *Symptom Severity Score, intervention vs. placebo (n = 116)*
- Figure 6.7.1 *RoB2 output, traffic light plot*
- Figure 6.7.2 *RoB2 output, summary plot*

Commonly Used Abbreviations

(Inclusive of shortened works, symbols and units):

± Plus, or Minus

< Less Than

> More Than

≤ Less-than or Equal to

≥ Greater-than or Equal to

% Percentage

$\eta^2_{partial}$ Partial eta squared

ABTS™ 2, 2'-Azino-di-(3-ethylbenzthiazoline sulphonate)

AIS Australian Institute of Sport

AMP Anti-microbial Peptides

ANOVA Analysis of Variance

AXIS Appraisal tool for Cross-Sectional Studies

BSA Bovine Serum Albumin

cm Centimetres

CV Coefficient of Variance

DNA Deoxyribonucleic Acid

EBV Epstein Barr Virus

EBNA Epstein Barr Virus nuclear antigen

ELISA Enzyme-Linked Immunosorbent Assay

g Grams

HRP Horseradish Peroxidase

I² Heterogeneity statistic
Ig Immunoglobulin
IgA Immunoglobulin A
IL Interleukin
Kg Kilograms
Km Kilometres
LD Limit of Detection
LL-37 Cathelicidin
LLOQ Lower Limit of Quantification
LOCF Last Observation Carried Forward
m Metres
µg.mL⁻¹ Micrograms per millilitre
µL Microlitres
mg Milligrams
min Minutes
mL Millilitres
mmol.L⁻¹ Millimoles per litre
NOCB Next Observation Carried Backward
OD Optical Density
OPD o-phenylenediamine
QC Quality Control
PBS Phosphate Buffered Saline
RCT Randomised Control Trial

RNI Reference Nutrient Intake
RoB2 Cochrane Risk of Bias 2
ROBINS-I Risk of Bias in Non-randomized Studies of Interventions
RPE Rating of Perceived Exertion
s Seconds
SC Standard Curve
SD Standard Deviation
SE Sleep Efficiency
SIgA Salivary Immunoglobulin A
SPSS Statistical Package for the Social Sciences
TL Training Load
TMB 3,3',5,5'-Tetramethylbenzidine
TST Total Sleep Time
TTIB Total Time in Bed
TNF- α Tumour Necrosis Factor alpha
UI International Units
UK United Kingdom
ULOQ Upper Limit of Quantification
URI Upper Respiratory Illness
URS Upper Respiratory Symptoms
URTI Upper Respiratory Tract Infection
VCA Viral-capsid Antigen
VDR Vitamin D Receptor

Vitamin D2 ergocalciferol

Vitamin D3 cholecalciferol

WASO Wakes After Sleep Onset

25(OH)D 25-hydroxyvitamin

1,25(OH)₂D 1,25-Dihydroxyvitamin D

CHAPTER 1

Introduction

Successful competitive performance for an elite athlete is determined by the narrowest of margins (Colbey et al., 2018); specifically, there was 0.34 of a second separating gold, silver and bronze medal winners in the men's 50m Freestyle at the 2020 Summer Olympic Games in Tokyo. At the 2016 Olympic Games, in 81% of all swimming events the difference between winning and finishing 4th was less than a second (Pollock et al., 2019). Whether the goal is to qualify to represent their country, or win an international medal, numerous years of training are needed in order to achieve success as an elite athlete. In swimming, sprinters typically race for between 20 seconds to 1 minute, then middle-distance swimmers typically endure races of 2-5 minutes. However, the training schedule of elite swimmers starts in adolescence and includes 3-6 hours of training per day. This would usually include two pool sessions and one dry land session, involving resistance, plyometric and flexibility training (Hellard et al., 2015; Papacosta & Nassis, 2011). The importance of avoiding missed training days was highlighted by Raysmith and Drew (2016), when athletes were seven times more likely to achieve a performance goal if they completed >80% of planned training weeks. Due to small margins for athletic success, chance of performance success has been shown to significantly reduce for every week of missed training (Raysmith & Drew, 2016). On top of this, a national and international swimmers' competition schedule includes racing 10-30 times per year, thus the importance of staying illness-free is evident.

Despite this, during international competition it has been reported that up to 8% of elite athletes experience illness, with $\leq 64\%$ of those illnesses affecting the respiratory system (Alonso et al., 2010; Engebretsen et al., 2013; Mountjoy et al., 2010; Soligard et al., 2015). Interestingly, 18-46% of these illnesses resulted in absence from training and competing (Alonso et al., 2010; Engebretsen et al., 2013; Soligard et al., 2015; Soligard et al., 2017), and were reported most by swimmers (Mountjoy et al., 2010). This is important because self-reported mild illness symptoms have shown to have a small harmful effect on male swimmers (Pyne et al., 2005), being detrimental on performance by reducing alertness,

reaction times and prompting low mood (Smith, 2012; Smith et al., 1998). Naturally, athletes who manage to avoid illness during taper and competition, tend to swim faster than others who experienced upper respiratory illness (URI) (Pyne et al., 2001). Yet in one study the majority of illnesses occurred within the final two months prior major competition (Raysmith & Drew, 2016). The above findings highlight the impact of URI on missed training and subsequent competitive performance (Engebretsen et al., 2013; Neville et al., 2008).

Risk of URI increases with additional stressors suffered by athletes, such as; repeated bouts of unusually heavy training, participating in competitive events, overseas travel, lack of sleep and nutritional deficits (Bermon et al., 2017; Walsh, 2019). Most commonly, athletes suffer from increased risk of URI because they endure heavy training loads. In elite swimmers who were monitored over a 4-year period, risk of URI was 50-70% higher during intensive training, especially during winter (Hellard et al., 2015; Svendsen et al., 2016). With this, falls in mucosal immunity following prolonged, heavy training bouts has been found alongside increased risk of URI (Neville et al., 2008). In light of this, Gleeson et al. (2017) proposed identifying individual thresholds of training load, that may put an athlete at increased risk of illness. Thus, support has been given for routine athletic monitoring of mucosal immunity through measurement of salivary Immunoglobulin A (sIgA) and associated URI, during periods of physical and psychological stress (Pyne et al., 2001).

In seminal research, Gleeson et al. (1995) reported a small proportion of athletes to experience reoccurring upper respiratory symptoms (URS), at significantly high rates. Further investigation found, 55% of athletes suffered with recurrent URI, commonly exceeding the general population average of 2.7 episodes per year (Reid et al., 2004). The most common condition identified amongst athletes with reoccurring illness, was Epstein Barr Virus (EBV) (Reid et al., 2004). It has been suggested that symptoms of URI may be associated with EBV viral reactivation, rather than a primary infection. Gleeson et al. (2002) observed a relationship between EBV and URI, reporting EBV seropositive elite swimmers to be significantly more likely to suffer with symptoms. This is notable,

because studies have found 60-79% of athletes to be EBV seropositive (Blume et al., 2018; Gleeson et al., 2002). Therefore biological testing for evidence of EBV would allow identification of athletes at risk of recurrent URI; in order to adopt preventative strategies, including training and lifestyle modifications for optimal training and performance (Gleeson & Pyne, 2016; Gleeson et al., 2017).

Few studies have highlighted the role that sleep plays in recovery and prevention of illness. However, a recent review found athletes were often unable to achieve the minimum recommendation of ≥ 7 hours sleep (Roberts et al., 2019). In swimmers specifically, average sleep durations have been reported as low as 5 hours and 24 minutes on nights prior to training (Sargent et al., 2014a). Increased training loads have shown to further negatively impact sleep quantity (Schaal et al., 2017). Overall, athletes have shown to generally struggle to achieve the recommended $\geq 85\%$ sleep efficiency, during training periods or competition (Roberts et al., 2019). These findings are empirical because inadequate sleep can lower immunity and increase risk of URI (Cohen et al., 2009; Peake et al., 2017). Therefore, there is a growing urgency to understand sleep patterns in elite athletes (Sargent et al., 2014a; Sargent et al., 2014b).

Inevitably, the impact that illness can have on training and competition, could lead to sporting drop-out and so research is required to provide knowledge on potential illness preventative strategies. With increased incidence of URI in winter, it could be assumed that seasonal differences contribute to illness risk; perhaps due to lower serum concentration of vitamin D. During winter months more than half of athletes have insufficient vitamin D status (He et al., 2016), with one study showing as many as 35% of elite athletes being deficient (Close et al., 2013). Lower vitamin D status is associated with increased incidence of URI (Sabetta et al., 2010). Therefore, governments and some sporting bodies have advised supplementation of vitamin D (AIS, 2021; Public Health England, 2019). A recent meta-analysis found that vitamin D supplementation reduced risk of upper respiratory tract infection (URTI) specifically, and gave some protection against illness (Jolliffe et al., 2021). Positive findings with vitamin D supplementation have particularly been found in those suffering with low baseline status (Martineau et al., 2017).

Despite this, few well-designed, randomised control trials (RCTs) have been conducted in elite athletes, monitoring the effects of this on incidence of URI and associated symptoms.

CHAPTER 2

Literature Review

2.1 Overview of the Immune System

The main role of the immune system is to protect the body against foreign pathogens that enter the body (Michael Gleeson, 2006) and can be broadly separated into two main strands; innate and adaptive. The innate immune system is the first line of defence; physical (skin, mucosal surfaces) and chemical (anti-microbial peptides (AMPs), phagocytes) defences offer an immediate response. Under this system falls the production of AMPs, neutrophils, macrophages, natural killer (NK) cells, dendritic cells, and immunoglobulins, which tend to ingest and kill pathogens (Walsh, 2018). The innate immune system responds in the same way to all foreign substances, hence being named “non-specific”. Unlike the adaptive immune system, response does not strengthen upon repeated exposure (Walsh et al., 2011). If the innate immune system cannot resist the pathogen, the adaptive immune system would be activated (“specific”). This consists of T- and B- lymphocytes, which have several roles; antibody production, cytotoxic T cell killing and development of T memory cells (Walsh, 2018). Despite this simplistic separation of the two systems, they work collaboratively to combat pathogens (Gleeson et al., 2013).

2.1.1 Mucosal Immunity

The mucosal immune system is often considered independent from other immune processes in the body (Colbey et al., 2018). This is because it refers to the response at mucosal membranes specifically; surfaces that are in contact with the external environment such as the mouth, nose, and throat (Walsh et al., 2011). As these surfaces are constantly exposed to foreign pathogens, the airway epithelium regulates saliva secretion to protect the respiratory tract (Weitnauer et al., 2016). Saliva composition consists of a large number of protein compounds, immunoglobulins, hormones, peptides and various enzymes (Gatti & De Palo, 2011; Papacosta & Nassis, 2011).

Salivary α -amylase is the most dominant enzyme in saliva, which is responsible for degradation of starch and glycogen to maltose (Papacosta & Nassis, 2011). However, this enzyme can also interrupt growth of some bacteria within saliva (Li & Gleeson, 2004). Another enzyme that can contribute to the innate mucosal immune system is lysozyme, which has antimicrobial properties and destroys bacteria by breaking down the polysaccharide wall of the bacterial cell (Bishop & Gleeson, 2009).

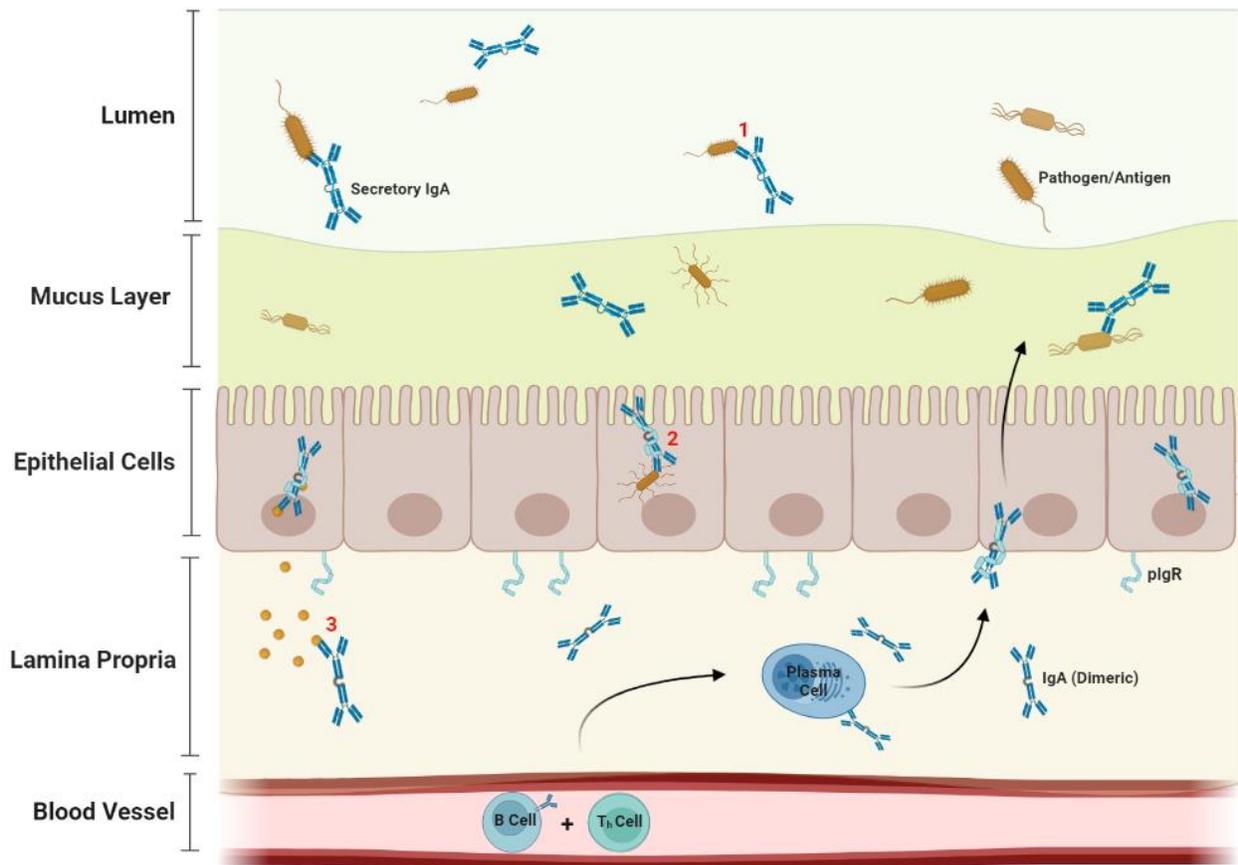
The main AMPs investigated within sport immunology include but are not limited to, lactoferrin and cathelicidin (sometimes referred to as LL-37) (West et al., 2006). In addition to their antimicrobial properties, AMPs can prevent foreign antigens from breaching immune defences (Colbey et al., 2018). Acting as a barrier for the innate mucosal immune system, AMPs prevent microorganisms from causing infection by inhibiting growth and activity, or by preventing them from initiating an inflammatory response (West et al., 2006). Firstly, lactoferrin can prevent bacteria growth and subsequently preventing a number of viruses responsible for respiratory infections (Bishop & Gleeson, 2009). Secondly, cathelicidin plays an essential role for immune responses by killing pathogens and stimulating cytokines and chemokines (Bucki et al., 2010). Human cathelicidin is secreted by neutrophils, epithelial cells and salivary glands and can be detected in whole saliva (He et al., 2016), but this peptide additionally circulates within plasma. The role that cathelicidin plays for illness has been shown, as one study reported a negative correlation between number of Upper Respiratory Tract Infections (URTI) and salivary cathelicidin (Usui et al., 2012). It should be mentioned that secretion of AMPs increases significantly during inflammation, as they influence the secretion of proinflammatory cytokines, such as of tumour necrosis factor (TNF- α), interleukin (IL)-8 (West et al., 2006). Together, Immunoglobulin A (IgA) with innate AMPs and mucosal enzymes help provide the 'first line of defence' against pathogens in saliva (Colbey et al., 2018; Gleeson & Pyne, 2000; Gleeson et al., 1995; Walsh et al., 2011).

2.1.2 Immunoglobulin A

Immunoglobulin A is the most abundant protein found at the mucosal surfaces and plays a vital role in innate immune response (Bosch et al., 2002; Gleeson & Pyne, 2000). Two subclasses of IgA exist; IgA1 in monomeric form is mainly found within circulating blood and IgA2 in dimeric form which is most prominent within mucosal secretions (Turner et al., 2021; Yel, 2010). Transportation of IgA from the blood vessel to the mucosal surface can be seen in Figure 2.1. Firstly, differentiation of B-lymphocytes occurs with the help of T-helper cells, into IgA producing plasma cells. Once in the lamina propria, activated plasma cells produce IgA in dimeric form where IgA monomers are joined by a polypeptide J chain (Li & Rush, 2009). Transcytosis of dimeric IgA from the lamina propria into the lumen are mediated by the polymeric immunoglobulin receptor (pIgR); one dimeric IgA is bound to the pIgR at the basolateral side of the epithelium and is transported to the lumen (Li et al., 2020). Then, pIgR fragment becomes the secretory component on secretory IgA (Bishop & Gleeson, 2009; Pyne et al., 2001). There are three basic methods in which IgA can protect the mucosal surfaces; secretory IgA can bind and neutralise antigens at the mucosal surface (1), if an antigen has infiltrated the epithelial cells, dimeric IgA can bind within the cell and export it (2), or lastly, dimeric antibodies can bind to any antigen present in the lamina propria, then bind to pIgR and be transported out into the lumen (exporting antigens and pathogens out of the lamina propria) (3) (Figure 2.1).

Figure 2.1

Transportation of IgA to the mucosal surface

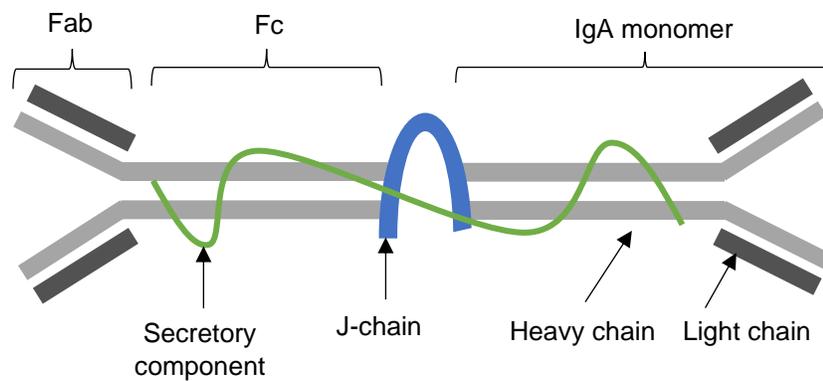


Note. Three basic methods in which IgA can protect mucosal surfaces are: 1) Secretory IgA can bind and neutralise antigens at the mucosal surface; 2) Dimeric IgA can bind to an antigen within the epithelial cell and export it out into the lumen; 3) Dimeric antibodies can bind to any antigen present in the lamina propria, then bind to pIgR and be transported out into the lumen. pIgR, polymeric immunoglobulin receptor. Created with BioRender.com (2022).

Overall, IgA functions as multi-layered mucosal defence responding to pathogens by; providing specific antibodies, preventing penetration of the mucosal epithelium, interrupting replication, and acting as a transport mechanism for the elimination of antigen in the submucosa (Gleeson et al., 1999; Li & Rush, 2009). This is important because the vast majority (>90%) of infections originate at the mucosal surfaces (Brandtzaeg, 2003), where defence is largely mediated by secretory IgA (Figure 2.2). Therefore, secretory IgA is commonly examined within saliva as salivary IgA (sIgA) and has been considered as

the best indicator of mucosal immunity (D'Ercole et al., 2016). Historically, high incidence of respiratory infection has been found in individuals with selective IgA deficiency (Cunningham-Rundles, 2001), highlighting the link between IgA and illness. Since then, several studies have highlighted an association between sIgA and susceptibility to upper respiratory illness (URI) (Gleeson et al., 1995; Gleeson et al., 1999; Ihalainen et al., 2015; Nieman et al., 2002), and therefore has become a meaningful biomarker.

Figure 2.2

Secretory IgA in dimeric form

Note. Each IgA monomer comprises of two heavy chains and two light chains. In dimeric form, two IgA monomers are joined by the J-chain and bound to a secretory component. Fab, fragment antibody binding portion; Fc, fragment crystallisable portion. Adapted from Bishop & Gleeson (2009).

2.2 Upper Respiratory Illness

Common symptoms of URI include a sore throat, cough, headache, fatigue, runny nose, sneezing and watery eyes (Gleeson & Pyne, 2016; Thomas & Bomar, 2022). It has been suggested that the most common acute illness is URI, occurring around twice as frequently to any other (Monto, 2002). It has been reported that adults suffer from URI around three times each year (Arroll, 2011). This was supported by a survey conducted in Germany between 2011-2012, which found individuals aged ≥ 15 years reported an average of 1.3-3.2 URI illnesses (Bayer et al., 2014). Incidence of URI creates a large burden on society, socially and economically. It has been estimated that URI accounted

for ten million outpatient appointments each year (Thomas & Bomar, 2022). Individually, this could be disruptive for socioeconomic reasons, because of increased missed work days.

Fricker et al. (2000) found that swimmers have a similar incidence of URI to the general population (approximately 2.7 episodes each year). Elsewhere however, it has been reported that some elite athletes suffer from higher incidence of URI, than recreational athletes and those who are sedentary (Gleeson & Walsh, 2012; Spence et al., 2007; Walsh & Oliver, 2016). Gleeson et al. (1995) reported a small proportion of athletes to experience reoccurring illness, at significantly high rates. Further investigation found, 55% of athletes suffered with recurrent URI, commonly exceeding the general population average of 2.7 episodes each year (Reid et al., 2004). Regardless of population comparisons, URI accounts for 35-65% of illnesses presented by athletes (Pyne et al., 2001), and the impact that can have on an individual is evident. In addition to the burdens of URI already identified, for an athlete it could be hugely detrimental to training, and subsequent performance. Upper respiratory illness could detriment performance by reducing alertness, causing slower reaction times (Smith et al., 1998; Smith, 2012). Therefore, it would come as no surprise that athletes who manage to avoid illness during taper and competition, swim faster. Given this, athlete monitoring of URI has become popular, in order to identify athletes at risk of frequent URI and to examine possible interventions to reduce risk. However, there are many confounding variables that influence illness risk, including; age, nutritional status, seasonal variation, psychological wellbeing, pathogen exposure (e.g., close contact with infected individuals), psychological stress and exercise (Dhabhar, 2014; Walsh et al., 2011).

2.2.1 Monitoring URI with Self-Report Questionnaires

Regardless of cause of URI, the impact of associated symptoms could still be disadvantageous to athletic performance, by altered or missed training days or competition. In addition, self-reported mild illness symptoms have shown to have a small harmful effect on male swimmers (Pyne et al., 2005). Therefore, it is important to consider how an individual perceives URI symptoms and their impact. Self-reported questionnaires

are routinely used in studies to monitor incidence, duration and severity of symptoms associated with URI. This is due to ease of use for the athletes, making additional factors easier to monitor (Fricker et al., 2005). Examples of commonly used questionnaires include the Wisconsin Upper Respiratory Symptom Survey (WURSS), the Jackson Score questionnaire (Jackson & Dowling, 1959), and the monthly illness log developed by the Australian Institute of Sport (AIS), which has been adapted elsewhere (Fricker et al., 2005). Usually, they require participants to record daily or weekly symptoms of URI (e.g., headache, runny nose, coughing), over the course of the study period.

That said, how a URI episode has been defined and the way results from self-reported questionnaires are interpreted has differed between the literature. As an example, Da Boit et al. (2015) classified a single URI episode as a symptom score ≥ 12 . Where symptoms were not significant enough to be a URI, total number of symptom days was recorded as days with a symptom score ≥ 5 . This was in contrast to Fricker et al. (2005), who reported an illness where symptoms were present on 2 or more consecutive days, or when the severity was rated highly enough to modify training (moderate-severe). Here, symptoms separated by two days or less were regarded as a recurrence or continuation of the initial illness. Whereas elsewhere, an asymptomatic period of at least 7 days was needed for a subsequent URI episode to be classified as new (Bishop, 2006; Rama et al., 2013; Spence et al., 2007; Svendsen et al., 2016). In addition to these variances, the presence of perceived symptoms can be recorded according to their severity in a multitude of different ways. Severity of symptoms could be recorded via a scale of light (L), moderate (M), or severe (S) (Gleeson et al., 2013), or by whether there are training implications (1 = no change in training program, 2 = training program modified, 3 = complete cessation of training) (Fricker et al., 2005). Either way, these were both given a score of 1, 2, or 3 for quantitative data analysis (Gleeson et al., 2013; Fricker et al., 2005). Elsewhere for example, symptom severity was calculated as the average score on a scale of 1–7; “very mild,” “mild,” “moderate,” and “severe”, aligned with numbers 1, 3, 5 and 7 (Barrett & Barrington, 2005; Dubnov-Raz et al., 2015). Differences in URI classification and symptom scoring mean that comparisons between studies can be difficult and should be undertaken with caution.

Not only do questionnaires suffer methodological issues, it should be considered that paperwork may add an additional time burden on participants. Several studies have reported low levels of compliance and missing data points (Dubnov-Raz et al., 2015). Therefore, instead of daily reporting, some studies have looked at retrospective questionnaires (Ekblom et al., 2006; Fahlman & Engels, 2005), or using a team physician to interview for URI (Francis et al., 2005), to reduce missing data. In addition, symptoms of allergies, cold dry air inhalation and inflammation are often difficult to differentiate (Reid et al., 2004; Spence et al., 2007). That said, even minor symptoms could result in altered training for an athlete, and so highlights the importance of monitoring. Therefore, the majority of longitudinal studies use self-reported symptom questionnaires or illness logs (Neville et al., 2008); they provide a useful assessment of individual athletic monitoring to investigate trends for researchers and coaches. Most recently, Harrison et al. (2021) highlighted the importance of the daily assessment of URI symptoms, regardless of whether pathogen recognition is available. In addition to monitoring symptoms, use of a questionnaire could be useful in gaining self-perceived sleep and level of athlete fatigue (Neville et al., 2008). Overall, there may always be problems with self-reported subjective data. However, if physician diagnosis is not possible, then gaining an idea of perceived symptoms is useful for athletic monitoring; this provides rationale for the monitoring of upper respiratory symptoms (URS) and other factors, such as sleep, through use of self-report questionnaires in the athletic population.

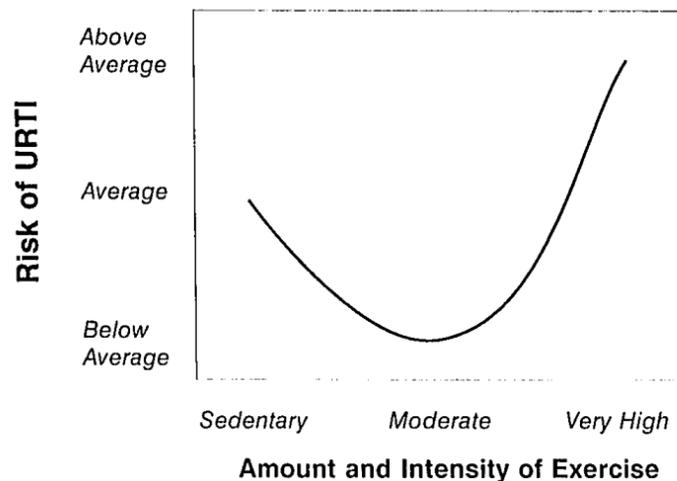
2.3. Exercise and URI Risk

Exercise can have both a positive and negative effect on risk of URI, dependent on exercise intensity and duration (Pedersen & Hoffman-Goetz, 2000). Seminal work modelled the relationship between URTI and exercise in the form of a “J-shaped” curve (Figure 2.3, (Nieman, 1994)). It has been suggested that moderate to vigorous exercise, defined by an intensity threshold of 60% of oxygen uptake and a duration threshold of <60 minutes, has the ability to improve immune function (Nieman & Wentz, 2019). In earlier research, Nieman (1994) reported that 30 minutes of brisk walking most days for previously sedentary individuals, was associated with an improved immune function.

Regular involvement in moderate-vigorous activity was associated with a 20–30% reduction in risk of URTI (Matthews et al., 2002). In addition, those who partook in aerobic exercise, 5 or more days a week, had 43% reduced days of URTI and 32-41% reduced severity and symptoms, compared to those who were largely sedentary (Nieman et al., 2011). It has been suggested that stress hormones such as cortisol, which can suppress immune function and pro-inflammatory cytokines, do not reach high levels during moderate exercise bouts lasting <60 minutes. In a recent review by Dhabhar (2014), it was noted that short-term acute stress enhanced immune-protective responses. In support, one meta-analysis found that regular, moderate-intensity exercise had an effect on prevention of colds (Lee et al., 2014).

Figure 2.3

The “J-shaped” curve



Note. The proposed relationship between varying amounts of exercise and risk of URTI (D C Nieman, 1994).

Mechanisms for the protective effect of moderate to vigorous exercise include: enhanced macrophage activity, increased circulation of immunoglobulins, pro-inflammatory cytokine production, neutrophils, NK cell cytotoxicity, T cells and B-cells (Gleeson et al., 2013; Nieman & Wentz, 2019; Walsh, et al., 2011). Neutrophils specifically have been reported to continually increase up to 6 hours following moderate exercise (Peake, 2002). Monocytosis has been observed 1-2 hours after an acute bout of exercise, typically

returning to baseline within 6 hours after exercise (Walsh et al., 2011). Number and function of lymphocytes have dramatically increased following exercise (Campbell & Turner, 2018; Walsh et al., 2011). It has been suggested that exercise-induced increases in lymphocytes for example, could enhance health (Nieman & Wentz, 2019). Furthermore, research exploring a shorter duration of exercise (≤ 30 minutes) at higher intensity, found T cells to increase following interval swimming (Wilson et al., 2009). Lastly, increased sIgA concentration has been shown during 12 weeks of moderate exercise (30 min, three times per week), with a significant reduction in URI symptoms (Klentrou et al., 2002). Therefore, it has now generally been accepted that regular involvement in moderate to vigorous exercise has shown enhanced immune function, compared to those that live a sedentary lifestyle (Gleeson et al., 2013). Thus, United Kingdom (UK) government guidelines currently recommend at least 150 minutes of moderate, or 75 minutes vigorous aerobic activity, each week (Public Health England, 2020).

In contrast to the widely accepted notion that immune function is improved by regular moderate to vigorous exercise (<60 minutes), the effect of a single bout of intense and longer duration exercise remains disputed (Campbell & Turner, 2018). With the 'J-shaped curve', it was hypothesised that high volume and intense acute exercise could elicit an above average risk of URTI (Nieman, 1994). The first study to show this was conducted in 140 ultramarathon runners, where a survey was completed before and two weeks after a 56 km ultramarathon (Peters & Bateman, 1983). They found that two weeks following the ultramarathon, 33% of runners experienced URI compared to only 15% of the control group (Peters & Bateman, 1983). To support, another study randomly selected 2,311 runners and monitored illness symptoms following the 1987 Los Angeles marathon; 13% of runners showed URI compared to 2% of the control group (Nieman et al., 1990). Thus, this led to the belief of an 'open window' for infection, where an individual may become more susceptible to illness for 3-72 hours following prolonged, intense exercise (Nieman & Pedersen, 1999).

If acute exercise is prolonged, immunosuppression could last several hours or days (Gleeson, 2007; Gleeson & Bishop, 2005; Nieman & Pedersen, 1999). Specifically, sIgA

and other immune biomarkers have been altered during recovery from prolonged and intensive endurance exercise (Nieman & Wentz, 2019; Peake et al., 2017). Despite an initial increase with exercise, Shek et al. (1995) reported a 40% decrease in NK cells count, for <7 days after the cessation of long intensive exercise. In addition, reduced number of circulating lymphocytes has been shown (Clifford et al., 2017; Shek et al., 1995). Specifically, marathon and ironman races promoted T-cells to fall 1-hour post exercise (Clifford et al., 2017; Perry et al., 2013), which could subsequently reduce production of inflammatory cytokines (Shaw et al., 2017). Even if immunosuppression is too brief to have any major influence on immediate URI risk, it should still be considered that there could be cumulative effect for athletes who endure this several times a week (Shephard & Shek, 1999).

Within a recent review, Campbell and Turner (2018) disputed the 'J-shaped curve', suggesting that several studies now challenge this phenomenon. One large cohort study consisting of 1,509 participants, found that higher physical activity levels led to 18% reduced risk of self-reported URI (Fondell et al., 2011). However, the population in this study, classified as having a high physical activity level ($>55 \text{ MET}\cdot\text{h}\cdot\text{d}^{-1}$), were described as someone with a sedentary job, who is moderately active (participating in jogging/gym for one hour each day); which does not align with the activities of an athletic population. Therefore, it appears the study actually supports the 'J-shaped curve', showing reduced risk of illness with regular moderate-vigorous exercise, and the argument given within the review, does not stand.

That said, it should be considered that it is difficult to control all factors that may influence an increase in illness risk and the above studies suffered from several limitations. Perhaps increased risk of URI could be due to psychological stress and anxiety (Dhabhar, 2014; Edwards et al., 2018). Through activation of the sympathetic nervous system and hypothalamus-pituitary-adrenal (HPA) axis, circulating stress hormones increase; these hormones alter immune function and could further explain the increased risk of URI (Dhabhar, 2014). Alternatively, the potential cause of increased illness seen following marathon or running events, may in fact be the attendance to a mass participant event,

where risk of acquiring infectious pathogen is higher (Campbell & Turner, 2018). The effect of mass participant events was evident during the recent COVID-19 pandemic; even spectating mass sporting events had to stop, to reduce risk of transmission. Interestingly, Ekblom et al. (2006) reported that only marathon runners who reported pre-race infection, showed infection after the marathon, suggesting that illness was influenced by increased weekly mileage before the marathon (Fricker et al., 2005). Lastly, the main fundamental limitation of each of the aforementioned studies was that none verified URTI presence by PCR analysis on nasopharyngeal and throat swabs (Walsh & Oliver, 2016), so there would be no way to confirm the occurrence of illness.

2.3.1 URI Risk for Athletes

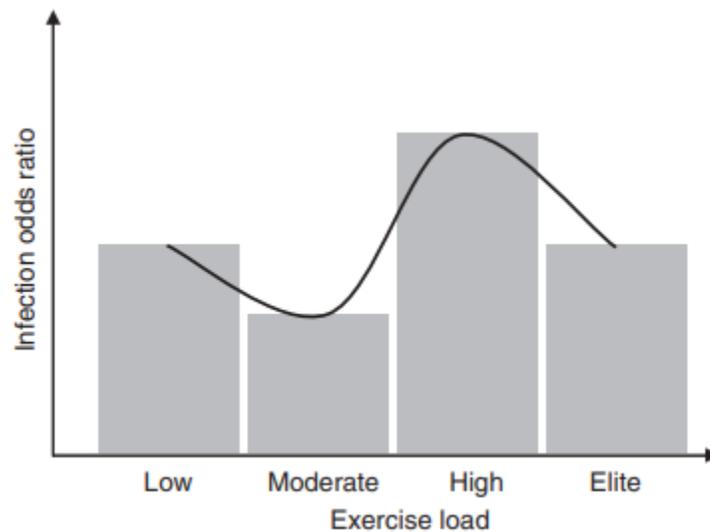
A key study by Spence et al. (2007) observed elite athletes, recreational athletes and sedentary individuals over 5-months. This research was important, because URI episodes were tested for common viral and bacterial pathogens by nasopharyngeal and throat swabs, which research has been criticised for previously for not doing. Over an entire competitive season, results supported the 'J-shaped curve'; the lowest rates of URI were identified in recreational athletes, next the sedentary controls, with the highest presenting in the elite athletes (Spence et al., 2007). These findings were reinforced, as it has been suggested that fitter adults such as recreational athletes, were less likely to experience URI (Coad et al., 2015), and endured shorter duration of symptoms (Dimitriou et al., 2017), compared to those of a lower fitness level and are sedentary. Likewise, elite athletes who engage in repeated high volume and intense endurance training have shown to continually suffer from higher incidence of URI (Spence et al., 2007; Walsh & Oliver, 2016). Moreira et al. (2013) examined elite Brazilian futsal players over a 4-week period, and found that athletes were more susceptible to increased URI symptom severity, due to high intensity training.

In contrast, Hoffman and Krishnan (2014), found ultramarathon runners to be healthier, reporting fewer days missed at work and school, compared to the general population. Authors reported an average of 1.5 sickness days for the year (Hoffman & Krishnan, 2014), compared to an average of three URI episodes recorded in the general population

(Arroll, 2011; Bayer et al., 2014). Furthermore, analysis of 11 national and international level cross-country skiers, biathletes and long distance runners, showed that total number of training hours was inversely correlated with reported illness days (Mårtensson et al., 2014). With this in mind, researchers have formed another hypothesis and modelled an 'S-shaped curve' (Figure 2.4); suggestive of the idea that elite athletes are more well adapted to high training demands (Malm, 2006).

Figure 2.4

The "S-shaped" curve



Note. The proposed relationship between training load and infection (Malm, 2006)

The 'S-shaped curve' suggested that elite athletes were at a lower risk of infection compared to a lower level athlete, whilst undertaking intense training and competition (Malm, 2006). Whilst the 'J-shaped curve' may be true for some, it has been suggested that the curve may flatten for elite athletes. This is because an important distinction between national and international level athletes was found. Despite having a small sample population, results indicated that international athletes suffered fewer URI episodes than national level athletes (Hellard et al., 2015). It could be argued that in order to become a successful elite athlete, the individual must have an immune system capable of fighting infection during prolonged periods of intense training (Mårtensson et al., 2014). So, genetic factors may account for this (Walsh & Oliver, 2016), where some athletes

have a naturally higher tolerance to high training loads (Malm, 2006). A genetic predisposition to pro-inflammatory cytokine responses has been found, which may influence risk of URI in athletic populations (Cox et al., 2010). Another plausible explanation could be behavioural factors, as international athletes may be more likely to ensure quality management of diet, hygiene, stress, supplementation and sleep (Hellard et al., 2015; Walsh et al., 2011). Alternatively, international athletes may receive funding, and as a result are less likely to have a job or financial worries (Walsh & Oliver, 2016). Overall, evidence suggests that moderate to vigorous exercise training in sedentary or less active individuals reduces risk of URI. While some studies have shown improved protection against URI in athletic cohorts, this is limited and most have shown increased risk of URI (Spence et al., 2007). Regardless of population comparisons, elite athletes who engage in very high volumes of training, over prolonged periods of time would be at greater risk of URI due to over-reaching or intensified training (Gleeson, 2007; Meeusen et al., 2013). That said, some consideration should be given to the cause of symptoms associated with URI.

2.3.2 Causes of URI

Within the general public, viral infection is the most common cause of URI (Gleeson et al., 2002). Generally, viruses enter the body through the respiratory tract, via the mouth or nose, then infect selected cell types and replicate to further infect other cells. This response to fight the infection, causes inflammation, aches, fatigue and the symptoms listed previously (Gleeson et al., 2002). Interestingly however, when causes of URS have been monitored in athletes, only 27-30% were seen to be caused by an infectious agent (Reid et al., 2004; Spence et al., 2007), suggesting respiratory infections are uncommon in athletes. Even though a small proportion of URI symptoms were caused by an infectious agent (Cox, et al., 2008; Spence et al., 2007), the majority of studies still use the term infection and URTI. Unfortunately, very few studies have verified URTI presence by PCR analysis on nasopharyngeal and throat swabs (Spence et al., 2007; Walsh & Oliver, 2016). Some studies use clinical diagnosis, however the validity of a physician-verified URTI has now been questioned (Cox et al., 2008). Without definite classification, it could be argued that using terminology such as infection or URTI should not be used.

Multiple aetiologies can cause difficulty in accurate classification of URI, because symptoms commonly overlap other illnesses (Thomas & Bomar, 2022). For example, as URI can affect any part of the mucosa, sometimes they are referred to as a “cold” or “sore throat” (Arroll, 2011). Difference in URI classification has been widely discussed as a major limitation of many studies, causing an inability to compare studies and draw conclusions. Evidence from a moderate sized cohort was suggestive of URI having multiple aetiologies in athletes (Colbey et al., 2018), and would most likely involve both infectious and non-infectious causes (Cox et al., 2008). Underlying causes of URI may not be due to infectious respiratory pathogens, but instead due to allergies or asthma (Gleeson & Pyne, 2016; Spence et al., 2007). Undiagnosed or inappropriately treated asthma and allergies are common findings in clinical assessments for those experiencing recurrent URI (Gleeson & Pyne, 2016). One study revealed that through investigation of this, new diagnoses of exercise induced asthma were made for many athletes (Reid et al., 2004). In addition, exercise-induced bronchospasm could resemble URI and prompt the potential misclassification (Cox et al., 2008). Exercise-induced bronchoconstriction for example, is often a consequence of hyperventilation which would stimulate an inflammatory response (Anderson & Holzer, 2000). Damage to airway lining from hyperventilation, cold air or pollution exposure, has also been recognised as a cause of URI for athletes (Kippelen et al., 2012). Thus, understandably, some researchers have reported URS (Ihalainen et al., 2016), instead of URTI.

One study investigated 41 competitive athletes and even though no consistent pattern was found for causes of URI, 68% of athletes had pre-existing conditions with the potential to cause recurrent infection and fatigue (Reid et al., 2004). These included primary or unresolved infection, immune deficiency, and Epstein Barr Virus (EBV) reactivation. The most common condition identified amongst athletes specifically, was evidence of Epstein Barr Virus (EBV) (Reid et al., 2004). Since then, few studies have examined the link between EBV and URI. However it has recently been suggested that reactivation of EBV may be a novel marker for risk of URI in athletes (Perkins & Davison, 2021).

2.3.2.1 Epstein Barr Virus

Epstein Barr Virus is a human gamma-1 herpesvirus, which is most commonly associated with infectious mononucleosis (IM), otherwise known as glandular-fever. Primary infection with EBV usually happens at a young age, and is usually always asymptomatic (Rickinson et al., 2014). In the case of it presenting itself as IM, symptoms could be mild or severe, and include fever and sore throat. Once infected however, the virus lays dormant for the rest of an individual's life. In relation to the immune system, primary infection affects B-lymphocytes, thereafter memory B-cells circulate in a dormant state (Clancy et al., 2006). Two large scale studies with access to the UK biobank and Public Health England Sero-epidemiology Unit (SEU), found that 85.3-94.7% of healthy individuals ($n = 12,020$) were EBV seropositive (Kuri et al., 2020; Mentzer et al., 2022). Additionally, they found that prevalence of EBV was higher in females, and increased with age. Prevalence of EBV seropositivity was reported in 94% of athletes, which was higher than controls (Pottgiesser et al., 2012). Lifelong persistence of EBV and the high prevalence shown, promotes the monitoring of EBV serostatus. Furthermore, a relationship has been observed between previous infection of EBV and incidence of URI. Gleeson et al. (2002) reported that seropositive elite swimmers were significantly more likely to suffer with symptoms of URI; highlighting the importance of observing EBV serostatus. Thus, identification of EBV status could help to identify athletes with an increased susceptibility to illness (Hoffmann et al., 2010; Pottgiesser et al., 2012).

It is possible to distinguish acute from past infection of EBV using only three parameters; viral capsid antigen (VCA) IgG, VCA IgM and EBV nuclear antigen (EBNA) IgG (Paschale & Clerici, 2012). Determination of evidence for recent or past infection with EBV is usually performed with human serum or plasma, on commercially available Enzyme Linked Immunosorbent Assay (ELISA) kits (Cox et al., 2004; Gleeson et al., 2002; Kuri et al., 2020). The three following ELISA kits serve as a marker of recent or historic EBV infection; 1) detection of IgG antibodies for VCA appear in the acute phase of infection, peak at 2 to 4 weeks and then persists for life (showing previous infection), 2) detection of IgM antibodies for VCA, appear early in infection and disappear within 4 to 12 weeks (showing current or recent infection), 3) IgG antibodies to EBNA develop 6 to 8 weeks

after the time of infection and are present for life (also showing past infection) (Kuri et al., 2020). In addition to EBV-specific antibodies, EBV viral loading can be examined, allowing the detection of increased EBV activity in whole blood or saliva (Yamauchi et al., 2011). As an example, Gleeson et al. (2002) measured EBV-DNA in saliva. These measurements are important to note, as it has been proposed that reactivation of EBV may occur following immune depression, and contribute to cases of URI (Walsh et al. pt1, 2011).

Increased incidence to URI may be due to increased viral EBV-DNA expression, which has been detected in saliva before the appearance of symptoms (Gleeson et al., 2002). These findings were supported by Yamauchi et al. (2011), who found increased expression of EBV-DNA in collegiate rugby players with URS than those with no symptoms. Reid et al., (2004) found that 22% of athletes had EBV-DNA, and it has been suggested that the detection of this prompts dysregulation of cytotoxic T cells (Rickinson & Moss, 1997). Another possible explanation could be that sIgA plays a role in the process of EBV transport into the mucosal epithelium via secretory component (Sixbey & Yao, 1992). With this, EBV-DNA was associated with decreased sIgA concentration (Gleeson et al., 2002), showing a significant decrease in sIgA the day before EBV-DNA reactivation (Yamauchi et al., 2011). These findings may further account for the association found with increased risk of URS, as falls in sIgA have been associated with increased URS risk.

That said, even though EBV reactivation presented the highest number of URI in athletes elsewhere, findings lacked statistical significance (Blume et al., 2018). Furthermore, a recent study in 15 professional footballers being observed over 16 weeks, found that EBV-DNA in saliva was not a useful marker for URS (Perkins & Davison, 2021); EBV DNA results were similar between those with symptoms and those without. It should be considered that few studies have determined EBV serostatus in athletes, plus findings on EBV-DNA in saliva are conflicting; therefore, more research is needed to determine the link with EBV and URI. Interestingly, Perkins and Davison (2021) confirmed that sIgA was

a more useful marker of URS risk, with 40% decreased sIgA secretion from baseline before the occurrence of symptoms.

2.4 SlgA and URI

Earlier research highlighted the association between sIgA and illness; Gleeson et al. (1995) observed national swimmers over seven months, and found that pre-season resting sIgA concentration could be used as a predictor of URI frequency. Pre-season sIgA results showed that swimmers with a low sIgA concentration averaged 1.8 - 4.2 infections, compared to no infections for those with high sIgA concentration. More recently, low baseline resting sIgA concentration predicted URS risk in elite male hockey players (Keaney et al., 2021). Overall, increased incidence of URI and associated symptoms has been identified with low levels of sIgA, decreased IgA secretion rates and a decline in sIgA concentration over a training period (Gleeson et al., 1995; Gleeson et al., 1999; Ihalainen et al., 2016; Nieman et al., 2006). In one example, decreased sIgA by $\geq 65\%$ showed that elite rugby union players, were at greater risk of URI within the following two weeks (Tiernan et al., 2020). Furthermore, a recent systematic review by Rico-González et al. (2021), examined 23 studies on team sports, and confirmed that a low level of sIgA (concentration and secretion rates) was correlated with higher incidence of URI; therefore, supporting the use of sIgA for monitoring URI in athletes. Authors promoted the use of sIgA as a marker of mucosal monitoring to help analyse risk of subsequent URI, and advised that training load management was mandatory to health to avoid immunosuppression (Rico-González et al., 2021).

Despite this, some research has shown conflicting results; no change in sIgA (Pyne et al., 2001) and increased sIgA has additionally been reported alongside URI (Gleeson et al., 2000; Gleeson et al., 1995; Papadopoulos et al., 2014). That said, these studies showed infrequent sampling and tested at times where there is naturally lower incidence of illness (taper). Differences could also be due to differences in study design or many factors that affect measurement of sIgA. As an example, significantly lower sIgA concentrations have been reported 48 hours (Mackinnon et al., 1993) and one week before URS (Neville et al., 2008), which may explain the lack of findings for some studies.

Despite some resistance (Moreira et al., 2008; Orysiak et al., 2017), the association between use of sIgA for determination of URI has been well documented (Ihalainen et al., 2015; Tiernan et al., 2020). Overall, collection of sIgA has become an important marker for measurement of mucosal immunity and subsequent URI. Secreted at the mucosal surfaces, sIgA acts in defence against URI and so a strong relationship between them has been established; presenting as one of the most promising markers for athletes prone to URI (Pyne & Gleeson, 1998).

2.4.1 Collection of sIgA

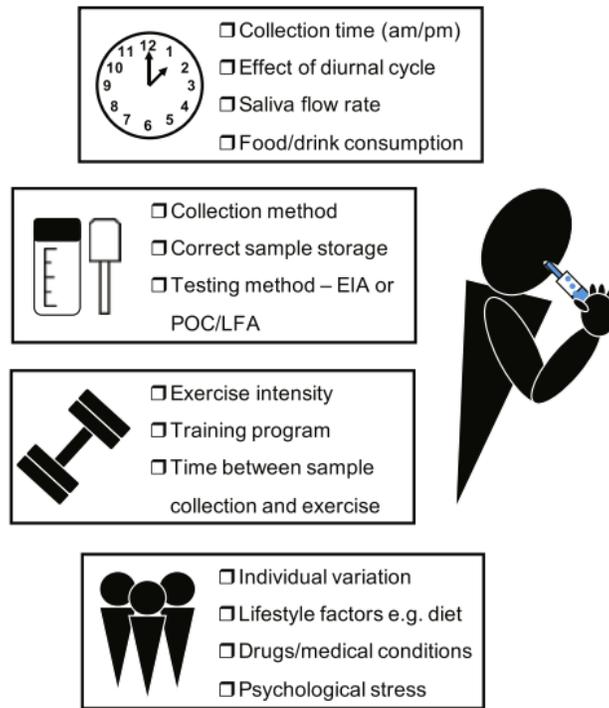
Saliva collection can be carried out with or without stimulation (Gröschl, 2008). Methods include spitting, using swabs, pipettes or sponges, although these have been found to be disadvantageous (Bhattarai et al., 2018; Salimetrics, 2015). Mainly, collection of stimulated samples has been said to be unreliable, giving inaccurate concentrations of mucosal markers (Strazdins et al., 2005). Therefore, the current 'gold standard' for measuring mucosal immune markers is through unstimulated saliva samples (Hanstock et al., 2016) using the passive drool method. This involves participants to be in a seated position with their head tilted slightly forward, while making minimal orofacial movement (Gomes et al., 2013). Some reports have suggested avoiding food and drink for at least 2 hours prior to sampling (Papacosta & Nassis, 2011), and others, 30 minutes (Granger et al., 2012). Despite several guidelines for saliva sampling, it does offer some practicality over other biological sampling methods.

Collection of saliva provides a non-invasive alternative to the collection of serum or plasma, allowing rapid and frequently sampling, without 'needle stress' (Gröschl, 2008; Papacosta & Nassis, 2011). This requires minimal training and allows for self-sampling, which could be especially useful in field studies. Despite reported lower biomarker concentrations in saliva compared to blood, it has been considered a reliable reference for their respective blood concentrations (Cadore et al., 2008). Interestingly, researchers have also demonstrated use of tear secretory IgA as a non-invasive biomarker of mucosal immunity (Hanstock et al., 2016). However, evidence on this is limited and external influences on IgA in tear fluid such as, age, diet, training etc., are unknown. Therefore,

use of sIgA would be preferable, allowing for comparisons between literature to be made. Overall, collection of saliva is stress free, non-invasive, and comparable (Gröschl, 2008). Despite this, it would be important to control and standardise saliva collection in any study design, because many factors affect sIgA and lead to variance in results (Bhattarai et al., 2018). A summary of this can be seen in Figure 2.5, which was presented in a recent review of sIgA (Turner et al., 2021).

Figure 2.5

Factors affecting measurement of sIgA



Note. EIA, Enzyme immunoassay; LFA, lateral flow assay; POC, point of care system (Turner et al., 2021).

The currently accepted ‘gold standard’ methodology for measurement of sIgA involves the use of an ELISA (Turner et al., 2021). This is because ELISAs are considered to be most accurate and reliable, which is why the majority of studies employ this method. Portable lateral flow devices (LFD) are now commercially available as point of care systems, which can provide rapid results (Gleeson et al., 2017). Specifically, the IPRO

LFD was seemingly reliable when compared to the gold standard ELISA (Coad et al., 2015). In one review however, these point of care systems were promoted with caution as they were relatively new and needed further validation (Miočević et al., 2017).

2.4.2 Variability of sIgA

One of the main problems surrounding research in sIgA, is the considerable within- and between-subject variation found. Concentration of sIgA varies widely when comparing between individuals, even in healthy populations (Francis et al., 2005; Kugler et al., 1993). Although it should only be used as a guide, normal range values are 93 – 974 $\mu\text{g/mL}$ (Salimetrics, 2015). As an example, Nehlsen-Cannarella et al. (2000) reported sIgA concentrations of 58- 548 $\mu\text{g}\cdot\text{mL}^{-1}$ amongst 39 participants. Variability is usually demonstrated by presenting coefficient of variation (CV) as a percentage (%). Thus, studies have reported high between-subject variation for sIgA with examples of group CV ranging from 68-71% (Dwyer et al., 2010; Neville et al., 2008). Salivary IgA could be affected by many factors such as sex differences, diet, or ethnicity (Gleeson, 2000). This was exemplified by significantly higher sIgA concentration and secretion rates found in males, compared to females (Gleeson et al., 2011). In addition, poor oral hygiene was a common problem found for 278 athletes, leading into the London 2012 Olympics (Needleman et al., 2013); which may be another factor to consider for the variation found in sIgA between-subjects (Marcotte & Lavoie, 1998). Not only is there large variation between-subjects, but also within individuals.

Notably, in regard to monitoring an individual, it would be easier to measure quantifiable change with smaller within-subject variation (Hopkins, 2000). However, large within-subject variation of sIgA has been reported; with reported individual CV ranged between 28-48% (Dwyer et al., 2010; Francis et al., 2005; Neville et al., 2008). Specifically, Dwyer et al. (2010) showed individual CV to be 48%; authors recommended establishing an accurate baseline for each individual on a case-by-case basis, rather than using population reference ranges. It should be stated, authors additionally found high CVs for sIgA secretion rates for between (75%) and within (43%) subjects (Dwyer et al., 2010). Further sources of heterogeneity may include environmental factors, dietary intake,

alcohol or caffeine, stress, and illness status (Francis et al., 2005; Gleeson, 2000; Salimetrics, 2015). Alongside these, circadian variations in mucosal markers are evident (Leicht et al., 2018). Specifically, concentration of sIgA is significantly higher in the morning (06:00-08:00 hours) and has been reported to be stable from 12:00 hours (Dimitriou et al., 2002; Li & Gleeson, 2004). Therefore, serious consideration should be given for timing of saliva sampling for measurement of sIgA, perhaps with testing after midday with restrictions on food and drink. Furthermore, the less samples taken, the less likelihood to detect real change (Hopkins, 2000), so regular sample collection has been recommended.

As secretion of sIgA is controlled by the parasympathetic and sympathetic nervous system, psychological stress would also influence sIgA levels (Bosch et al., 2002; Engeland et al., 2016; Phillips et al., 2006). Specifically, stressors such as exercise have also been reported to have marked changes on sIgA (Gleeson et al., 2004; Li & Gleeson, 2004). Most commonly, sIgA concentration has been shown to decrease immediately following an intense bout of exercise. However, this may be because exercise can greatly impact the dehydration status of an individual, effecting saliva flow rate (Gleeson et al., 2004). Therefore, if monitoring sIgA over a longer time period, collection of sIgA should be done on a day of rest. Overall, careful consideration should be given to timing of sample collection, analytical conditions and study design (Gleeson, 2000). Therefore, regular saliva samples should be collected at standardised times of day, before training to avoid post-training effects on markers or dehydration.

2.4.3 Expression of sIgA

An important consideration when interpreting findings, would be that differences in expression of sIgA make comparisons difficult. In earlier studies, only absolute sIgA concentrations were expressed in results (Tharp & Barnes, 1990; Tomasi et al., 1982). Then, sIgA was expressed not only in absolute concentration, but also alongside albumin ratios. No changes in albumin, meant it could be confirmed that decreased sIgA concentrations were not due to dehydration or alterations in saliva flow rate (Gleeson et al., 1995). Now more favourably however, results express sIgA in terms of secretion rate

(saliva flow rate multiplied by IgA concentration) (Dwyer et al., 2010; Fahlman & Engels, 2005). Research monitoring sIgA and sIgA secretion rate, provided methodological evidence that these measures were adequate without the need to perform additional analysis of salivary protein or osmolality (Fahlman & Engels, 2005; Sari-Sarraf et al., 2008). The benefit of expressing sIgA secretion rate would be that saliva flow rates are taken into consideration.

Naturally, variation in saliva flow rate would be expected under different conditions such as exercise, due to dehydration, with within- and between-subject variation also. As an example, females have lower saliva flow rates compared to men (He et al., 2014), due to smaller salivary glands (Inoue et al., 2006), which may explain differences found in sIgA secretion rates previously identified. That said, any biomarker concentration should take flow rate into account, as this could heavily affect secretion of mucosal markers and subsequent results (Wood, 2009). Saliva flow rate can be calculated by weighing a collection tube, before and after saliva collection, plus recording total unstimulated saliva collection time; these two methods can be used to calculate secretion rate. Secretion rate of sIgA accounts for saliva flow rate, but sIgA concentration does not (Leicht et al., 2018). As an example, research conducted in cyclists presented increased sIgA concentration following 2 hours cycling (Walsh et al., 2002). However, when expressed as secretion rate, sIgA was lower due to decreased saliva flow rate (Walsh et al., 2002). Brandtzaeg (2007) concluded that both sIgA concentration and secretion rate should be reported to ensure saliva flow rate is accounted for, in addition to the high variance seen between individuals.

2.5 Exercise and sIgA

2.5.1 Acute Exercise and sIgA

Moderate exercise has been found to increase sIgA secretion (Bosch et al., 2002; Klentrou et al., 2002). This could be because acute stress typically increases sIgA (Trueba et al., 2012), which likely happens due to increased secretion of IgA from B-lymphocytes or increased transport across the epithelium to saliva (Bosch et al., 2002).

That said, intensity and duration of the exercise bout could have a major effect on sIgA response; intense exercise could alter activity of the HPA axis (Walsh et al., 2011). Activation of the HPA axis from intense exercise (increased stress) would produce cortisol, and these elevated stress hormones stimulate a fall in sIgA (Hellard et al., 2015). Previously, cortisol has shown to decrease expression of pIgR messenger RNA (mRNA) in rabbit culture studies (Rosato et al., 1995). Furthermore, decreased sIgA was found following a treadmill run to exhaustion in rats and was associated with lowered pIgR mRNA expression (Kimura et al., 2008). Alternatively, it has been shown that intense exercise can reduce number of circulating lymphocytes (Clifford et al., 2017; Shek et al., 1995) and inhibit monocyte and T-cell activation (Tvede et al., 1989), which is vital because it is thought to impact on the production of immunoglobulins (Walsh et al., 2011).

Therefore, opposed to moderate exercise it has been well documented that sIgA concentration decreases immediately following an intense bout of exercise. One of the earliest studies to suggest this was by Tomasi et al. (1982), who reported a 20% reduction in sIgA after cross-country skiing. This was additionally found following intense cycling (Mackinnon et al., 1989), swimming (Gleeson et al., 1999; Gleeson et al., 1995), and marathon running (Nieman et al., 2002). These findings, in turn, led to decades of research examining the relationship between exercise and sIgA. More recently, Mariscal et al. (2019), reported a significant drop in sIgA concentration in 21 national handball players following a match, where larger changes were observed for those playing more than 30 minutes.

A criticism of early studies however, includes the issues around expression of sIgA already identified. Expression of sIgA concentrations alone has been criticised as it does not control for amount of saliva produced (saliva flow rate). Differences in flow rate could heavily affect biomarker results (Wood, 2009), so now researchers have expressed sIgA in terms of secretion rate (Fahlman & Engels, 2005). Li and Gleeson (2004) reported that cycling at 60% maximal oxygen consumption ($\dot{V}O_2\text{max}$) for 2 hours, increased sIgA concentration. However, the increase observed could have been a result of the significantly reduced saliva flow rate since no changes in sIgA secretion were observed.

This could be down to dehydration status, reducing the amount of saliva (Proctor & Carpenter, 2007). It was suggested that the only consistent biomarker for identifying and monitoring athletes at risk of URS is through use of sIgA secretion (Walsh, et al., 2011).

One study observing seventeen nationally ranked tennis players, demonstrated that sIgA secretion rate decreased significantly after 1 hour of intense tennis (Novas et al., 2003). However, the level of suppression in sIgA secretion observed here was less pronounced than elsewhere (Nieman et al., 2002; Steerenberg et al., 1997). Perhaps the shorter duration or type of exercise may have contributed to this. Following a marathon, 98 runners showed sIgA secretion rate to significantly decrease by 25% (Nieman et al., 2002). Also, triathletes completed 1500 m swimming, 40 km cycling and 10 km running (taking 2 to 2.5 hours) and found significantly reduced sIgA secretion rates (Steerenberg et al., 1997). This is in agreement with other research conducted in rowing (Nehlsen-Cannarella et al., 2000), suggesting that longer duration, endurance exercise may be more likely to lower sIgA and secretion rates.

Interestingly, in one study examining incremental cycling tests to exhaustion, sIgA secretion rate increased (Allgrove et al., 2008). However, cycling bouts were on average 22.3 minutes and despite being high intensity, they were short in duration. It could be argued that the short duration of exercise was not enough to elicit sIgA depression. Saying that, Blannin et al. (1998) reported increased sIgA secretion rate following cycling bouts to exhaustion (80 % VO_2 max until exhaustion or 55 % VO_2 max for 3 hours or to fatigue). Here, saliva flow rate decreased and authors proposed that “exercise may detrimentally affect the quantity of saliva produced, but not the quality of saliva” (Blannin et al., 1998). Whereas some others have reported no change in sIgA secretion rate following sprint interval training (Davison, 2011), or intermittent bouts of soccer (Sari-Sarraf et al., 2008). As well as the intensity and duration of training load, changes in sIgA have been suggested to be dependent on the fitness level of the athlete (Neville et al., 2008); which could explain differences in findings for sIgA following acute exercise.

One possible explanation for the differences seen between studies could be due to the large between-subject variability in sIgA, which is evident. Dwyer et al. (2010) reported large between-subject variability in sIgA secretion (75%), suggesting that group mean data may not provide an accurate representation of findings. Furthermore, it makes comparisons between studies and different groups very difficult. Other rationale for inconsistencies concerning sIgA could be from differences among studies in both timing of saliva collection and sample size. Either that, or perhaps increased frequency of training may accentuate sIgA suppression. Mackinnon & Hooper (1994) found that sIgA secretion rate decreased 20 to 50% following 3 consecutive days of 90-minute treadmill running. Importantly, secretion rates were significantly lower on days two and three, compared to one (Mackinnon & Hooper, 1994). Intense daily exercise appeared to have a cumulative effect on sIgA and mucosal immunity. Therefore, studies observing the acute sIgA response to exercise may present different findings for those conducted on athletes vs. healthy individuals. Thus, research has now been conducted in monitoring sIgA over training seasons.

2.5.2 Effect of Training Load on sIgA in Athletes

Training for an elite athlete commonly consists of enduring periods of intense training to induce physiological adaptation, followed by light or moderate training for recovery. Due to the positive effect of moderate exercise found on mucosal immunity, decreased training loads are used (taper) when leading into a competition (Papacosta & Nassis, 2011). Increased concentration of sIgA has been found during taper periods for competitive swimmers (Papadopoulos et al., 2014). With this, it has been proposed that increased sIgA secretion could act as an illness preventative measure (Gleeson et al., 2000b; Klentrou et al., 2002). Therefore, decreases in training load have been used, to increase sIgA levels and reduce subsequent risk of URI. However, as most elite swimmers train at least twice a day, it has been proposed that the combined effect of training on immune function and inadequate recovery time, could result in a chronic immunodepression (Mackinnon, 2000). Key research described previously, found cross-country skiers to have lower resting sIgA concentration compared to controls (Tomasi et al., 1982). Despite the majority of literature indicating no difference in athletes compared to the general

population, athletes often endure periods of prolonged and intense training. For some athletes, these intense periods are completed in 4-year cycles, with some also juggling education and jobs, showing why monitoring of sIgA and associated URI is so important for success.

Researchers have reported that chronic stress was found to suppress immune response (Dhabhar, 2014) and was associated with decreased sIgA (Bosch et al., 2002), potentially due to decreased expression of pIgR as mentioned previously. Therefore, earlier findings in swimmers indicated that prolonged, intense training could chronically depress sIgA concentration (Gleeson et al., 1995, Gleeson et al., 1999; Tharp & Barnes, 1990). Tharp and Barnes (1990) reported a significant decrease in sIgA in competitive swimmers over a 3-month training programme. Since then, numerous longitudinal studies have identified periods of intense training to decrease sIgA concentration and secretion rates (Fahlman et al., 2017; Moreira et al., 2008). Fahlman and Engels (2005) observed significantly decreased sIgA and secretion rate, over a 12-month training season in American football players. Moreover, 149 military recruits reported lower sIgA secretion during intense training (Scott et al., 2019). Suppression of sIgA after prolonged exercise or heavy training is itself a probable consequence of altered T lymphocyte function (Clancy et al., 2006). That said, many longitudinal studies have also shown no association between repeated bouts of intense training and sIgA (Tiernan et al., 2020). A recent review by Turner et al. (2021), presented mixed findings for longitudinal studies examining sIgA and exercise.

One study observing national swimmers, reported that intensive training over 5-months found no significant change in sIgA (Pyne et al., 2001). However, saliva samples were only taken at two points during this study period, thus validity of this should be questioned due to infrequent sampling (Neville et al., 2008). Likewise, Novas et al. (2003) observed no significant trends for exercise induced changes in sIgA over a 12-week period in elite tennis players. Despite finding significantly reduced sIgA after 1 hour of intense practice, no differences or trends were found within the groups, over time. One possible explanation for lack of significant findings for this study, could be that the response was

delayed. Tiernan et al. (2020) found no significant difference in sIgA following increased training loads. However, upon further analysis, it was found that sIgA decreased 1-2 weeks after training load was increased. Moreover, one study examining two bouts of intermittent soccer reported decreased concentration of sIgA, but 24 hours after exercise (Sari-Sarraf et al., 2008). Finally, although Moreira et al. (2013) found intensive training to increase risk of URI, no significant difference in sIgA was found. However, it should be mentioned that some athletes showed lower levels of sIgA; highlighting the fact that individual monitoring of sIgA may be useful. As a result, timing of sampling and monitoring individuals is likely to be an important consideration during longitudinal observations.

All things considered, a recent systematic review investigating team sports showed lower values of sIgA after greater training loads of high intensity and volume (Rico-González et al., 2021). Differences in findings could be down to several factors including large between- and within-subject variability, athlete fitness level, duration and intensity of exercise, expression of sIgA, and timing of sampling. The great within-subject variability seen in elite athletes, may indicate that some athletes are more susceptible to stress and illness than others (Francis et al., 2005; Neville et al., 2008), perhaps due to a genetic predisposition to increased infection resistance (Cox et al., 2010), or having higher individual training thresholds, where the risk of illness increases (Foster, 1998). In one study, 30% of athletes experienced recurrent URS over winter months (Gleeson et al., 2012). These illness prone athletes had higher training load and significantly lower sIgA secretion rate, than illness-free individuals. Not only does large individual variation question the validity of studies with infrequent sampling, it creates support for regular athlete monitoring and determining individual baseline measures (Neville et al., 2008). Therefore, rather than displaying group means, it be important to monitor individual changes and track changes in sIgA over time.

Overall, monitoring sIgA has been argued to be crucial for identifying risk of URI for athletes, providing a quick and effective marker of mucosal immunity over a training season (Papacosta & Nassis, 2011). However, timing of sampling is critical to note, and all influencing factors on sIgA discussed should be considered for any future research. It

has been suggested that measurement of mucosal markers should not be used in isolation, a subjective monitoring questionnaire may provide further information into the illness status of the athlete (Neville et al., 2008). Harrison et al. (2021) advised use of a blended approach incorporating immune measures with subjective real-world URS data through self-reported questionnaires. That way, a log could be kept for external factors that may influence sIgA and subsequent illness. This is because even stressful life events have been associated with decreased sIgA concentration (Phillips et al., 2006). One longitudinal study of 852 German elite reported increased illness alongside high stress levels and sleep deprivation (König et al., 2000); highlighting the impact that other factors can have on mucosal immunity and risk of illness.

2.6 Overview of Sleep

2.6.1 Stages of Sleep and Definitions

Under the current classification, there are four stages of sleep which involve different levels of consciousness and brain activity (Berry et al., 2015). These can be categorised into two main types, rapid eye movement (REM) and non-REM. Non-REM consists of the first three stages, representing 'light' sleep in stage 1-2, and 'deep' sleep in stage 3 (sometimes called slow-wave sleep) (Walsh et al., 2021). The deepest stages of sleep are usually where growth hormone is released (Halson, 2014a). The final stage is REM and during this, the brain is highly activated. A greater duration of REM has been shown to enhance recovery and lead to improved wakefulness (Walker & Stickgold, 2005). Dependent on sleep duration, this process could cycle through and repeat up to 6 times, which would contribute to good sleep quality (Marshall & Turner, 2016). Overall, 7- 9 hours of sleep for a young adult consists of around 61% 'light' sleep, 16% 'deep' sleep and 23% REM (Ohayon et al., 2004). Outside of these stages of sleep, most researchers investigate sleep parameters that are comparable for research, such as quantity and quality of sleep. Table 2.1 identifies common sleep parameters used by most studies, and the appropriate definitions.

Table 2.1

Definitions of Common Sleep Parameters

Common Sleep Parameters	Definition
Latency (minutes)	The period of time between bedtime and sleep onset time, or how long it takes to fall asleep.
Efficiency (%)	Number of TST divided by the total number of TTIB, expressed as a percentage.
Total Time in Bed (TTIB) (minutes)	The amount of time spent in bed attempting to sleep between bedtime and get-up time.
Total Sleep Time (TST) (minutes)	The total amount of sleep obtained.
Wakes After Sleep Onset (WASO) (minutes)	The total number of minutes the subject was awake after sleep onset occurred.
Number of Awakenings	The number of different awakening episodes throughout sleep, as scored by the algorithm.
Average Time Awake (minutes)	The average length, in minutes, of all awakening episodes.
Bedtime (hours: minutes)	The self-reported time at which a participant went to bed to attempt to sleep, e.g., when 'turning off the light'.
Get-up time (hours: minutes)	The self-reported clock time at which a participant got out of bed and stopped attempting to sleep.

Note. Definitions were used as defined by ActiGraph (Wyatt, n.d.) and (Sargent et al., 2014).

2.6.2 Recommendations

The National Sleep Foundation's duration of sleep recommendation is 7 to 9 hours for young adults (Hirshkowitz et al., 2015). Within a worldwide survey conducted by Philips Healthcare (2021), the average amount of sleep achieved by adults was 7 hours 18 minutes. However, in the Chemist4U (2022) survey conducted on 2,000 participants from the UK, they found a lower average sleep duration of 6 hours 6 minutes. It must be considered that survey data was used, as opposed to quantifiable sleep data but can be used as a guide for the general population. Furthermore, it has been recommended that

adults should achieve a sleep efficiency of at least 85% (Ohayon et al., 2016). Sleep efficiency <85% is considered abnormal (Cohen et al., 2009), with $\leq 74\%$ indicating bad sleep quality.

2.6.3 Impact of Sleep Inadequacy

Meeting sleep requirements are essential for both the brain and body, as it can have a major impact on a person's daytime functioning (Halson, 2014). Sleep affects physiological processes, learning, memory and cognition (Dattilo et al., 2011). Studies have shown sleep restriction (3-5 hours per night) to lead to impaired speed and reaction times (Belenky et al., 2003), learning and memory consolidation (Walker & Stickgold, 2005), and mental and physical wellbeing (Haack & Mullington, 2005). Therefore, the effect of poor sleep could be highly impactful for athletes.

At a basic level, sleep restriction would increase athletes pre-training feelings of fatigue (Sargent et al., 2014). As a result, this fatigue could compromise the quality of the session and training performance. In addition to the associated fatigue with sleep restriction, detriments in performance are evident. Following three days of sleep restriction (three hours), Reilly and Piercy (1994) reported significantly reduced bench press and deadlift performance. However, the likelihood of any athlete having this magnitude of sleep restriction is unlikely. Plus, a review by Fullagar et al. (2015) summarised that results were conflicting for the effect of sleep restriction, as some markers of performance were maintained. Even with this in mind though, it would come as no surprise that athletes reported poorer mood and higher exertion during training than normal, following sleep restriction (Reilly & Piercy, 1994). Particularly during intense training loads, it would be likely that associated fatigue and low mood would impact motivation for training and performance.

Furthermore, exercise performance does seem to be negatively affected during periods of sleep deprivation (Fullagar et al., 2015). Research has shown slower sprint time (Skein et al., 2011), and decreased treadmill endurance performance (Oliver et al., 2009). Both studies examined the effect of 30 hours with no sleep, which seem to have more of a

detrimental effect. If this was to continually happen, it could have more chronic long-term effects. This could in turn, additionally place the athletes at greater risk of developing URI and other health problems (Cohen et al., 2009).

2.6.3.1 Risk of URI

People who obtain an insufficient amount of sleep over several consecutive days are at increased risk of impaired immune function (Vgontzas et al., 2004). Research has shown that manipulation of sleep can affect immune markers, including leukocyte activity and distribution, cytokine production, and antibody levels (Besedovsky et al., 2019). One study found that individuals with less than 7 hours sleep were 2.94 times more likely to develop a cold than those that had 8 hours' sleep (Cohen et al., 2009). In addition to this, another study in adolescents suggested that short sleep duration was associated with a higher number of illnesses, included cold and flu (Orzech et al., 2014). Elsewhere in unpublished work, Harrison (2020) suggested that runners were twice as likely to have a post-marathon URTI if poor self-perceived sleep quality was recorded. In contrast, Ghilotti et al. (2018) found no association between physical activity, sleep duration and quality, and the incidence of URI in a large Swedish cohort. However, the majority of results were self-reported, with no quantifiable data such as sleep actigraphy or illness classification from nasal swabs. The majority of findings here suggest sleep inadequacy could impair immune function and increase risk of illness; therefore, the importance of meeting recommended requirements of sleep is evident and supports the use of sleep monitoring within athletic cohorts.

Although being preliminary findings, a possible mechanism behind this could be a reduction of circulating IgA (Ruiz et al., 2012). Following two nights of total sleep deprivation monitored by polysomnography, decreased IgA was found which did not return to baseline following sleep recovery. In contrast, another study showed increased IgA following one night without sleep (Hui et al., 2007). Authors concluded that sleep deprivation enhanced changes in serum humoral immunity. Perhaps the additional day of sleep deprivation between the studies, could give rationale for the contrast in findings.

Therefore, more work is needed to determine the effect of sleep deprivation on IgA and the link with illness risk.

2.7 Athletes and Sleep

Only a small number of athletic studies examining sleep have included non-athletic controls. However of those that have, evidence has suggested that sleep quality is poorer in athletes, than a non-athletic population (Bender et al., 2018; Gudmundsdottir, 2020; Leeder et al., 2012). Leeder et al. (2012) analysed 46 national athlete habitual sleeping habits for 4 days during training, and compared to 20 gender matched, healthy controls (Leeder et al., 2012). Through use of wrist worn Actigraphy, they found that athletes spent more time awake in bed, had higher sleep latency and lower sleep efficiency, when compared to the healthy subject controls (Leeder et al., 2012). Furthermore, Surda et al. (2019) reported a higher percentage of 'poor sleepers' (48% vs. 18%) and excessive daytime sleepiness in elite swimmers (16% vs. 2%), compared to controls.

2.7.1 Quantity of Sleep Achieved

Despite the importance of sleep, restricted sleep quantity has been continually reported amongst athletes (Knufinke et al., 2018). A recent review found that athletes were often unable to achieve the recommended ≥ 7 hours of total sleep time (Roberts et al., 2019), supporting existing findings in swimmers specifically (Leeder et al., 2012; Sargent et al., 2014a; Sargent et al., 2014b). Sargent et al. (2014b) analysed two weeks (926 sleep periods) of habitual sleep during a normal training phase, from 70 elite athletes from seven different sports (including swimming); authors reported that 60% of athletes slept below the national minimum recommendation. In a recent study conducted on 108 adolescent swimmers, a total sleep time (TST) of 6 hours 14 minutes was recorded (Gudmundsdottir, 2020). Plus, in Olympic swimmers similar findings have been found (de Mello et al., 2020). This is a major problem because research has suggested a requirement of < 10 hours sleep for well-trained athletes, in order to recover and recuperate (Calder, 2002).

One main factor to influence sleep duration is early morning training. In swimmers specifically, average sleep durations have been reported as low as 5 hours and 24 minutes on nights prior to training (Sargent et al., 2014a). This study of ten Olympic swimmers, found that early morning training negatively influenced sleep duration; 5 hours 24 minutes with a 05:18 get up time with early training, compared to 7 hours 24 minutes with a 07:06 get up time if training later in the day. One study by Gudmundsdottir (2020) further supported this, who found decreased TST with early morning training. The problem being that retiring to bed earlier the night before training, does not seem to significantly help the reduction in sleep duration (Sargent et al., 2014a). Despite earlier bed times, individual sport athletes still slept on average 30 minutes less, compared to team sport athletes (Lastella et al., 2015). As early morning trainings are routine in elite swimming, it could be argued that swimmers are at greater risk of poor sleep quantity, than other athletes.

2.7.2 Quality of Sleep Achieved

In one systematic review, average sleep efficiency for athletes was 86%, and many studies overlapped the threshold value of 85% (Gupta et al., 2017). These findings were similar to other research observing sleep efficiency (85.6%) in military recruits (Bulmer et al., 2022). However, some athletes have poor markers of sleep quality, with up to half of all elite athletes being categorised as 'poor sleepers' (Gupta et al., 2017). Interestingly, athletes from individual sports obtained less sleep and had poorer sleep efficiency, than athletes within team sports (Lastella et al., 2015). In swimmers specifically, Lastella et al. (2015) reported a sleep efficiency of 84.4%, below the threshold that would be considered normal (85%) (Cohen et al., 2009). Similarly, de Mello et al. (2020) reported an average sleep efficiency of 82% in 14 Olympic swimmers. One study however, reported 85.5% sleep efficiency, suggesting not all swimmers suffer from abnormal sleep (Lastella et al., 2012). It has been suggested that TST and efficiency decrease with age (Juliff et al., 2015; Ohayon et al., 2004), however it is unlikely for this to be the case here (22 vs. 27 years). Alternatively, perhaps differences in the level of athlete could influence sleep efficiency findings; although, this is speculative and other factors should be considered.

Ohayon et al. (2016) suggested an important indicator for good sleep quality included a sleep latency of ≤ 15 minutes. While Surda et al. (2019) examined 20 elite swimmers and reported an average latency of 18.3 minutes, close to the recommendation, most studies show a longer latency onset period. Interestingly, swimmers reported the highest latency (40 minutes) compared to other sports (e.g. rugby sleep latency was 8 minutes) (Lastella et al., 2015). Latency of long duration (35 minutes) was also found in Olympic swimmers (de Mello et al., 2020). Pre-sleep arousal or anxiety during intense training periods, may play a role in taking longer to fall asleep. Alternatively, some individual sports require two bouts of training a day, with higher training load which could affect results. Regardless, differences between individual and team sports indicate that athletes in individual sports, such as swimming, need more help (Erlacher et al., 2011). Furthermore, WASO could dramatically impact sleep quality through fragmentation of the phases of sleep, which has been reported in athletes (Gupta et al., 2017). Large variations in WASO has been reported in swimmers, ranging from 14.1- 64.7 minutes (de Mello et al., 2020; Gudmundsdottir, 2020; Lastella et al., 2015; Surda et al., 2019). Overall, there is no clear consensus on whether a causal relationship exists between athletic training and sleep inadequacy (Walsh et al., 2019). However, sport specific risk factors known to impair sleep quantity and quality include, high training loads (Dumortier et al., 2017; Hauswirth et al., 2014), early morning training (Sargent et al., 2014), travel, and competition (Erlacher et al., 2011; Juliff et al., 2015).

2.7.3 Impact of Training Load and Competition on Sleep

It has been proposed that sleep disturbances may be an important symptom of over-reaching, as a result of increased training load or changes in training schedule (Lastella et al., 2018). As an example, higher training loads influenced a decrease in TST, in gymnasts (Dumortier et al., 2017), and elite synchronised swimmers (Schaal et al., 2017). Likewise, in a study conducted on 22 collegiate swimmers, longest sleep duration was found when training load was low (Astridge et al., 2021). These findings suggest that high training loads are impactful on sleep parameters.

Furthermore, athletes have rated sleep as vital for successful competitive performance. In a study of 576 elite Brazilian athletes, poor sleep quality decreased the odds of winning at competition (Brandt et al., 2017). Despite the apparent importance of sleep, few studies have examined the effect of competition on sleep parameters (Halson, 2013). However, those that have reported reduced sleep quantity and quality on the night before competition (Erlacher et al., 2011; Lastella et al., 2014). Results have shown reduced TST on the night prior to competition, with almost 70% of athletes experiencing poorer sleep than normal. In addition, Walsh et al. (2019) reported extended latency for national and international swimmers during competition, when compared to training phases. It has been suggested that this could be caused by pre-competition anxiety and stress, which makes sense as similar findings have been found for university students before an exam (Ahrberg et al., 2012). This has been supported by Erlacher et al. (2011), observing the sleep patterns of 632 athletes before competition and found 65.8% experienced poor sleep. Similarly, 64% of athletes experienced poor sleep before competition in another large cohort of 283 elite Australian athletes (Juliff et al., 2015). Both studies reported the main issue being not being able to fall asleep. Here, factors such as nervousness and thoughts about the competition were rated highest for causing the sleep problems (Juliff et al., 2015), and was found most in those in individual sports (Erlacher et al., 2011). Due to the large cohort studies having similar findings, it could confidently be said that competition is a risk factor for inadequate sleep for athletes and should be monitored closely.

2.7.4 Strategies to Improve Sleep

Due to the negative impact found on sleep inadequacy and immune function, it is not surprising that the hypothesis emerged that sleep could play an important role in recovery from illness. Animal studies have shown that infections can increase non-REM sleep, which is needed to help fight infection (Besedovsky et al., 2019). Therefore, sleep has been identified as one of the best recovery strategies available to elite athletes (Sargent et al., 2014b). One proposed method to improve sleep, is habitual sleep enhancement. Previously, encouragement to achieve a minimal 10 hours' sleep per night in basketball players was explored, and presented positive findings for performance measures. Mah et

al. (2011) reported enhanced sprint times, decreased reaction time and improved mood, following extended sleep. Furthermore, a small increase in habitual sleep duration from 7 to 8 hours per night, was shown to reduce reaction time, and feelings of sleepiness and fatigue (Kamdar et al., 2004). Although extending habitual sleep periods would be ideal for athletic performance, napping could be a useful alternative (Marshall & Turner, 2016).

2.7.4.1 Napping

In addition to extending habitual sleep duration, napping has been suggested to be a useful method to enhance recovery for athletes (Marshall & Turner, 2016). Research has explored the effect of napping following a night of partial sleep loss and found positive outcomes in national karate (Daaloul et al., 2019) and judoka athletes (Romdhani et al., 2020). Earlier, Waterhouse et al. (2007) reported that following partial sleep loss (4 hours), a post-lunch nap improved alertness, sleepiness and sprint time in ten healthy males. Longer nap time (90 minutes) has been suggested to enhance antioxidant defence (Romdhani et al., 2020); however, decreased sprint performance and increased sleepiness (Romdhani et al., 2021). Therefore, dependent on the requirement of training in the afternoon, it would seem that duration of nap could make a difference in training performance. In further support, napping has shown beneficial changes in cortisol and IL-6, and could therefore reduce inflammation and stress (Halsen, 2014b). It would seem that napping could dramatically improve an athlete's ability to perform skills within training, whilst ensuring adequate recovery.

It should be considered that even when napping, swimmers in one study still obtained significantly less sleep on training days (Sargent et al., 2014a). That said, Besedovsky et al. (2019) suggested that napping appeared to support recovery in immune changes. Napping and extension of sleep duration may counteract immune changes induced by sleep deprivation and is therefore recommended. This could be vital for an athlete where early morning training is unavoidable; arguably coaches should enforce napping as part of an athlete's routine, especially if they have high incidence of illness.

To conclude, sleep interventions that extend sleep duration and napping, have shown promising results in improving performance and immune markers. Although evidence is lacking, sleep seems to play an important role in recovery and prevention of illness too. Therefore, elite sport would benefit from identifying athletes at risk of sleep inadequacy which could be achieved with appropriate sleep monitoring. Interestingly, one study conducted in 14 Olympic swimmers showed that by teaching sleep hygiene methods over 10 consecutive days, significantly improved sleep quantity and quality leading into competition (de Mello et al., 2020). Simple things such as, use of eye masks and ear plugs, afternoon napping, avoiding television, computers and phones before sleeping and avoiding caffeine 4-5 hours before sleeping, are effective methods to improve sleep for athletes. As a result of sleep monitoring, sleep education or management from coaching staff (by reducing training load or changing scheduled training times), could help in improving sleep adequacy in athletes.

2.7.5 Methods of Monitoring Sleep

Use of sleep monitoring in elite sport has previously been supported (Lastella et al., 2015; Sargent et al., 2016). Sleep disturbances may be an important symptom of over-reaching (Lastella et al., 2018). By monitoring, it may allow coaches to modify training loads or training schedules, for athletes most at risk of sleep inadequacy. The ‘gold standard’ method for sleep measurement is used of Polysomnography (PSG), and consists of electrodes monitoring brain activity, eye movement, and muscle activity (Besedovsky et al., 2019). However this method is expensive and typically performed within a laboratory, which is an unnatural sleep setting (Walsh et al., 2021). Although the best at determining sleep stages, PSG is impractical for multiple day use and for field-based studies, such as for athletes. When analysis of sleep stages is unnecessary, wrist-worn actigraphy devices have been praised.

2.7.5.1 Actigraphy

The most commonly used method for sleep assessment in athletes, is research-grade actigraphy (Walsh et al., 2021). These are wrist-worn devices that continuously record

body movement, which is usually stored in 60 second epochs (Halson, 2014a). When the interest is sleep duration and quality, rather than sleep stage assessment, activity monitors are a reasonable alternative to PSG. Wrist-worn actigraphy has additionally been validated against PSG, providing comparable and accurate data when compared against the 'gold standard' method (Quante et al., 2018; Zinkhan et al., 2014). Further strengths include the fact actigraphy is less invasive than PSG, and accurately measures bed time, sleep duration, latency and awakenings, being easily performed at home (Besedovsky et al., 2019). Therefore, this method presents several benefits over PSG, for field studies wanting to monitor over time.

That said, it has been reported that a minimum of five nights measurement (usable data) is required to obtain reliable actigraph measures of sleep, for adolescents (Acebo et al., 1999). It is likely that Actigraphy equipment and software has improved in the last 20 years, however no update on this can be found and should be considered for current research. Plus, analysis using software does need some expertise, as they require use of an accelerometer-based algorithm to estimate total sleep time. As an example, common sleep algorithms have included Sadeh (Sadeh et al., 1994) and Cole-Kripke (Cole et al., 1992), for the GT3X+ ActiGraph device (ActiGraph LLC, Florida, US). The Sadeh algorithm has been considered most appropriate for younger populations because it was developed using subjects ranging from 10 to 25 years of age, plus was more accurate in detecting wake time (Quante et al., 2018). Something else to consider would be that devices cannot log sleep with a push of a button. Although the device can estimate sleep times due to lack of movement, this can sometimes be inaccurate so a sleep log would be needed to determine 'in bed' and 'out of bed' times.

2.7.5.2 Sleep Diaries, Logs and Questionnaires

Subjective methods of sleep assessment include sleep diaries or logs, which require individuals to complete upon awaking and before going to bed. These would allow for estimates of sleep duration, latency and quality of sleep (Besedovsky et al., 2019). However, these add a time burden for those filling them out, and often have shown overestimates of sleep duration and efficiency when compared to PSG (Walsh et al.,

2021). Therefore, questionnaires are commonly used as an alternative, as it allows the individual to fill out information on self-perceived sleep quantity and quality retrospectively, over one week (Besedovsky et al., 2019). Self-reported sleep questionnaires such as the Pittsburgh Sleep Quality Index (PSQI), which assesses sleep quality and disturbances over a 1-month time interval have been widely used and validated (Buysse et al., 1989). It has been suggested that subjective methods of sleep assessment such as questionnaires or diaries could be used in conjunction with actigraphy, increasing reliability when used together (Halson, 2014a).

2.8 Vitamin D and URI Risk

All things considered, other factors present increased URI risk for athletes such as nutritional deficiencies (Bermon et al., 2017; Walsh, 2019). Overall, the symptoms associated with URI are hugely detrimental to training and competition for athletes. As a result, research investigating how to limit the URS suffered by athletes has grown.

There are two main forms of vitamin D which have different molecular structures, vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol) (Farrokhyar et al., 2017). They both effectively increase serum concentrations in the body, but D₂ comes from plants, and D₃, from animals. Production of vitamin D₃ from exposure to sunlight containing ultraviolet B (UVB) radiation, accounts for 80-100% of the body's requirements (Farrokhyar et al., 2017; Walsh, 2019); UVB radiation produces pre-vitamin D₃ from 7-dehydrocholesterol (Wilson-Barnes et al., 2020). Limited quantities of vitamin D can be obtained from diet alone (10-20%) (Owens et al., 2018). Once circulating within the body, both forms are transformed in the liver to be hydroxylated and activated to 25-hydroxyvitamin (25(OH)D), the best indicator of status (Bermon et al., 2017; Wilson-Barnes et al., 2020). This is most commonly measured in studies by serum blood sampling, as it is the major circulating vitamin D metabolite. This can be hydroxylated further in the kidney, or other tissues, to produce the active metabolite 1,25-Dihydroxyvitamin D (1,25(OH)₂D). The active metabolite is responsible for most actions of vitamin D, with the main effect being to increase calcium absorption; however, measurement is reportedly difficult and time

consuming, due to lower serum concentrations and shorter life span (Vanderschueren et al., 2013).

Serum measurement of 25(OH)D has shown the fundamental role vitamin D can play in skeletal muscle function (Close et al., 2013) and inflammation (Larson-Meyer et al., 2012). The advantageous effect found on musculoskeletal health especially, has led to worldwide dietary recommendations (Holick, 2017). However, the benefits of vitamin D are now understood to be much further reaching. Vitamin D receptors (VDRs) have been discovered in almost all immune cells (Baeke et al., 2010). Monocytes, macrophages, neutrophils and T and B lymphocytes all contain VDR, and also express an enzyme (1- α hydroxylase) which is responsible for assisting hydroxylation of 1,25(OH)₂D (Owens et al., 2018). Additionally, vitamin D plays a vital role in regulation of AMPs and protein production at the mucosal surfaces, such as cathelicidin and β -defensin (Liu et al., 2006). More specifically, Cathelicidin (an AMP) has shown to be direct target of the VDR and is strongly up-regulated by 1,25(OH)₂D (Gombart et al., 2005). This has been supported, showing 25(OH)D levels to be significantly correlated with cathelicidin concentration (Bhan et al., 2011). Vitamin D can also prevent overly exuberant immune responses by activating T and B lymphocytes (adaptive immunity) (Bermon et al., 2017; He et al., 2016). Plus, it has shown anti-inflammatory effects, by influencing pro-inflammatory cytokines and neutrophil activation (Quesada-Gomez et al., 2020; Walsh, 2019).

Vitamin D reduces inflammation by decreasing production of pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and IL-6 (Liu et al., 2006; Yagüe et al., 2020). For example, IL-6 has shown to increase following intense training, which is thought to be related to the appearance of muscle damage (Yagüe et al., 2020). Linked to this, low levels of vitamin D in the general population and in athletes (after intense exercise) have shown increased IL-6 and TNF- α (Yagüe et al., 2020). Therefore, confirming that vitamin D improves the inflammatory response (Gombart et al., 2005; Walsh, 2019). Consequently, vitamin D has been found to play an important role for immune function and inflammation, which could impact risk of URI.

Research has shown a negative association between 25(OH)D level and incidence of URI, in the general population (Berry et al., 2011; Sabetta et al., 2010), military recruits (Laaksi et al., 2010), and athletes (Cox et al., 2008; Halliday et al., 2011; He et al., 2013; He et al., 2016). Thus, Sabetta et al. (2010) was the first prospective study in the general population which found serum 25(OH)D concentration to be correlated with incidence of URI in adults; where concentrations of $>95 \text{ nmol}\cdot\text{L}^{-1}$ were associated with a two-fold decrease in incidence of URI. Elsewhere, advantageous 25(OH)D levels have been defined as around $75 \text{ nmol}\cdot\text{L}^{-1}$ (Bischoff-Ferrari, 2008). In support, another study conducted over a 4-month winter period in athletes, found that 25(OH)D optimal athletes ($>120 \text{ nmol}\cdot\text{L}^{-1}$) reported significantly less URI, than 25(OH)D deficient ($>30 \text{ nmol}\cdot\text{L}^{-1}$) athletes (He et al., 2013). Plus, those that were deficient had significantly longer duration and increased severity of URS (Cox et al., 2008; He et al., 2013).

With this, some researchers have argued that previous 25(OH)D guidelines were made with only bone health in mind (Owens et al., 2018). Therefore, it should be considered that a higher 25(OH)D could be needed in other tissues, such as for immune health. Thus, He et al. (2016) proposed that higher 25(OH)D concentrations may be required to positively impact immune function, than what has previously been defined for bone health. Close et al. (2013) defined 25(OH)D concentration of $50\text{-}120 \text{ nmol}\cdot\text{L}^{-1}$ to be adequate and $>120 \text{ nmol}\cdot\text{L}^{-1}$ to be most optimal for illness. As explained, vitamin D has been found to play an important role for immune function (He et al., 2016), displaying the importance of 25(OH)D measurement to identify those with insufficiencies.

2.8.1 Deficiency

The current 'gold standard' measurement of 25(OH)D concentration is through use of liquid chromatography tandem mass spectrometry (Snellman et al., 2010). However, a clear definition on what constitutes vitamin D insufficiency is debated (Owens et al., 2018). Recommendations by the Institute of Medicine (2011) refer to total serum 25(OH)D; with individuals $\geq 50 \text{ nmol}\cdot\text{L}^{-1}$ ($>20 \text{ ng}\cdot\text{mL}^{-1}$) being sufficient, and individuals with $<30 \text{ nmol}\cdot\text{L}^{-1}$ ($<12 \text{ ng}\cdot\text{mL}^{-1}$) being considered deficient. There is some conflict on insufficient vitamin D levels for health, with some reporting it as $\leq 50 \text{ nmol}\cdot\text{L}^{-1}$ (Institute of

Medicine, 2011) and others, $<80 \text{ nmol}\cdot\text{L}^{-1}$ (Farrokhyar et al., 2015). However, the Institute of Medicine (2011) guideline discussed above were agreed with in a recent immunonutrition consensus statement (Bermon et al., 2017).

Large variations exist between 25(OH)D concentrations (Close et al., 2013). One major factor that increases risk of 25(OH)D deficiency includes darker skin pigmentation. A cross sectional study in the UK (449,942 participants) by Lin (2021), found that Asian (53.7%) and black (34.9%) individuals had a higher proportion of vitamin D deficiency than white (12%). This can be explained by the fact that individuals with darker skin pigmentation require 5-10 times longer exposure to UVB for the same absorption of radiation, compared to paler skin pigmentations (Holick, 2017). In addition, higher risk of deficiency has been identified for those with lack of adequate sun exposure (Close et al., 2013), use of sunscreen (Faurischou et al., 2012), and for those for those that live at higher latitudes (>37 degrees North or South) (Barcal et al., 2016), which promote significantly restricted vitamin D synthesis.

As previously identified, vitamin D₃ can be derived from sunlight (Farrokhyar et al., 2017), thus, seasonal variations are evident. One Spanish study suggested that most individuals would not obtain the daily vitamin D recommendation from exposure to solar UVB radiation alone, during winter months (Serrano, 2018). Unsurprisingly then, proportion of 25(OH)D deficiency has been reported to be higher during winter months (Lin et al., 2021; Scullion et al., 2018). Specifically in the UK, national diet and nutrition survey statistics showed that 56% of adults aged ≥ 19 years had serum 25(OH)D below $25 \text{ nmol}\cdot\text{L}^{-1}$, during January-March (Public Health England, 2019). This was compared to 8% of adults in the same study population, July-September (PHE, 2019). This stresses the significance of seasonal impact on 25(OH)D concentration.

2.8.1.1 Deficiency in Athletes

In addition to the general population, low serum 25(OH)D status has been identified in athletes, with similar trends being shown. One meta-analysis observing 23 studies (2,313 athletes), found that 56% had vitamin D inadequacy (Farrokhyar et al., 2017). They found

that risk was significantly higher for $>40^{\circ}\text{N}$ latitudes. In two studies at latitude 41°N and 43°N , 94-100% of wrestlers reported 25(OH)D insufficiency during winter months (Barcal et al., 2016; Fikratkerimov et al., 2019). As previously identified however, definitions of insufficiency differ, so some findings should be taken with caution. As an example, two of these studies reported insufficiency as 25(OH)D $<80\text{ nmol}\cdot\text{L}^{-1}$ (Barcal et al., 2016; Farrokhyar et al., 2017), and the other reported $<75\text{ nmol}\cdot\text{L}^{-1}$ (Fikratkerimov et al., 2019). Saying that, lower 25(OH)D levels have been reported in trained athletes, compared to sedentary controls (Jerome et al., 2017); stressing the potential importance of supplementation for athletes. Despite the variation in guidelines, risk of vitamin D insufficiency in athletes is evident, especially for those who train indoors (Constantini et al., 2010), and in winter (Lin et al., 2021; Scullion et al., 2018). With the high prevalence of vitamin D insufficient athletes and its positive influence on URI risk, it comes as no surprise that regular supplementation has been advised by the UK government, and sport governing bodies (PHE, 2019; AIS, 2021).

2.9 Supplementation of Vitamin D

The European Food Safety Authority (EFSA, 2012; EFSA, 2015) have recommended safe guidelines of oral D_3 supplementation, which increases serum 25(OH) without risk of sunburn. The recommended daily allowance (RDA) of dietary vitamin D for adults ranges between 400-600 IU (Walsh, 2019). Recommendations are based upon skeletal health benefits, so previously it has been questioned whether daily 400-600 IU vitamin D supplementation is enough to provide immune benefits. It would be important to note, a EFSA upper tolerable limit guideline was identified (4,000 IU), because risk of toxicity has been found in doses exceeding 10,000 IU (EFSA, 2012).

Most recently, interest in vitamin D supplementation for health has grown, due to the emergence of positive findings on reducing coronavirus disease 2019 (COVID-19) symptoms (Lanham-New et al., 2020). It has been suggested that supplementation of vitamin D may reduce COVID-19 symptoms due to the previously described anti-inflammatory properties (Laird et al., 2020). Thus, UK government guidance now suggests that everyone should consider a daily vitamin D supplement of 400 IU,

especially during winter months (PHE, 2019). So much so, that free vitamin D supplements were provided in England to COVID-19 'at-risk' groups during the pandemic; 900,000 supplements were given to vulnerable with limited access to the outdoors. Alongside this, recommendations for athletes have been made by sport governing bodies (AIS, 2021).

2.9.1 Effect on URI

Large scale meta-analyses conducted in the general population have reported Vitamin D supplementation to have a protective effect against illness, reducing the risk of experiencing URI (Bergman et al., 2013; Jolliffe et al., 2021; Martineau et al., 2017). Despite this, findings in healthy individuals that partake in regular exercise, such as military recruits and athletes, present conflicting results. Some randomised control trials (RCTs) have shown no difference in number of illnesses following supplementation in swimmers (Dubnov-Raz et al., 2015; Lewis et al., 2013). Whereas Jung et al. (2018) reported a reduction in URS episodes in Taekwondo athletes, following supplementation of vitamin D. Recently however, it has recently been proposed that supplementation may reduce burden of URI rather than prevent incidence (Harrison et al., 2021).

Interestingly, Cannell and Hollis (2008) proposed that vitamin D supplementation (daily dose of 2,000 IU for three days) may produce enough cathelicidin to cure URI, influenza and the common cold. When examining military recruits, Harrison et al. (2021) found that vitamin D supplementation in winter enabled $\geq 95\%$ of individuals to become 25(OH)D sufficient. Alongside this, participants reported reduced duration of illness and lower peak severity of symptoms, when compared with a placebo group. This was in agreement with findings in athletes, which found reduced lower number of URS days (Da Boit et al., 2015) and reduced symptom severity scores, following supplementation of vitamin D (Jung et al., 2018). Possible mechanisms include the fact that vitamin D could limit inflammation, thus reducing symptoms severity and duration (Walsh, 2019). With this in mind, maintaining vitamin D sufficiency with supplementation could reduce number of lost training days due to URI (Laaksi et al., 2010). Saying that, other research has shown no change in duration or severity of symptoms (Dubnov-Raz et al., 2015), causing difficulty

to draw conclusions on the influence supplementation may have on URI in active populations.

2.9.2 Effect on Mucosal Immunity

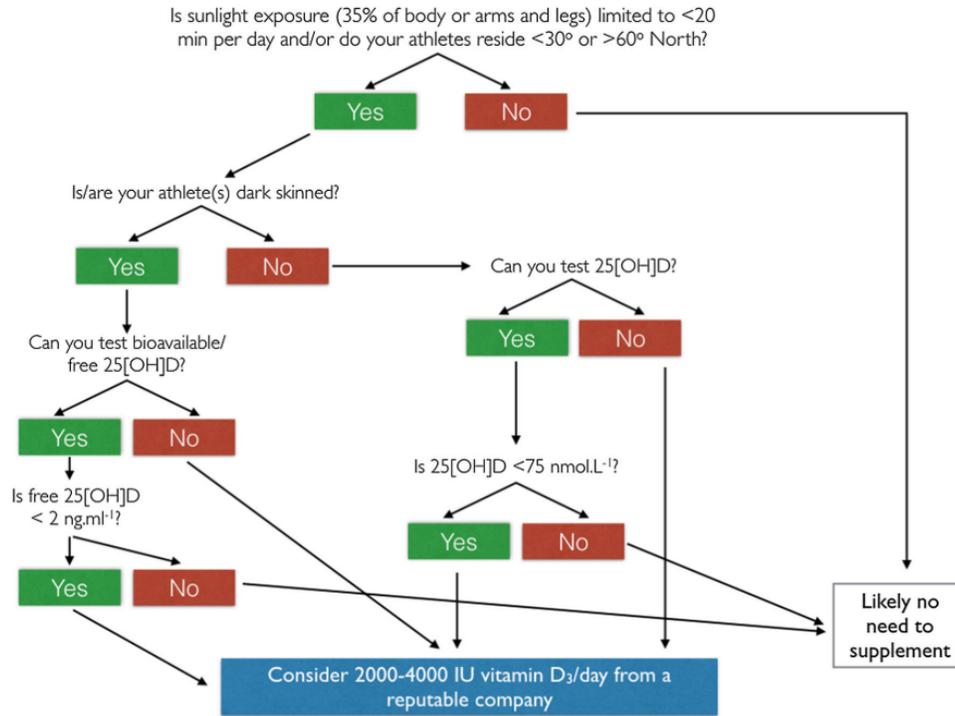
Some improvements in mucosal immunity have been shown in military recruits (Scott et al., 2019) and athletes (He et al., 2016), following vitamin D supplementation. Both studies found that supplementation of vitamin D was associated with increased sIgA secretion rates (He et al., 2016; Scott et al., 2019). In addition, vitamin D has been suggested to facilitate production of the human cathelicidin (Todd et al., 2015). Therefore, this AMP has been shown to be correlated with vitamin D status, with reported increases following vitamin D supplementation (Bhan et al., 2011; He et al., 2016). It should be mentioned however, Scott et al. (2019) found a small increase in sIgA secretion at week 4 only, during the most stressful period of military training. They found no influence on sIgA at any other time points, plus no change in cathelicidin at any time between supplementation groups (Scott et al., 2019). This was compared to He et al. (2016), whom supplemented male athletes with 5000IU of vitamin D, which is above the tolerable upper limit advised (EFSA, 2012). One possible explanation for this could have been the difference in vitamin D dosage (1000IU vs 5000IU); perhaps lower doses were not sufficient to promote more prominent effects on mucosal markers, because not only was sIgA secretion significantly higher following supplementation by He et al. (2016), but also cathelicidin secretion.

Despite the findings above, supplementation of vitamin D has also shown no change in mucosal immunity in both military recruits (Harrison et al., 2021) and athletes (Jung et al., 2018). Despite supplementation of 5000IU for 4-weeks during winter training, no change in sIgA concentration was found (Jung et al., 2018). Previously though, measurement of sIgA concentration alone has been criticised due to the lack of consideration for flow rate, making comparisons more difficult. Another study conducted in military recruits additionally found no change in sIgA or cathelicidin secretion rates (Harrison et al., 2021). Authors concluded that the lack of influence found for mucosal immunity, supported the notion that supplementation would not reduce incidence of URI but instead reduce burden (decreased duration and severity due to inflammation effects) (Harrison et al., 2021).

Importantly, it has been found that protective effects from vitamin D supplementation against illness were stronger in those with baseline deficiency (Martineau et al., 2017). Specifically, supplementation had more of an effect for individuals with serum 25(OH)D <50 nmol/L (Harrison et al., 2021). Therefore, differences in study findings could be due to baseline 25(OH)D concentrations and should be considered when comparing findings. Due to this finding, 'blanket' supplementation of vitamin D has been recently questioned. In a recent review by Owens et al. (2018), a decision tree was made to encourage effective and safe supplementation for those dealing with a broad range of athletes (Figure 2.6). Despite promising results conducted within the general population, the effect of vitamin D supplementation on URI and mucosal immunity for athletes remains unclear. Therefore, more well-designed studies are to confirm the effectiveness of vitamin D in prevention of URI and the effect on mucosal immunity and inflammatory cytokines, in athletes (Cicchella et al., 2021; Walsh, 2019).

Figure 2.6

Decision tree for athletic supplementation of vitamin D (D. J Owens et al., 2018)



2.10 Thesis Aims

A variety of factors influence mucosal immunity and subsequent URI, but research examining the influence of these in elite swimmers is lacking. With this in mind, the overall thesis aims were: 1) to monitor mucosal immunity via sIgA and URS, alongside training load, EBV status and sleep parameters to explore potential relationships in elite swimmers (**Chapter 4**); 2) to develop and optimise an in-house ELISA for the detection of sIgA (**Chapter 5**); 3) investigate the effect of vitamin D supplementation on immune markers and URI in athletes, via a meta-analysis and systematic review (**Chapter 6**).

CHAPTER 3

General Methods

This chapter will outline common methodology used within the current thesis and serves to function as a reference for **Chapter 4** and **Chapter 5**. Other more specific methods can be found in the relevant chapters.

3.1 Ethical Approval

Ethical approval was granted by The University of Hertfordshire, Health Science Engineering & Technology ECDA (Ethics protocol number: aLMS/PGR/UH/02940(1,2,3)).

3.2 Participant Recruitment

Study one (**Chapter 4**) recruited male and female national and international level swimmers, from the Loughborough University team. Development of an in-house ELISA assay in **Chapter 5**, required recruitment of diagnosed IgA-deficient male and female participants. Participants were recruited through methods including verbal informing, hand-outs (**Chapter 4**) and research advertisement on an immune deficiency Facebook page (**Chapter 5**). Following ethical approval, participants received written and verbal instructions via an information sheet, about what the study would entail and were made aware of the risks and benefits of participating. Prior to commencing each study, participants provided written informed consent and completed health screens.

3.3 Saliva Collection and Storage

Unstimulated saliva was collected using a passive drool method (Hanstock et al., 2016). All participants were instructed to passively drool, head tilted forwards, into a saliva test tube. Saliva samples were collected at least 30 minutes after eating, drinking, or tooth brushing.

In **Chapter 4**, saliva samples were collected weekly over the 8-month period in standardised conditions, every Wednesday at 12:00 hours to control for effects known to alter sIgA (Chapter 2, Figure 2.5). On Wednesdays, swimmers did not partake in morning training and had the longest rest time between sessions. Swimmers were asked to passively drool for three minutes; however, if sufficient saliva was collected, participants were asked to drool for an extra two minutes and record total time. Samples were transported in a triple sealed box on ice, back to the University of Hertfordshire. For IgA deficient participants (**Chapter 5**), saliva sample pots were sent via post with instructions (Appendix A) for saliva collection and storage before first class postal return to the University of Hertfordshire. Samples were taken in the afternoon (pm), on a day they had completed no exercise. Participants were asked to aim to fill the tube with 10ml of saliva (lines are indicated on the sample pot). No timing was needed for this, due to the nature of the requirement for sample. As soon as samples had been collected, they were immediately put on ice packs provided, for postal transport. All samples were double centrifuged at $4000 \text{ rev}\cdot\text{min}^{-1}$ for 10 minutes (Sorvall ST 8R, Thermo Fisher Scientific, USA), to make acellular and remove supernatant. Once the supernatant had been removed, samples were made into $300 \mu\text{L}$ aliquots and stored at -80°C until analysis.

3.3.1 Saliva Flow Rate

A sample collection time limit was used (**Chapter 4**), so that saliva flow rate could be calculated and accounted for in later analysis. Saliva flow rate ($\text{mL}\cdot\text{min}^{-1}$) was calculated by dividing the volume of saliva by the collection time. The density of saliva was assumed to be $1.0 \text{ g}\cdot\text{mL}^{-1}$ (Cole & Eastoe, 1988). Secretion rate ($\mu\text{g}\cdot\text{min}^{-1}$) of sIgA was then determined by multiplying IgA concentration by the saliva flow rate (Li et al., 2009).

3.4 Commercial ELISA

After thawing, samples were centrifuged for five minutes at $3000 \text{ rev}\cdot\text{min}^{-1}$. Samples were run on a commercially available Secretory IgA ELISA Kit (Salimetrics LLC, Irvine, CA). Following manufacturer instructions, all samples were analysed in duplicate, with an individual's 8-months of saliva run on a single plate to avoid inter-assay variation. Ready-

made standards (concentrations: 0, 2.5, 7.4, 22.2, 66.7, 200, 600 $\mu\text{g mL}^{-1}$) were used to create a seven-point standard calibration curve. Specifically, the commercial ELISA followed the 'competitive assay' methods, where standards, controls and samples were added to pre-coated wells. After the addition of goat anti-human IgA conjugated to horseradish peroxidase (HRP) and substrate solution (TMB), colour change was analysed on a plate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, USA) at 450nm, to obtain the optical density of each well. Absorbance values of each well could then be analysed; the zero-absorbance value was subtracted from the unknown samples' absorbance value and concentration was then interpolated from the calibration curve using a 4-parameter logistic curve method (GraphPad Prism, version 8). Inter-assay CV was 8%, Intra-assay CV was 2%.

3.5 General Statistical Analysis

All data was compiled using Microsoft Excel and evaluated using both SPSS software (version 26.0; SPSS Lead Technologies Inc, Chicago, IL) and GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). Data is presented as mean values and standard deviation (mean \pm SD). The Shapiro-Wilk test was used to check the normal distribution of all variables, before choosing the appropriate parametric or nonparametric test for analysis. In the event of non-normally distributed data; analysis was sometimes performed on logarithmic transformed data, and analysis of variance (ANOVA) was still conducted, as the model is sufficiently robust to detect statistically significant differences between means with type 1 error (Blanca et al., 2017). Where appropriate, data was also checked for homogeneity of variance and sphericity before statistical analysis. Significance was accepted at the $p \leq .05$ level.

CHAPTER 4

Immune Monitoring Alongside Factors Associated with Upper Respiratory Illness in Elite Swimmers, Over an 8-month Training Period

4.1 Introduction

The majority of research suggests that intensified training leads to a greater degree of immune suppression, and increased risk for URI and fatigue (Peake et al., 2017). Thus, most illnesses occur during the heaviest training periods (Rama et al., 2013; Spence et al., 2007). In addition to this, following a four year prospective study, higher incidence of URI was found for swimmers when training in winter (Hellard et al., 2015). Dependent on the year, the British Swimming Championships or the Commonwealth Games usually fall in April, meaning that high volumes and intensity of training occur throughout winter months, which would further accentuate risk of URI. Despite the differences in aetiology discussed, presentation of URS in athletes negatively effects training and subsequent sporting performance (Colbey et al., 2018). Plus, the most frequent reason for elite athletes to visit a sports medicine clinic which was non-injury related, was URI (Fricker et al., 2000; Gleeson et al., 2017). The high and intense training volumes necessary to compete successfully at an elite level, can only be achieved with fewer illness symptoms (Mårtensson et al., 2014). It should be mentioned, given the uncertainty regarding the aetiology of URI and the inability to clinically determine cause for the current study, it has been favoured to report URS instead of URI or URTI (Walsh & Oliver, 2016), and therefore URS will be reported in this chapter.

Research investigating use of sIgA as an illness monitoring biomarker and the relationship with URS has previously been discussed in Chapter 2 (page 22). Overall, increased incidence of URS has been identified with low levels of sIgA (one laboratory defined as $<40 \mu\text{g}\cdot\text{mL}^{-1}$), decreased sIgA secretion rate, and a decline in sIgA

concentration over a training period (Gleeson et al., 1995; Gleeson et al., 1999; Ihalainen et al., 2016; Nieman et al., 2006). Specifically, depressed sIgA and increased incidence of URS has been shown in swimmers (Gleeson et al., 1995; Hellard et al., 2015). Despite some conflict on this (Papadopoulos et al., 2014; Pyne et al., 2001), a recent systematic review by Rico-González et al. (2021) examined 23 studies and confirmed that a low level of sIgA (concentration and secretion rates) was correlated with higher incidence of URI. While some longitudinal studies have identified periods of intense training to decrease sIgA concentration and secretion rate with increased risk of URS (Mårtensson et al., 2014; Walsh et al., 2011), others have not (Novas et al., 2003; Pyne et al., 2001). Therefore, there is no clear consensus and mixed results could be reflective of the large within- and between-subject variation found with sIgA (Chapter 2, page 25). Specifically, within-subject variability seen in elite athletes (Dwyer et al., 2010; Francis et al., 2005), may indicate that some athletes are more susceptible to stress and illness than others (Francis et al., 2005; Neville et al., 2008a); highlighting the need for individual monitoring. Typically though, sources of heterogeneity for sIgA may include diurnal variation, environmental factors, dietary intake, stressors such as exercise, and illness status (Francis et al., 2005; Gleeson, 2000; Salimetrics, 2015). Some research failed to observe an association between IgA and URS due to lack of strict control of these factors.

Overall, the major concern for coaches and athletes are the accompanying illness symptoms that can limit training and prevent successful competition (Fricker et al., 1999; Reid et al., 2004). Therefore, Spence et al., (2007) suggested further study was required to uncover causes of unidentified URS and risk factors in athletes. Epstein Barr Virus was identified as one of the most likely causes of URS (Reid et al., 2004), suggesting viral reactivation rather than primary infection (Chapter 2, page 20). Gleeson et al. (2002) reported a significant relationship between EBV seropositivity and URS. Therefore, identification of at-risk athletes via serum testing indicative of present or past infection with EBV, would be an important marker to examine.

An association has been found between short sleep duration (< 7 hours) and increased number of illnesses, including cold and flu (Cohen et al., 2009; Orzech et al., 2014). In

addition, it has been suggested that athletes may need more than the national recommendation (NR) of sleep to adequately recover and have optimum health (Calder, 2003). These findings are empirical because athletes do not obtain a sufficient amount of sleep, regularly sleeping less than the NR of 7-9 hours of sleep per night (Leeder et al., 2012; Sargent et al., 2014; Sargent et al., 2014). Of a large group of mixed sport athletes, 60% were reported to sleep below the NR (Sargent et al., 2014), with swimmers sleeping an average of only 5 hours and 24 minutes on nights prior to training (Sargent et al., 2014). The cause of sleep disturbance for athletes were reportedly due to three main factors; training, competition and travel (Gupta et al., 2017). Specifically, early morning training and increases in training load have shown to be increased risk factors for impaired sleep. Competitive swimming commonly implements early morning training and high training loads, it could be argued that swimmers especially, are at increased risk of sleep inadequacy. Furthermore, inadequate sleep has been reported leading into competition (Erlacher et al., 2011). Therefore, there is a growing urgency to understand sleep patterns in elite athletes (Sargent et al., 2014a; Sargent et al., 2014b). Due to the impact that sleep could have on immune function, it was deemed important to analyse sleep parameters over three different training loads, plus into competition, in the current study.

For some athletes especially, there appears to be a dose-response relationship between risk of URI and training load, and therefore would warrant further investigation to potentially identify those at increased risk and explore factors that influence this. Monitoring an individual's sIgA secretion rate and changes in sIgA concentration throughout a training programme, could help identify athletes at risk of URS and predict number of illnesses (Gleeson & Pyne, 2016). Despite not finding an association between lowered sIgA and illness, Pyne et al., (2001) supported this idea of routine assessment of mucosal immunity in athletes during periods of physical and psychological stress, to reduce the risk and impact of URS. Through monitoring sleep via self-reported questionnaires plus wrist-worn actigraphy alongside these factors, data could be used to determine any trends for the current swimmers. Furthermore, despite being anecdotal, the British Swimming Physiologist questioned whether a small drop in body mass could be a potential early warning sign for illness in female swimmers. Previously, 5% weight

loss for competition influenced a reduction in immunoglobulins, impaired cell-mediated immune function and increased susceptibility to URS in wrestling and judo athletes (Hiraoka et al., 2019; Shimizu et al., 2011). That said, elsewhere no differences in immunological function has been found between energy availability (EA) groups (Ackerman et al., 2018). To explore this, body mass of all athletes was also monitored throughout the study to examine whether any association with illness existed. Overall, many factors could impact illness risk such as depression, anxiety, international travel, academic stress, lack of sleep and low diet energy intake (Nieman & Wentz, 2019); these were considered for the current study due to athletes also being university students. Consequently, due to the high demands on elite swimmers, both physiologically and psychologically, rationale for the current study is provided.

4.1.1 Study Aims

The aims of the present research were to carry out individual athlete immune monitoring and identify potential risk factors for URS in elite swimmers. Specifically, the study was designed to investigate whether there was a link between sIgA concentration, secretion rates and incidence of illness symptoms, alongside a) training volume, b) sleep quantity/quality, and c) EBV serostatus (Table 4.1).

Table 4.1

Study aims

	Aims
1	To investigate the effect of training load on incidence and severity of URS.
2	To determine whether a relationship exists between sIgA and URS.
3	To investigate the effect of training load on sIgA.
4	To determine whether a relationship exists between EBV serostatus, and incidence and severity of URS.
5	To investigate whether swimmers meet the national recommendation of 7-9 hours' sleep per night.
6	To investigate the effect of training load and competition on sleep parameters.
7	To determine whether changes in sleep parameters effect URS.
8	To investigate whether changes in female body mass would increase incidence of URS.

4.2 Methods

4.2.1 Participants

Twenty-three elite national or international swimmers were recruited, from Loughborough University Performance sprint and middle-distance/distance squads. Nine swimmers did not complete the study; five swimmers did not train on the chosen analysis day due to university commitments, and four swimmers retired before the commencement of the study. Therefore, fourteen swimmers were included in the final analysis. The current study investigated two differing training groups: the sprint group (Male, $n = 2$) and the distance group (Male, $n = 7$). Participant characteristics can be seen in Table 4.2.

Table 4.2

Participant characteristics

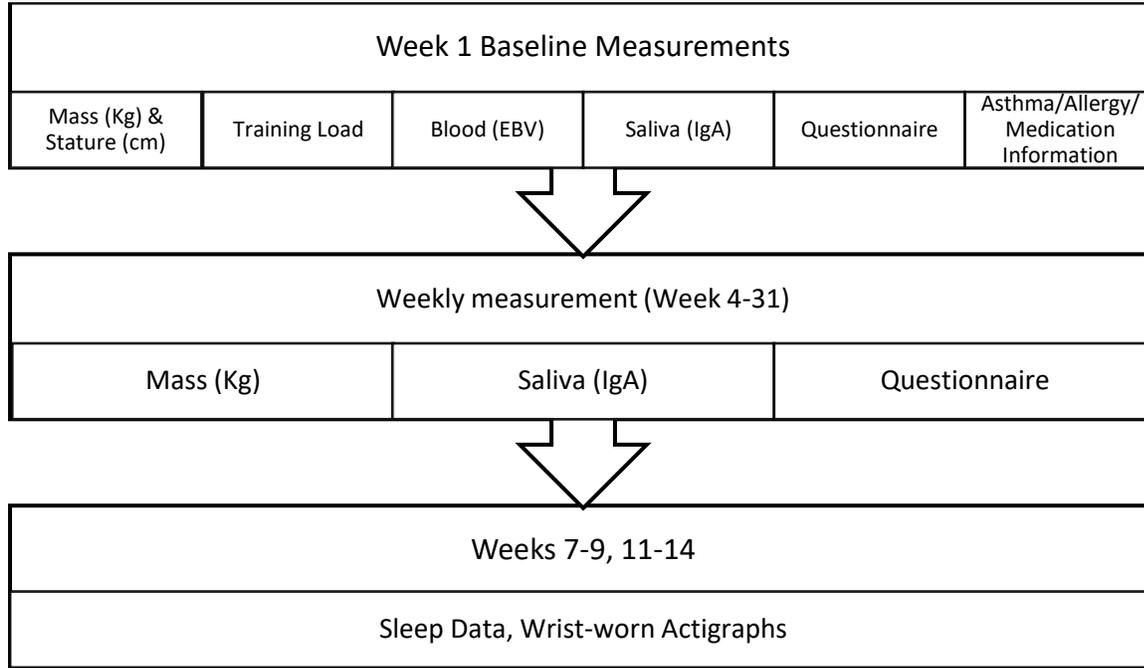
	Sex		Training Group	
	Male ($n = 9$)	Female ($n = 5$)	Sprint ($n = 6$)	Distance ($n = 8$)
Age (years)	20.1 ± 0.7	19.6 ± 0.8	19.8 ± 0.9	20.0 ± 0.7
Height (cm)	180 ± 7	176 ± 4	179 ± 6	179 ± 6
Body Mass (kg)	79 ± 6	69 ± 7	72 ± 9	77 ± 6

Note. Swimmer characteristics ($n = 14$), broken down by both sex and training group.

Study inclusion criteria included being a member of the sprint or distance swimming performance squads at Loughborough University for the whole swim season (August-April). Participants were asked to consider their health or injury status and were only included in the study if they had injuries they were capable training with. Furthermore, subjects were included in the study if they had a cold, flu, or sinus infection; due to the focus being to observe URS over the season. Figure 4.1 represents the measurements taken, and the timing during the 8-month surveillance study.

Figure 4.1

Study design diagram showing what measurements were taken and when



4.2.2 Baseline Measurements

In week 1, several measurements and samples were collected for baseline data. Each athlete completed a health screen recording history of medication and known cases of asthma or allergies. A stadiometer (Seca GmbH, Hamburg, Germany) and portable scales (Seca GmbH, Hamburg, Germany) were used to measure height and body mass. Additionally, blood samples were taken via venepuncture before the commencement of the study to determine Epstein Barr Virus (EBV) status.

4.2.3 Blood Collection and Storage

Participants attended a laboratory at Loughborough University before training, to provide a blood sample for detection of EBV. Whole blood samples were collected from consenting participants by standard venepuncture, from an antecubital vein. All samples were taken with butterfly needles, and 4 mL of blood was collected into evacuated collection tubes for serum (Vacurette, Greiner Bio-one, Kremsmunster, Austria). Serum

tubes were left for 30 minutes to allow for clot formation (21°C) (Tuck et al., 2010). Following clotting, samples were centrifuged at 4000 rev.min⁻¹ for 5 minutes. Samples were then stored on ice packs for transportation back to Hertfordshire, within 4 hours prior to freezing (Tuck et al., 2010). Subsequently, 0.5 mL serum aliquots were stored in labelled cryovials to reduce impact of freeze-thaw cycles, and frozen at -80°C for analysis.

4.2.3.1 EBV Serology

The detection of IgM and IgG antibodies for EBV viral-capsid antigen (VCA) and IgG antibodies for EBV nuclear antigen (EBNA) was completed by three separate Human ELISA kits (AbCam, Cambridge, UK). Following manufacturer instructions, all samples were analysed in triplicate on a single plate to avoid inter-assay variation. Intra-assay CV was 2%.

4.2.4 Salivary IgA Sampling and Analysis

Method of saliva collection and storage, plus analysis of sIgA via ELISA has been previously explained (Chapter 3, page 53).

4.2.5 Training Load

Training load was reported using two methods. Firstly, the training data for both swim squads was produced and provided by the coaches at Loughborough. This highlighted the periodised plan of load for each week, and was classified by the coaches as low, moderate, and high, so this was used for analysis. Athlete's weekly training load and attendance was monitored by the coaches at Loughborough University.

This was additionally observed alongside the swimmers' session ratings of perceived exertion (RPE) method of quantifying training load, developed by Foster (1998). This was because the swimmer may perceive training load differently depending on how they are feeling. This method involves multiplying RPE (intensity) by volume (Halson, 2014b); but as duration of training was always the same for swimmers, volume was taken as in

kilometres swum that week. Thus, training load was produced by multiplying the perceived intensity of swim training by the distance swum that week. Perceived intensity of training each week was identified using a rating of validated RPE scale. The OMNI and Borg CR10 scale are reliable and valid for use (Lagally & Robertson, 2006), as most elite athletes are familiar with this type of grading. Thus, for the current study, the 1 to 10 scale was used due to its simplicity of use (i.e., 1 = 'very low training intensity'; 10 = 'very high training intensity'). To determine whether there was any difference between the training load derived by the coaches and the swimmers, swimmer training loads were split into three groups; arbitrary units (a.u) were 0-200 (low), 200-400 (moderate), and 400+ (high). Then, correlational analysis was carried out to determine whether swimmer-derived training load could be compared to the training loads derived by the coaches.

4.2.6 Illness Symptoms Questionnaire

Swimmers were observed for 8 months using weekly reporting of training, sleep and injury/illness symptoms using an adaption of the Australian Institute of Sport (AIS) monthly illness log form, obtained from Professor Maree Gleeson. The weekly illness questionnaire (Appendix B) adapted and used elsewhere (Fricker et al., 2005) was used to collect data on illness symptoms, injuries, perceived intensity of both pool and land training, perceived quality, and quantity of sleep, which were all self-reported on a seven-day recall basis.

The type, severity and duration of illness were calculated using data collected from the questionnaires. The presence of perceived weekly symptoms was recorded according to their type and severity (1 = no change in training program, 2 = training program modified, 3 = complete cessation of training). An illness was defined as reporting URS on 2 or more consecutive days, or when the severity was rated highly enough to modify training (moderate-severe) (Fricker et al., 2005). For any subsequent episode to be classified as a new URS episode, there needed to be an asymptomatic period of ≥ 7 days (Spence et al., 2007). Symptoms separated by less than seven days were regarded as a recurrence or continuation of the initial illness episode. For every recorded URS episode, a total symptom severity score was calculated by multiplying the sum of symptom duration in

days, by the peak severity. Injuries were noted by the participants but not included in this analysis.

4.2.7 Sleep Analysis

Within the illness questionnaire, participant's perceived sleep quality and quantity was additionally monitored weekly. Firstly, participants were asked to rank their sleep quality on a scale of 1-10. In addition, participants were asked how many times they thought they had met the government recommendation of 7-9 hours each night and how many times had they woken up feeling fatigued.

To assess sleep-wake patterns, a wrist-worn activity monitor GT3X+ (ActiGraph, Florida, USA) was worn around the non-dominant wrist during night-time and nap sleep periods only. This was removed when participants arose from bed in the morning, or after napping. Instructions were given to participants before use (Appendix C). Parameters recorded included latency (mins), sleep efficiency (SE; %), total time in bed (TTIB; min), total sleep time (TST; min), wake after sleep onset (WASO; min), number of awakenings, and average time awake (min). These sleep patterns were monitored at three different times during the 8-month observation, to ensure that differing stages of training load (low, moderate, high) and a period of competition were examined. Individual sleep data reports were given to participants, see Appendix D as an example. The time when participants entered and finished a sleep period (nap or night-time sleep) was also recorded manually by participants, to ensure it corresponded with the time identified by the ActiGraph software during final analysis.

4.2.8 Statistical Analysis

All data was compiled using Microsoft Excel and evaluated using both SPSS software (version 26.0; SPSS Lead Technologies Inc, Chicago, IL) and GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). General statistical analysis has previously been discussed (Chapter 3, page 55). Missing values in these large data sets were input with the method Last Observation Carried Forward (LOCF) or Next Observation Carried

Backward (NOCB), which is commonly used for time series data. Normality was assessed using a Shapiro-Wilk test; upon violation of the normality assumption, analysis of variance (ANOVA) was still conducted as the model is sufficiently robust to detect statistically significant differences between means, with type 1 error (Blanca et al., 2017). Repeated measures ANOVA with post-hoc Bonferroni adjustment measured differences in number of URS episodes, symptom severity, symptom duration, missed training days, sIgA concentration and secretion rate, and sleep parameters between low, moderate, and high training loads. Greenhouse-Geisser correction was applied upon violation of Mauchly's test of sphericity for ANOVAs ($p < .05$). Mixed model ANOVA was used to determine differences in these outcomes, between training group, sex and EBV serostatus. Partial Eta-Squared (η^2_{partial}) was used to report effect sizes for ANOVA where effects were classified as small (0.01-0.08), moderate (0.09-0.25) and large (>0.25) (Cohen, 1988). A Wilcoxon signed-rank test was used upon violation of the normality assumption, to explore differences in sIgA concentration and secretion, between present URS and non-URS conditions. A paired-samples t-test was used to identify differences between individual sleep data between two different time points for competition. All results were presented as mean (\pm SD), and significance was accepted at the $p \leq .05$ level.

4.2.8.1 Individual Analysis

Due to the large variation found with IgA concentration between individuals, individual analysis was carried out. This was used to determine whether there were any visual relationships between IgA concentration, illness score, training load and sleep for each individual. By looking at individual data, patterns of sIgA could be assessed; for each swimmer, weekly sIgA concentration was subtracted from their overall 8-month healthy average, to give the amount that their weekly concentration was above or below. This indicated when sIgA concentrations were higher or lower than the healthy average sIgA concentration during the training season, for each individual. Additionally, sIgA values were normalised to each individual's mean, so relative sIgA percentage change could be observed over time.

4.3 Results

4.3.1 Training Load

A higher training distance was observed in swimmers in the middle-long distance group compared to the sprint group ($p = .001$). Average weekly training distance, minimum and maximum distances, plus weeks spent at each training load, can be seen for both training groups in Table 4.3. When reporting minimum training distances, the week before or of competition were not included as this was taper. Maximum training distances for both groups, were swum during a high intensity week. Over the whole season, it can be seen that the maximum weekly training distance recorded was 77 km, by a swimmer from the middle-long distance group.

Table 4.3

Average, minimum and maximum weekly training distances, plus weeks spent at each training load, for both training groups.

Training Group	Average Weekly Training Distance (km)	Minimum Training Distance (km)	Maximum Training Distance (km)	Weeks Spent at Each Training Load (%)		
				Low	Moderate	High
Sprint	29 ± 8	20	51	32	20	48
Distance	41 ± 11*	29	77	25	32	43

Note. Average, minimum and maximum weekly training distances, plus weeks spent at each training load, for both sprint and distance groups. *Denotes significance for training distance between the two training groups.

In the sprint group, the average swimmer-derived training load was 202 ± 74 (MIN = 10, MAX = 510), compared to the middle-long distance group which reported an average 253 ± 74 (MIN = 26, MAX = 693). There was no significant difference in mean training load between the groups ($p \geq .05$). Pearson correlational analysis showed there was a moderate, positive correlation between the coach derived training loads and swimmer's perceived training load, which was statistically significant ($r_{(34)} = .484$, $p = .003$). All swimmers competed in a total of 13 events during the season; however, they had two

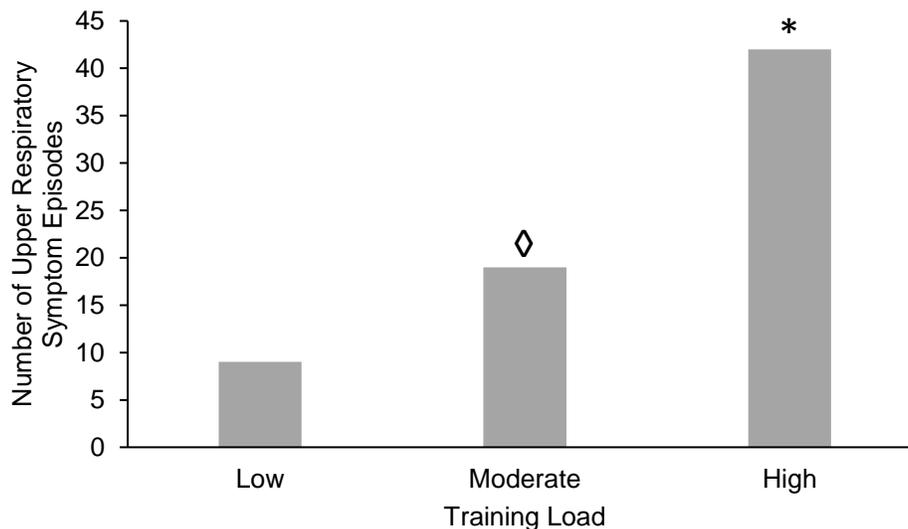
main target meets which they tapered for. These were the ASA Short course Winter Championships in week 16, then either the Commonwealth Games in week 32, or the Eindhoven Swim Cup in week 33.

4.3.2 Incidence, Duration and Severity of URS

Over the course of the 8-month observation, a total of 70 URS episodes were recorded for all swimmers. An average of five URS episodes was reported for each swimmer, ranging from no reported ($n = 1$), to 8 reported episodes. Through use of a repeated measures ANOVA (sphericity assumed), number of URS episodes reported by swimmers (Figure 4.2), showed a significant difference between training loads ($F_{(2,26)} = 19.626$; $p < .001$, $\eta^2_{\text{partial}} = .602$). A total of 60% of URS were reported by swimmers during high training loads. *Post hoc* analysis showed significantly higher number of URS from low to high training load ($< .001$), and from moderate to high training load ($p = .011$).

Figure 4.2

Total number of URS episodes over different training loads

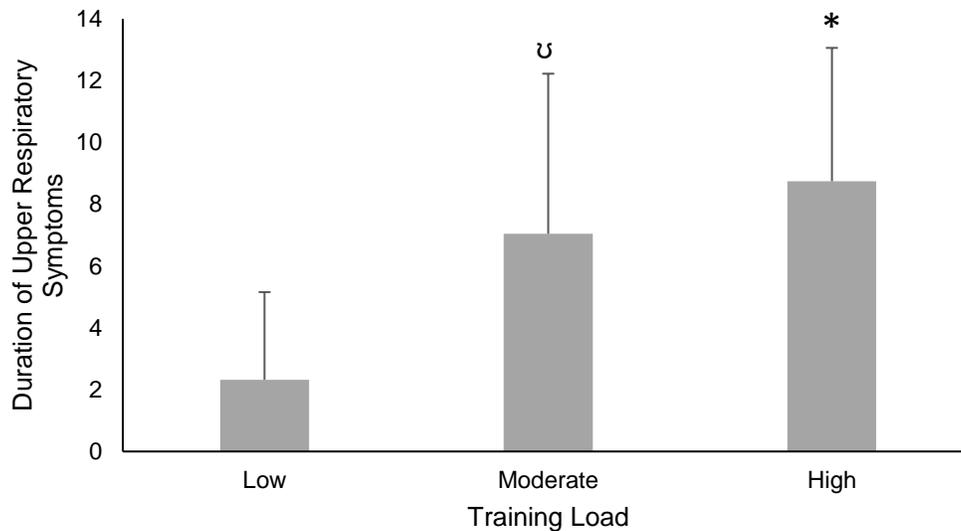


Note. Total number of URS episodes recorded by all swimmers ($n = 14$), during low, moderate, and high intensity training phases. ◇Denotes significance for number of URS episodes between moderate and high intensity training weeks. *Denotes significance for number of URS episodes between low and high intensity training weeks.

Overall average duration of URS episodes was eight days for all swimmers (range 2-28 days). Average duration of URS episodes was 2 ± 3 , 7 ± 5 , 9 ± 4 days for low, moderate, and high training loads retrospectively. Duration of symptoms was statistically significant between training loads ($F_{(2,26)} = 14.241$; $p < .001$, $\eta^2_{\text{partial}} = .523$), showing significantly longer symptom duration during moderate ($p = .021$) and high training loads (< 0.001), compared to low (Figure 4.3). There were 34 days of missed training due to reported URS, from nine swimmers (range 1-11 days for each swimmer). Despite no statistical difference between training loads ($F_{(1.132,14.717)} = 3.985$; $p = .061$, $\eta^2_{\text{partial}} = .235$), 76% of missed training days due to URS were during high training loads (Figure 4.4).

Figure 4.3

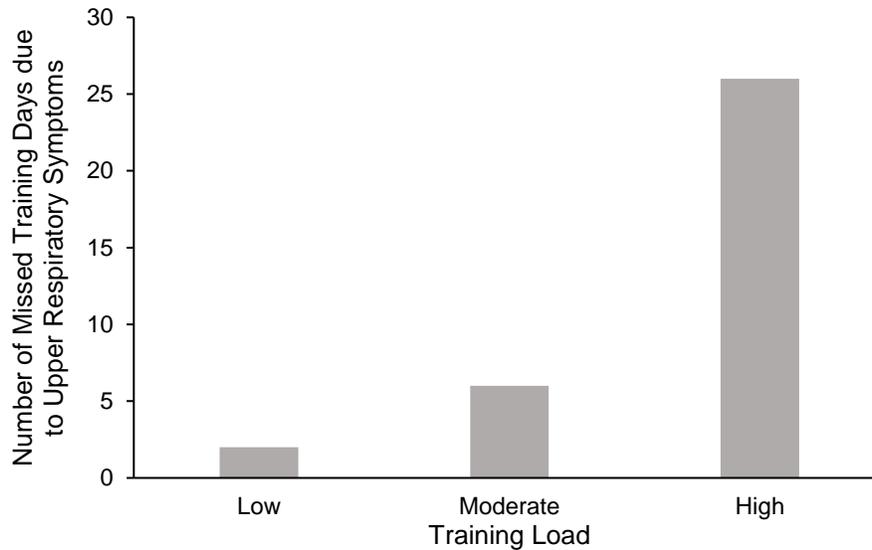
Mean duration of URS episodes over different training loads



Note. Average duration of URS episodes recorded by all swimmers ($n = 14$), during low, moderate, and high intensity training phases. u Denotes significance for duration of symptoms between low and moderate intensity training weeks. *Denotes significance for duration of symptoms between low and high intensity training weeks.

Figure 4.4

Total number of missed training days over different training loads

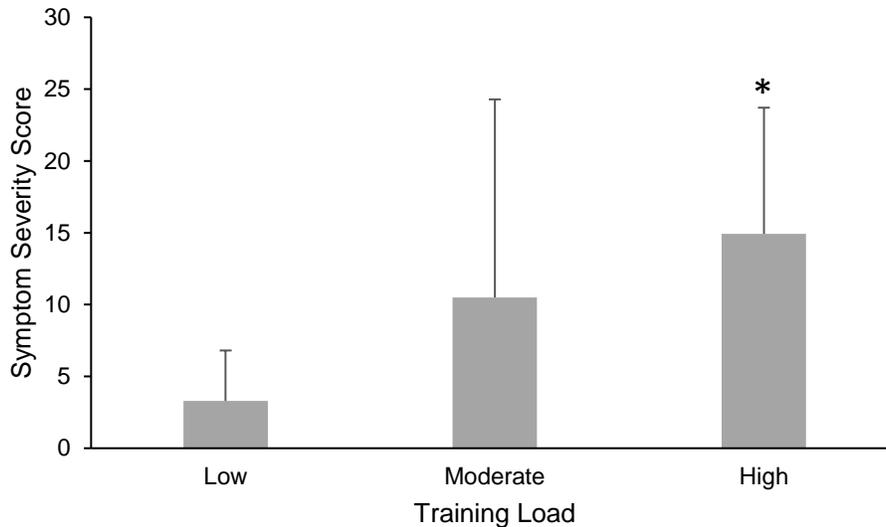


Note. Total number of missed training days due to reported URS, by swimmers ($n = 9$), during low, moderate, and high intensity training phases.

Average symptom severity score for low, moderate, and high training phases were 3 ± 4 , 11 ± 14 and 15 ± 9 respectively (Figure 4.5). Over the observed period, scores ranged from 2 to 63, with an average of 10. Through use of a repeated measures ANOVA with Greenhouse-Geisser correction, reported average symptom severity score demonstrated a significant interaction effect between differing training loads ($F_{(1.429, 18.581)} = 5.420$; $p = .022$, $\eta^2_{\text{partial}} = .294$). *Post hoc* analysis indicated a significantly higher symptom severity score for high training loads, compared to low ($p = .001$).

Figure 4.5

Mean symptom severity score over different training loads



Note. Symptom severity score recorded by all swimmers ($n = 14$), during low, moderate, and high intensity training phases. *Denotes significance for reported symptom severity scores between low and high intensity training weeks.

4.3.3 Salivary IgA

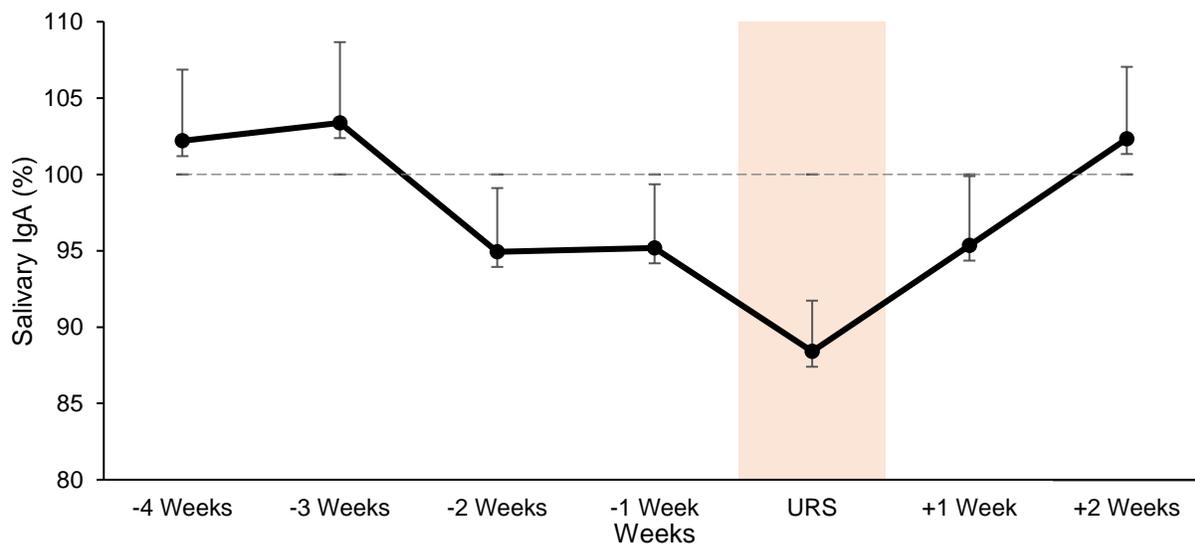
Over the 8-month study, a total of 392 saliva samples were analysed and mean sIgA concentration was $204 \pm 147 \mu\text{g}\cdot\text{mL}^{-1}$. All swimmers had detectable levels of sIgA in samples, which confirmed none had deficiency in saliva. Salivary IgA was variable within-subjects, with a mean CV of 33%. The difference in mean value between the lowest and highest individual sIgA concentration, was almost 29-fold ($31 \mu\text{g}\cdot\text{mL}^{-1}$ vs. $892 \mu\text{g}\cdot\text{mL}^{-1}$), and between-subjects CV was 72%.

A Wilcoxon signed-rank test showed that absolute sIgA concentration was significantly lower on weeks where swimmers reported URS, compared to weeks they did not ($Z = 2.132$, $r = -.418$, $p = .033$). No differences were found for saliva flow rate ($Z = 1.364$, $p = .172$) or sIgA secretion rate ($Z = 0.874$, $p = .382$), for URS weeks vs. non-URS weeks for all swimmers.

When sIgA values were normalised to each individual's mean, relative sIgA concentration was 12% lower during URS than when there were no symptoms present. Relative sIgA declined over two weeks prior to URS, being lowest during URS, before returning to above an individual's healthy average by 2 weeks following symptoms (Figure 4.6). Relative sIgA concentrations before, during and after URS were compared with repeated measures ANOVA (Greenhouse-Geisser correction) and no significance was found ($F_{(4.360,222.349)} = 1.926$, $p = .101$, $\eta^2_{\text{partial}} = .360$).

Figure 4.6

Seven weeks of salivary IgA (%), leading in and out of URS episodes



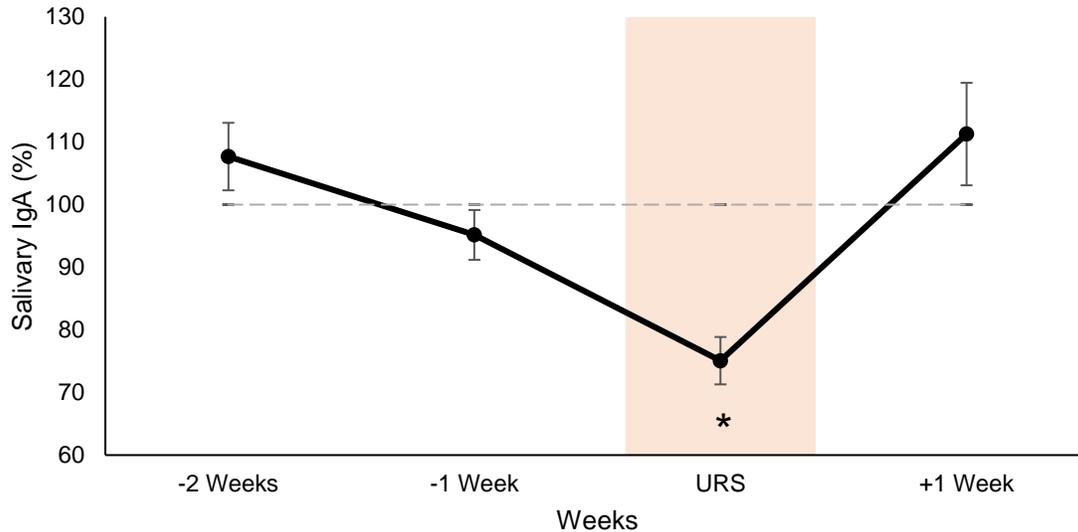
Note. Average relative sIgA (%) plotted over 7 weeks for all feasible URS episodes. Salivary IgA was normalised by each individual's mean; data presents 4 weeks before a URS episode, then 2 weeks after ($n = 59$).

That said, many URS episodes overlapped with all seven timepoints and therefore, this was reduced to four timepoints (-2 weeks to +1 week). Repeated measures ANOVA were repeated for episodes that had no overlapping data points ($n = 40$) and found that relative sIgA was 25% lower on weeks with URS and showed statistical significance ($F_{(1.971, 65.032)} = 7.417$, $p = .001$, $\eta^2_{\text{partial}} = .184$). *Post-hoc* analysis found that relative sIgA was lower on

URS weeks, than all three other time points (-2 weeks, -1 week and +1 week) (Figure 4.7).

Figure 4.7

Four weeks of salivary IgA (%), leading in and out of URS episodes



Note. Average relative sIgA (%) normalised by each individual's mean, plotted over 4 weeks for URS episodes; data presents 2 weeks before a URS episode, then 1 week after (n = 40).

*Denotes significantly lower relative sIgA for URS weeks, than all three other time points.

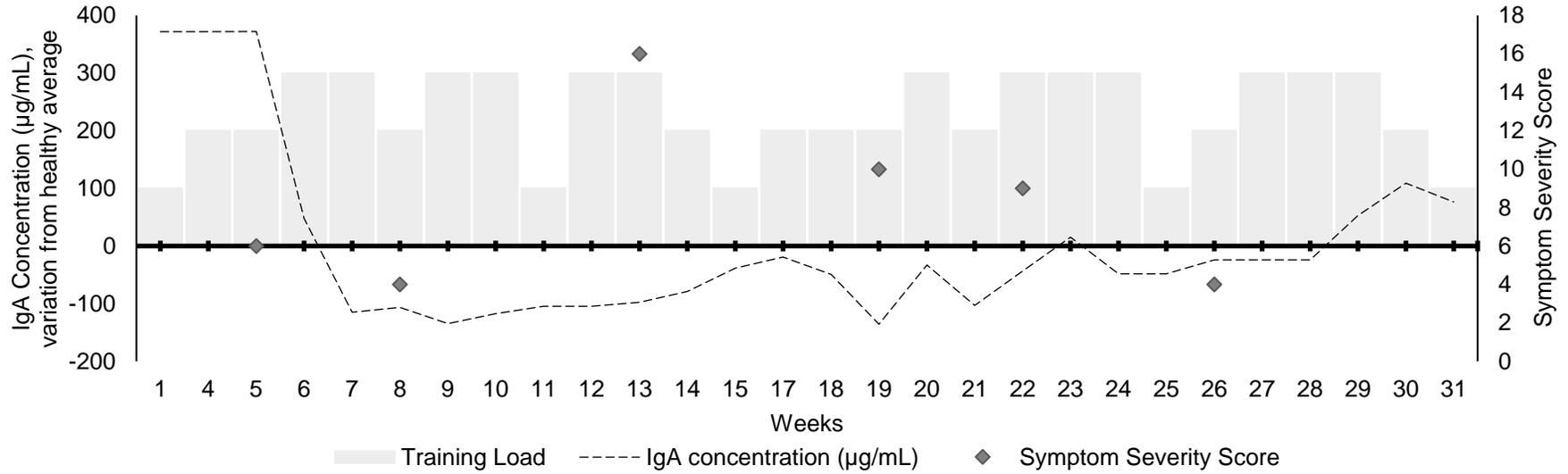
When looking at deviation from average healthy sIgA concentration, 13 out of 14 swimmers spent 50% or more time below this over the whole 8-month season. On average, swimmers spent 58% of the season being below their average healthy sIgA concentration; one swimmer spent 71% below average and reported above average URS episodes (total of 6) (Figure 4.8). Additionally, 10 out of 14 swimmers had above average sIgA concentration during and up to two weeks after competition, in week 16 and week 32.

Regarding training load, average absolute sIgA concentration for low, moderate, and high training phases was 186 ± 88 , 209 ± 112 , and $185 \pm 201 \mu\text{g}\cdot\text{mL}^{-1}$ respectively. Repeated measures ANOVA with Greenhouse-Geisser correction was used to determine whether average sIgA concentration differed significantly between low, moderate and high training

load bouts for all swimmers, of which no interaction was found ($F_{(1.020,13.262)} = .647$; $p = .438$, $\eta^2_{partial} = .047$). Furthermore, no interaction was found for sIgA secretion rate between training loads ($F_{(1.157,15.043)} = .315$, $p = .616$, $\eta^2_{partial} = .024$).

Figure 4.8

Variation from the average IgA concentration, plotted over the 8-month season for one swimmer.



Note. This data shows the swimmer spending 71% of the season, under their reported average (n = 1)

4.3.3.1 Group Analysis

Illness parameters and sIgA markers for the two training groups (Table 4.4) and both sexes (Table 4.5) were examined. For training groups over differing training loads, mixed model ANOVA analysis explored the existence of any differences between URS parameters. A significant difference occurred for symptom severity score ($F_{(2,24)} = 4.108$, $p = .029$, $\eta^2_{\text{partial}} = .255$); *post hoc* analysis showed significance between the two training groups during moderate training loads ($p = .18$) However, no differences were found for number of URS episodes ($F_{(2,24)} = .360$, $p = .701$, $\eta^2_{\text{partial}} = .029$), duration of symptoms ($F_{(2,24)} = 2.673$, $p = .089$, $\eta^2_{\text{partial}} = .182$) or missed training days ($F_{(1.121,13.458)} = .494$, $p = .515$, $\eta^2_{\text{partial}} = .040$). In addition, no significance was found for sIgA concentration ($F_{(1.021,12.250)} = 1.779$, $p = .207$, $\eta^2_{\text{partial}} = .129$) or sIgA secretion ($F_{(2,24)} = 2.945$, $p = .72$, $\eta^2_{\text{partial}} = .197$) between training groups over the three training loads. Visual representation of this can also be seen over time, for both the sprint (Figure 4.9) and middle-distance (Figure 4.10) groups.

Differences between sex over the three differing training loads for all URS parameters was also examined. Through use of a mixed model ANOVA, a significant difference was found between sex and training load for symptom severity ($F_{(2,24)} = 4.079$, $p = .030$, $\eta^2_{\text{partial}} = .254$). *Post hoc* analysis showed significance for sex, between moderate training load ($p = .028$). However, no differences were found for number of URS episodes ($F_{(2,24)} = .166$, $p = .848$, $\eta^2_{\text{partial}} = .014$), duration of symptoms ($F_{(2,24)} = 1.502$, $p = .243$, $\eta^2_{\text{partial}} = .111$), or missed training days ($F_{(1.140, 13.674)} = 1.075$, $p = .328$, $\eta^2_{\text{partial}} = .082$). Regarding sIgA, no significance was found for concentration ($F_{(1.022, 12.268)} = 1.310$, $p = .275$, $\eta^2_{\text{partial}} = .098$). A significant main effect was found for sIgA secretion ($F_{(2,24)} = 3.535$, $p = .045$, $\eta^2_{\text{partial}} = .228$) between sexes. However, *post hoc* analysis showed no significant difference between sex and training load ($p \geq .05$).

Table 4.4

Illness parameters and sIgA over different training loads between training groups

Training Load	Group	Swimmer Training Load (a.u)	Number of URS Episodes	Symptom Severity Score	Average Duration of URS	IgA Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$)	IgA Secretion ($\mu\text{g}\cdot\text{min}^{-1}$)	Saliva Flow Rate ($\text{mL}\cdot\text{min}^{-1}$)
Low	Sprint	149 \pm 91	5	4 \pm 2	3 \pm 0	215 \pm 114	149 \pm 53	0.79 \pm 0.20
	Distance	180 \pm 40	4	3 \pm 4	5 \pm 3	161 \pm 44	162 \pm 77	0.93 \pm 0.25
Moderate	Sprint	231 \pm 110	9	22 \pm 15 *	10 \pm 3	191 \pm 77	140 \pm 31	0.84 \pm 0.22
	Distance	192 \pm 150	10	2 \pm 3	8 \pm 5	222 \pm 130	173 \pm 88	0.85 \pm 0.19
High	Sprint	249 \pm 92	21	15 \pm 6	9 \pm 2	177 \pm 85	129 \pm 27	0.83 \pm 0.25
	Distance	345 \pm 128	21	15 \pm 10	10 \pm 5	203 \pm 107	171 \pm 80	0.89 \pm 0.22

Note. Illness parameters and measurements of sIgA for sprint ($n = 6$) and middle distance ($n = 8$) training groups, broken down by training load. Significance was found for symptom severity score between training groups and training loads, through use of a mixed model ANOVA. *Denotes significance for symptom severity score between training groups, during moderate training loads. Data is shown as mean \pm SD.

Table 4.5

Illness parameters and sIgA over different training loads between sexes

Training Load	Sex	Number of URS Episodes	Symptom Severity Score	Average Duration of URS	IgA Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$)	IgA Secretion ($\mu\text{g}\cdot\text{min}^{-1}$)	Saliva Flow Rate ($\text{mL}\cdot\text{min}^{-1}$)
Low	Male	6	4 \pm 4	5 \pm 3	163 \pm 47	167 \pm 69	0.99 \pm 0.21
	Female	3	3 \pm 3	3 \pm 0	223 \pm 121	138 \pm 62	0.66 \pm 0.11
Moderate	Male	11	4 \pm 6 *	9 \pm 4	216 \pm 123	178 \pm 77	0.93 \pm 0.21
	Female	8	19 \pm 16	9 \pm 4	196 \pm 86	124 \pm 43	0.70 \pm 0.06
High	Male	26	15 \pm 10	10 \pm 4	194 \pm 103	175 \pm 68	0.98 \pm 0.20
	Female	16	14 \pm 6	9 \pm 2	187 \pm 90	114 \pm 41	0.65 \pm 0.10

Note. Illness parameters and measurements of sIgA for male ($n = 9$) and female ($n = 5$) swimmers. *Denotes significance for symptom severity score between sex, during moderate training loads, plus a significant main effect was found for IgA secretion between sexes. Data is shown as mean \pm SD.

Figure 4.9

Illness measures presented alongside sIgA concentration for the sprint group. Training load for each week can be seen via bars; low, moderate, and high intensity over the whole testing period (n = 6)

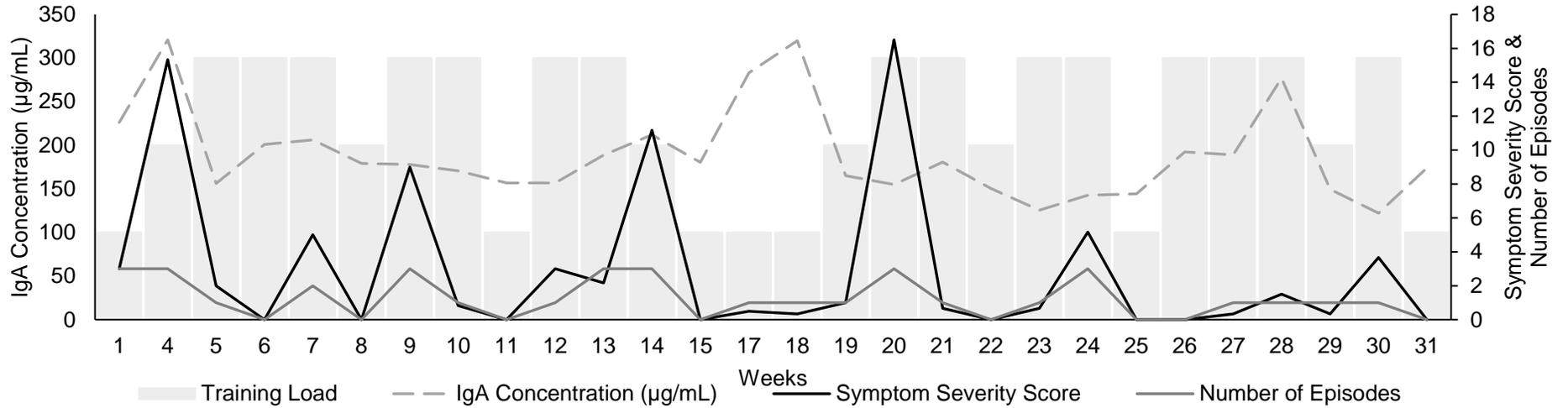
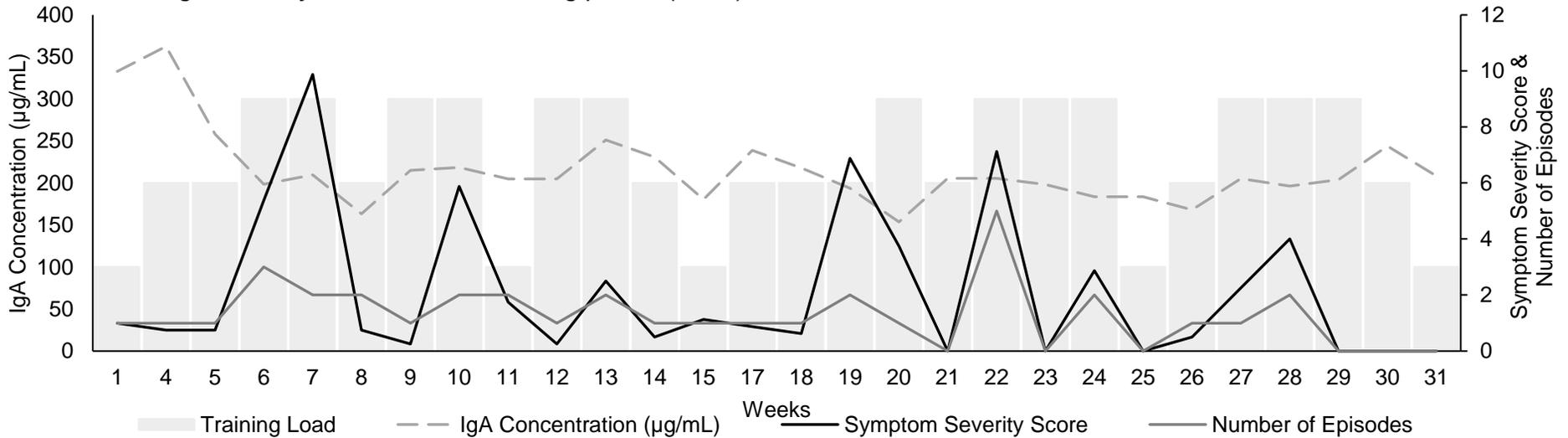


Figure 4.10

Illness measures presented alongside sIgA concentration for the distance group. Training load for each week can be seen via bars; low, moderate, and high intensity over the whole testing period (n = 8)



4.3.4 Sleep

4.3.4.1 Habitual Night-time Sleep

There is missing data for one swimmer, who found the wrist-worn device too uncomfortable during sleep and therefore, data presented is for 13 swimmers. When viewing all night-time sleep data, average TST was 6 hours and 30 minutes, while average latency was 14 minutes. Sleep efficiency for all swimmers, was on average 75%.

Sleep data was examined against training loads; low, moderate and high (Table 4.6) (nap time was excluded here). A repeated measures ANOVA found that latency (min), efficiency (%), WASO and average time awake did not show any significant differences between training load ($p \geq .05$). However, a significant interaction was found for TTIB (min) ($F_{(2,18)} = 17.140$, $p = .001$, $\eta^2_{\text{partial}} = .656$) between different training loads. Post-hoc analysis showed TTIB to be significantly less during moderate ($p = .002$), and high training loads ($p = .002$), compared to low. Moreover, a significant interaction was found with TST ($F_{(2,18)} = 14.434$, $p = .001$, $\eta^2_{\text{partial}} = .616$); significantly less TST was recorded during moderate ($p = 0.002$), and high training loads ($p = .008$) when compared to low. Finally, number of awakenings was significantly higher ($F_{(2,18)} = 4.542$, $p = .025$, $\eta^2_{\text{partial}} = .335$), during low training loads compared to moderate ($p = .048$).

Table 4.6

Average night-time sleep data

Training Load	Latency (min)	Efficiency (%)	TTIB (min)	TST (min)	WASO (min)	Number of Awakenings	Average Time Awake (min)
Low	17	71	553	445	90	30	3
Mod	12	82	451 *	366 *	72	22 *	3
High	12	72	449 ◇	363 ◇	74	25	3

Note. Night-time data for the whole group of swimmers ($n = 13$), during low, moderate, and high intensity training. *Denotes a significant difference between low and moderate training loads, for the variables marked. ◇Denotes a significant interaction between low and high training loads, for

the variables marked. TTIB, total time in bed; TST, total sleep time; WASO, wakes after sleep onset.

4.3.4.2 Napping

Napping was treated separately to night-time sleep during analysis. When napping, swimmers slept on average for 2 hours, and this was observed alongside training load (Table 4.7). Through use of repeated measures ANOVA analysis, it was found that efficiency, TTIB, TST, WASO, and number of awakenings did not differ significantly between differing training loads ($p \geq .05$). However, a significantly faster latency ($F_{(2,14)} = 4.387$, $p = .033$, $\eta^2_{\text{partial}} = .385$) was found during high training loads, compared to low ($p = 0.048$). Furthermore, with Greenhouse-Geisser correction, it was found that average time awake was additionally significantly lower during moderate and high training loads, compared to low ($F_{(1.203,8.423)} = 6.742$, $p = .027$, $\eta^2_{\text{partial}} = .491$).

Table 4.7

Average nap-time sleep data

Training Load	Latency (min)	Efficiency (%)	TTIB (min)	TST (min)	WASO	Number of Awakenings	Average Time Awake (min)
Low	10	70	170	115	45	7	6
Mod	8	70	152	122	21	7	3 *
High	4 \diamond	72	150	126	20	6	3 \diamond

Note. Nap-time data for the whole group of swimmers ($n = 13$), during low, moderate and high intensity training. *Denotes a significant difference between low and moderate training loads, for the variables marked. \diamond Denotes a significant interaction between low and high training loads, for the variables marked. TTIB, total time in bed; TST, total sleep time; WASO, wakes after sleep onset.

4.3.4.3 Self-Reported Sleep

Over the study period, swimmers reported an average sleep score of 6.7 (out of 10). A repeated measures ANOVA demonstrated no significant differences for sleep score

between differing training loads ($F_{(2,26)} = .575$; $p = .570$, $\eta^2_{partial} = .042$). Average sleep score for low intensity training was 6.8 ± 1.0 , compared to 6.5 for both moderate (± 1.1) and high (± 1.0) training intensities. Visual representation of self-reported sleep score can be seen against sIgA and number of URS episodes for both the sprint group (Figure 4.11) and distance group (Figure 4.12), over the 8-month study duration.

Figure 4.11

IgA concentration ($\mu\text{g}\cdot\text{mL}^{-1}$), sleep score and overall number of URS episodes for the sprint group. Training load for each week can be seen via bars; low, moderate, and high intensity over the whole testing period ($n = 6$)

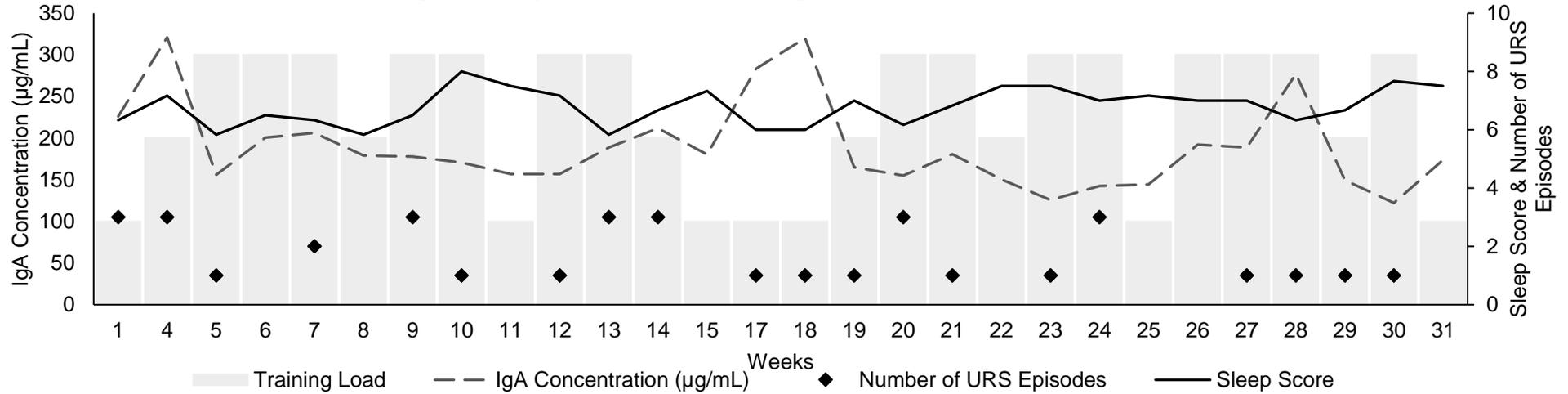
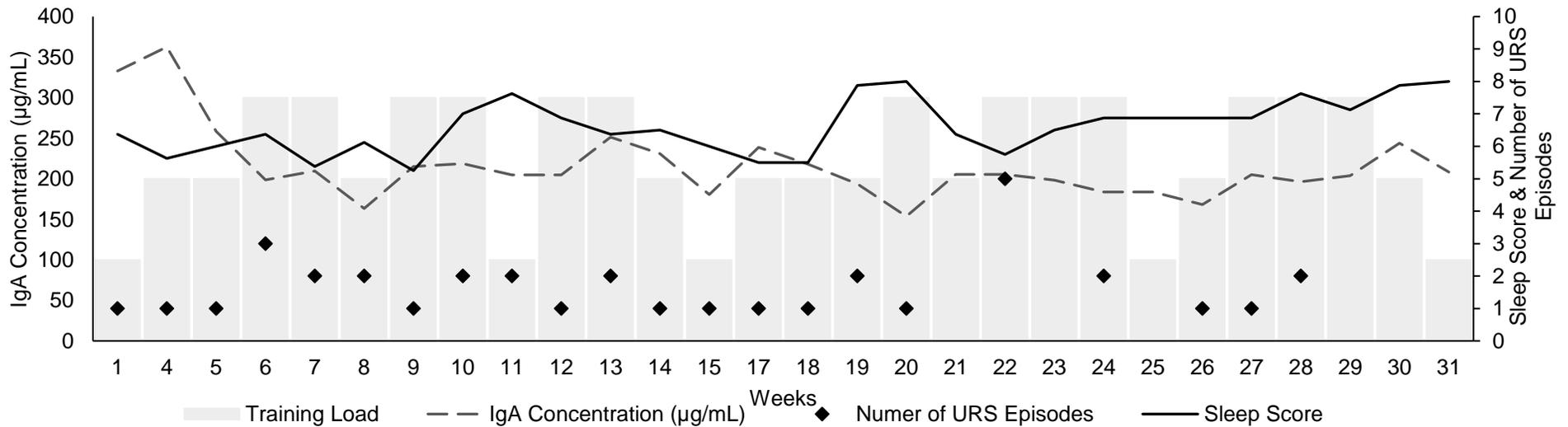


Figure 4.12

IgA concentration ($\mu\text{g}\cdot\text{mL}^{-1}$), sleep score and overall number of URS episodes for the distance group. Training load for each week can be seen via bars; low, moderate, and high intensity over the whole testing period ($n = 8$)



Over the 8-month observation, 43% of swimmers reported that they slept the NR of 7-9 hours per night, 5-6 times a week. This was followed by 3-4 times a week (24%), everyday (23%), 1-2 times (9%) and not once (1%), retrospectively. Percentage of time the NR was met, was analysed over the differing training loads (Table 4.8). Repeated measures ANOVA found an interaction for 5-6 days ($F_{(2,26)} = 10.944$, $p = .001$, $\eta^2_{partial} = .457$); swimmers thought they slept the NR 5-6 times a week, significantly more during moderate ($p = .014$) and high ($p = .001$) training loads, than during low. Another interaction was found for 3-4 days ($F_{(2,26)} = 9.051$, $p = .001$, $\eta^2_{partial} = .410$); swimmers reported that they slept the NR 3-4 times a week, significantly more during high ($p = .004$), than during low training load weeks.

Table 4.8

Percentages of time the swimmers slept the national recommendation of 7-9 hours per night, for differing training loads

	Low	Moderate	High
Everyday	31	21	48
5-6 Times	18	36 \diamond	46 *
3-4 Times	15	29	56 *
1-2 Times	20	13	67
Not Once	0	50	50

Note. \diamond Denotes a significant difference between low and moderate training loads, for the variables marked. *Denotes a significant interaction between low and high training loads, for the variables marked ($n = 14$).

Then, 41% of swimmers woke feeling fatigued, 1-2 times a week. This was followed by 3-4 times (35%), 5-6 times (18%), not once (4%) and everyday (3%), retrospectively. Percentage of time swimmers woke feeling fatigued, was analysed over differing training loads (Table 4.9). A repeated measures ANOVA found a significant difference for waking feeling fatigued 3-4 days per week between training loads ($F_{(2,26)} = 17.694$, $p = .001$, $\eta^2_{partial} = .457$).

= .576); significantly more during high training loads, than during low ($p = .001$), and moderate training load weeks ($p = .140$). Furthermore, a significant interaction was found for waking feeling fatigued 5-6 days a week ($F_{(2,26)} = 10.449$, $p = .001$, $\eta^2_{\text{partial}} = .446$), between training loads. *Post-hoc* analysis found times swimmers woke 5-6 times a week with fatigue, significantly more during high intensity training, than low ($p = .003$).

Table 4.9

Percentages of time the swimmers woke feeling fatigued each week, for differing training loads

	Low	Moderate	High
Everyday	22	22	56
5-6 Times	11	31	58 *
3-4 Times	17	29	54 * ∞
1-2 Times	26	28	46
Not Once	37	36	27

Note. *Denotes a significant interaction between low and high training loads, for the variables marked. ∞Denotes a significant difference between moderate and high training loads, for the variables marked ($n = 14$).

4.3.4.4 Pre- Competition

Average sleep data gathered from the ActiGraph, was observed for 1 and 2 weeks leading into competition (Table 4.10). Paired samples t-tests found a significant increase in TTIB ($t_{(34)} = 2.854$, $d = .482$, $p = .007$), TST ($t_{(34)} = 2.360$, $d = .399$, $p = .024$), and number of awakenings ($t_{(34)} = 2.364$, $d = .399$, $p = .024$), from two weeks to one week before competition. When this data is compared to average night-time sleep data across the season in Table 4.6, findings from two weeks before competition were similar to that seen during moderate training loads. Here, TST is significantly more one week before competition compared to moderate training loads (433 minutes vs. 366 minutes), yet there where increased number of awakenings (31 vs. 22).

Table 4.10

Average ActiGraph sleep data two weeks prior to major competition

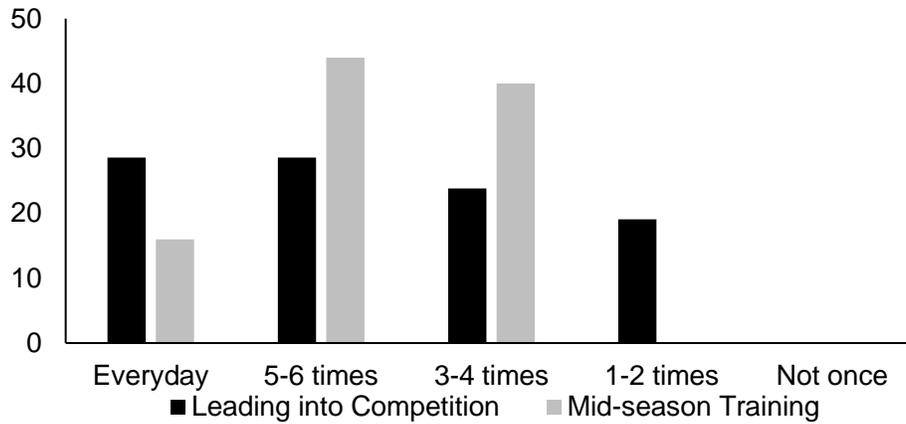
Weeks Before Competition	Latency (min)	Efficiency (%)	TTIB (min)	TST (min)	WASO (min)	Number of Awakenings	Average Time Awake (min)
Two	11	82	470	382	77	24	3
One	18	79	546 *	433 *	96	31 *	3

Note. *Denotes a significant interaction between one and two weeks before competition, for the variables marked ($n = 13$). TTIB, total time in bed; TST, total sleep time; WASO, wakes after sleep onset.

Times the NR was met (%) and times woken feeling fatigued (%), comparing two weeks before competition and two weeks mid-season training, can be seen in Figure 4.13 and Figure 4.14 retrospectively. Average sleep score before competition was reported as 6.2 (out of 10), compared to 7.2 during mid-season training. However, swimmers perceived sleep quantity to be improved leading into competition; swimmers reported they met the NR for sleep every day more leading into competition (29% vs. 16%). In addition, a higher percentage reported waking feeling fatigued only 1-2 times a week before competition (30% vs. 19%). Mixed model ANOVA analyses were used to explore whether a difference was seen in self-reported sleep scores between the two different training groups, of which no significant interactions were found ($F_{(2,24)} = .495$, $p = .616$, $\eta^2_{partial} = .040$).

Figure 4.13

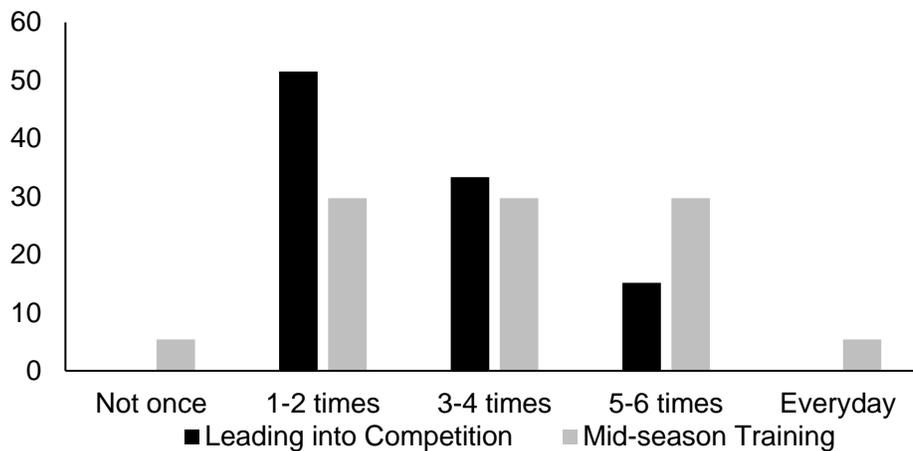
Percentage of time the national recommendation of 7-9 hours of sleep was met (%), during two weeks leading into competition compared to two weeks mid-season training



Note. Results shown are for all swimmers (n= 14).

Figure 4.14

Percentage of time swimmers woke feeling fatigued (%), during two weeks leading into competition compared to two weeks mid-season training



Note. Results shown are for all swimmers (n= 14).

4.3.5 Epstein Barr Virus

One swimmer did not consent to blood sampling via venepuncture and therefore, data presented is for 13 swimmers. Results of the EBV serology indicated that 8 out of 13 of the swimmers (61%) had evidence of past infection with EBV. All eight swimmers tested seropositive for anti-VCA IgG antibodies in serum, and this did not differ significantly between sex (male: 88%, female: 13%), or training group (sprint: 38%, middle-distance: 63%) ($p \geq .05$). Five out of the 8 seropositive swimmers (63%) showed presence of antibodies for Epstein-Barr Nuclear Antigen (EBNA). No swimmer had detectable levels of anti-viral capsid antigen (VCA) IgM antibodies in their serum, indicating none had current EBV infection (glandular fever) prior to the commencement of the study.

Serology status of EBV was compared against self-reported symptoms and IgA parameters (Table 4.11). Through use of Mann-Whitney U-tests and independent samples T-tests, no differences were found between EBV serostatus and any of the observed parameters ($p > 0.05$). Mixed model ANOVAs were used to explore whether differences in the main parameters could be seen over differing training loads. However, no interactions were found for sIgA concentration ($F_{(1,015,12,175)} = 1.1.09, p = .314, \eta^2_{\text{partial}} = .085$), symptom severity score ($F_{(2,24)} = .609, p = .552, \eta^2_{\text{partial}} = .048$), number of URS episodes ($F_{(2,24)} = 1.128, p = .340, \eta^2_{\text{partial}} = .086$), or sleep score ($F_{(2,24)} = 2.460, p = .107, \eta^2_{\text{partial}} = .170$), between EBV positive and negative swimmers over all three training loads. Despite lack of significance, it should be mentioned that a higher number of URS episodes and missed training days were presented by EBV positive swimmers, than EBV negative (Figure 4.15). This could be seen most, during high intensity training loads, where EBV positive swimmers reported 63% of URS.

Table 4.11

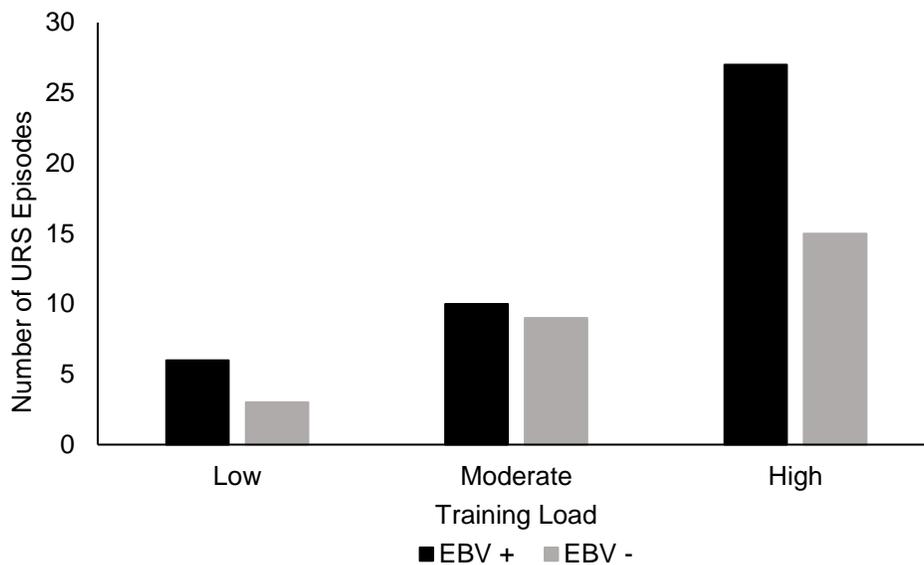
Comparisons of illness parameters by EBV serology status

EBV serology	Average IgA Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$)	Average Symptom Score	Highest Symptom Score	Average URS Duration	Missed Training Days	Number of URS Episodes	Swimmers EBNA Positive
Positive	187	10	63	7	25	43	63%
Negative	207	10	45	8	9	27	0%

Note. Average IgA concentration, average symptom severity score, lowest and highest symptom severity scores, average number of days of URS, number of URS episodes and percentage of swimmers with EBV-DNA detected, in seropositive ($n = 8$) and seronegative ($n = 5$) swimmers.

Figure 4.15

Reported number of URS episodes for EBV positive (+) and negative (-), over all three training loads



4.3.6 Body Mass

Over the 8-month study period, change in body mass for female swimmers ranged from 1.5 kg to 4.7 kg (2-6%) (Figure 4.16). Change in body mass for males ranged from 2.1 kg to 7.9 kg (3-9%) (Figure 4.17). Body mass fluctuations from both a) baseline and b) the average was not significantly correlated with URS incidence, symptom severity, or training load.

Figure 4.16

Change in body mass from baseline of female swimmers, over the 8-month season (n = 5)

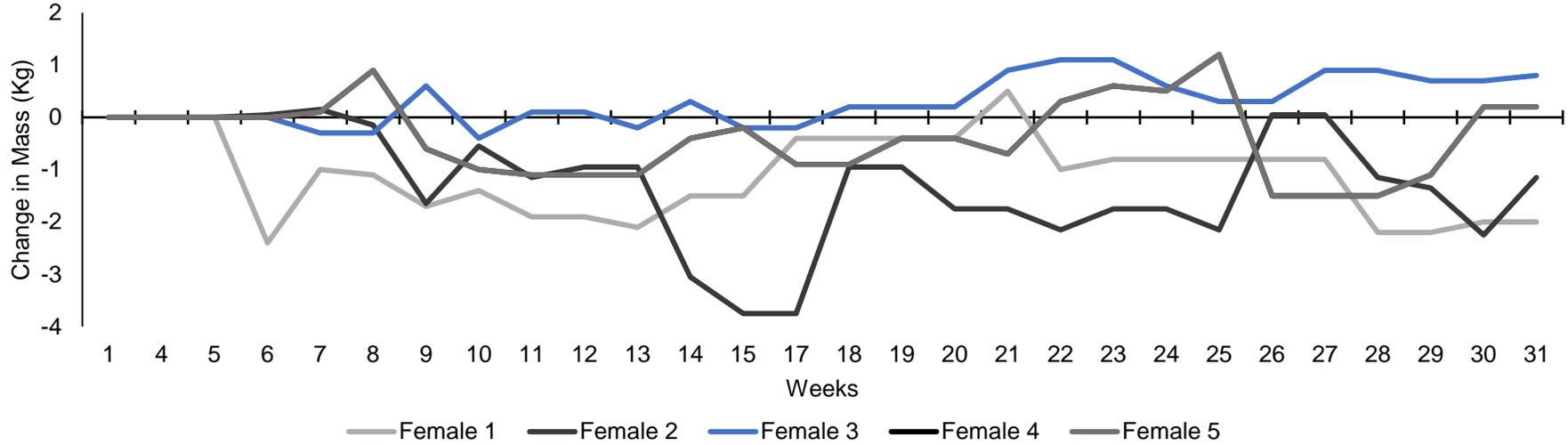
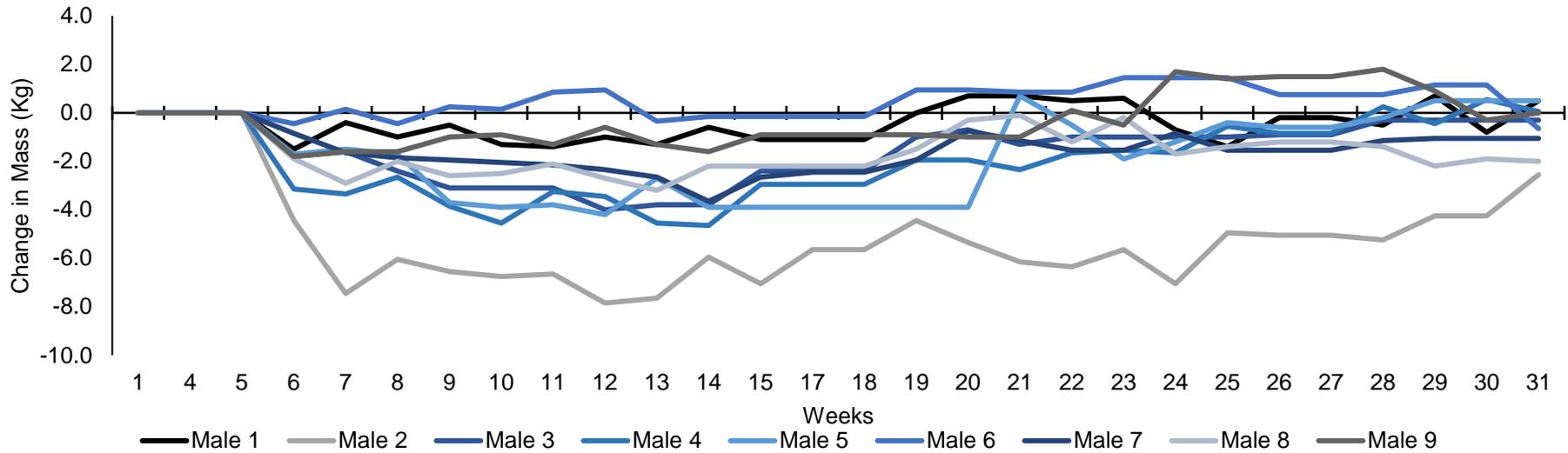


Figure 4.17

Change in body mass from baseline of male swimmers, over the 8-month season (n = 9)



4.4 Discussion

The current study was designed to investigate whether a relationship between sIgA concentration and URS exists. Alongside this, the effect of a) training load, b) sleep quantity and quality, and c) EBV serostatus was examined in regard to URS and sIgA in elite swimmers, over an 8-month season. The key findings of the current study showed several relationships between the investigated parameters and will be examined in order of the identified study aims. Firstly, it was found that increased training loads, increased the number of URS episodes, severity, and duration of symptoms (A1). Both absolute and relative salivary IgA concentration was significantly lower on weeks where URS were reported (A2) and displayed no change between training loads (A3). Despite lack of significance, EBV seropositive swimmers reported a higher incidence of URS episodes and higher number of missed training days, than those who were negative for EBV (A4). Lack of significance suggests this result could be due to chance, but was significantly underpowered, where population size was too small to have an effect. Regarding sleep, swimmers regularly slept below the national recommendation of 7-9 hours (A5), especially during increased training loads, with marked differences leading into competition (A6). No relationship was found between sleep patterns and risk of URS (A7). Lastly, despite female body mass fluctuations of up to 4.7 kg, they were not significantly correlated with URS incidence or symptom severity (A8).

4.4.1 Training Load

The sprint group completed a significantly lower weekly volume of training compared to the distance group (29 vs. 41 km). Average weekly training volume (35 km) was lower than that reported by the Australian Olympic team, of 55 km (range 40 – 80 km) (Mujika et al., 2002). Although, it has been suggested that elite British swimmers endure less training volume than other nations, reporting an average 48.4 ± 8.6 km per week (Mujika et al., 2002; Pollock et al., 2019). The average training volumes observed in the current study are less than that reported by Pollock (2019) and could be due to the difference in level of athlete. The current cohort of swimmers were national and international level and were not Olympic or world medallists, and so may not be comparable. However, sprint

swimmers trained significantly less than distance swimmers (on average 15 km less weekly) (Pollock et al., 2019), which is similar to the findings of the current study.

Different swimming events pose different physiological demands and so it came as no surprise there were differences in training volume; this allowed justification to divide the two groups for any analysis. It needed to be determined whether differences in training volume could influence immune response, or any other measured variables. Weekly volume of training was provided by the coaches, alongside their own version of proposed training load. Both methods of training load were used in the current study, so that it could be seen whether swimmers would perceive training load differently depending on how they are feeling, compared to coaches. However, swimmer perceived training loads were significantly correlated with coach derived training loads and therefore, throughout the analysis these were defined together.

4.4.2 Upper Respiratory Symptoms

4.4.2.1 Number of Episodes

Over the 8-month observation from September to April, an average of five episodes of URS were reported for each swimmer. These findings suggest that these swimmers have higher incidence of URS than that of the general population (3 episodes) (Arroll, 2011), but similar to other athletes elsewhere (2-5 episodes) (Bishop, 2006; Lin & Decker, 2010). In one study, Rama et al. (2013) reported an average of two URS episodes per swimmer. Despite the study population being similar, number of URS episodes was higher in the current study. Perhaps this was because an important distinction between performance level for illness incidence has been found (Hellard et al., 2015; Mårtensson et al., 2014); indicating that international athletes suffered fewer URS episodes than national level athletes (Hellard et al., 2015). This could be due to having a higher tolerance to high training loads (Malm, 2006), or that there is more emphasis on diet, hygiene, stress management, supplements, and sleep (Hellard et al., 2015; Walsh et al., 2011). Despite this, some swimmers in the current research were international level and yet still showed high incidence of illness. Therefore it was considered that additional psychological stress

could have influenced risk of URS in the current cohort of swimmers (Dhabhar, 2014). Edwards et al. (2018) found that perceived stress and anxiety before exercise can modify the immune response following exercise, which could have put the current swimmers at added risk of more URS. Despite not monitoring psychological stressors, financial hardship and studying could have influenced these findings as they were University students and should be considered.

Alternatively, differences in how URS episodes were classified should be considered as an explanation for contrasting findings; different methods were used for symptom logging and defining a URS episode. For example, one study reported that symptoms separated by ≥ 3 days were to be regarded as different URS episodes (Fricker et al., 2005). Whereas elsewhere, an asymptomatic period of ≥ 7 days was proposed for a subsequent episode to be classified as new (Spence et al., 2007). Most importantly, Rama et al. (2013) considered a new URS episode after a minimum interval of 10 days following the previous (Bishop, 2006). Therefore, the longer interval between episode classification may explain why fewer URS episodes were reported, when compared to the current study. Regardless of methodological differences, recent studies have shown increased susceptibility to URS with increased training load, especially during winter (Hellard et al., 2015; Svendsen et al., 2016).

4.4.2.2 Link with Training Load

The current study reported significantly more URS episodes (64%), higher symptom severity score and longer duration, during high training loads. In support, other literature has found most URS to occur during the heaviest training periods (Spence et al., 2007; Rama et al., 2013). Rama et al. (2013) monitored 19 elite swimmers for 7 months in winter, recorded daily illness symptoms and found that 67% occurred around high-volume training loads. Therefore, this finding strengthens and highlights the issue of increased risk of URS during intense training periods. Interestingly, non-athletic controls did not show similar URS occurrence at the same time points (Rama et al., 2013), suggesting the high training loads to be causal factor for illness. Due to no control group, the current study cannot comment on this; however, lowered immunity and increased rate of illness

has been repeatedly shown elsewhere, following high intensity training bouts (Fricker et al., 1999; Gleeson et al., 2000; Spence et al., 2007). In addition, similar to current findings, Moreira et al. (2013) examined elite Brazilian futsal players over a 4-week period, and found that athletes were more susceptible to high URI symptom severity during periods of high intensity training. Therefore, findings were consistent with previous literature, strengthening the relationship on incidence of illness, and symptom duration and severity in relation to training load.

There were 34 days of missed training due to reported URS, from nine swimmers. Despite no statistical difference between training loads, 76% of missed training days due to URS were during high training loads. One study found that athletes were seven times more likely to achieve a performance goal if they completed >80% of planned training weeks (Ray Smith & Drew, 2016). Furthermore, the importance of avoiding missed training days was highlighted when chance of success has been shown to significantly reduce for every week of missed training (Ray Smith & Drew, 2016). Interestingly, there was no significant difference in number of URS episodes between low and moderate training; supporting the method of load reduction to prevent illness symptoms. Despite lacking significance, EBV seropositive swimmers reported 25 missed training days compared to those who were negative (nine missed training days). These were important findings as it could be suggested to coaches to slightly reduce training load (high to moderate) for those at increased risk, or those that suffer frequent URS in order to prevent further symptoms.

4.2.2.3 Link with Body Mass Fluctuations

Another potential trigger or risk factor identified for increased incidence of URS could be weight loss. In 2014, the International Olympic Committee (IOC) defined a new syndrome, relative energy deficiency in sport (RED-S), expanding upon the original female athlete triad concept. This describes the impact of low energy availability (EA) on physiological functioning, performance, and general health of athletes of both sexes (Mountjoy et al., 2014). Previously, it has been said that athletes in prolonged states of low EA are at increased risk of infection, illness, fatigue, and nutrient deficiencies (Shimizu et al., 2012). Shimizu et al. (2012) found that sIgA secretion rates were significantly lower for female

distance runners with athletic amenorrhea, than those with adequate EA. Furthermore, higher number of illness symptoms were found for those with athletic amenorrhea, suggesting reduced mucosal immune function in athletes and enhance susceptibility to infection (Shimizu et al., 2012). Elsewhere researchers found that athletes that lost 5% body weight prior to competition, had significantly lower sIgA secretion and increased number of URS, compared to a control group (Hiraoka et al., 2019). Elsewhere, two weeks of weight loss impaired cell-mediated immunity and led to increased susceptibility to URS in judo athletes (Shimizu et al., 2011).

Despite female body mass fluctuations 2-6% in the current study, no significant associations were found between baseline or average data, and URS incidence, symptom severity, or training load. Current findings may be in contrast to the studies mentioned above because wrestling and judo athletes have to actively cut weight, and so may have more of an effect on risk of URI. Fluctuations in female body mass were evident in the current study, with one showing a 6% decrease in body mass. However, this was over the 8-month study period, unlike studies above that observed these falls over 2 weeks. In support of the current study findings, one study examined 1000 female athletes (ages 15–30 years) via questionnaires, to assess elements of the potential physiological and performance decrements associated with low energy availability (EA) (Ackerman et al., 2018). No differences in immunological function between EA groups were found, which may suggest that EA and fluctuation in body mass may not have any influence on immune function.

4.4.3 Salivary IgA

One of the main findings suggests that relative sIgA could help predict URS in elite athletes. When observing all feasible URS episodes over all seven time points (Figure 4.6), relative sIgA could be seen going below an individual's healthy average two weeks prior to URS and was 12% lower during URS. When data was explored over less time points to reduce number of overlapping URS episodes, relative sIgA was significantly lower during URS (25%) (Figure 4.7). These findings agree with Neville et al. (2008) who reported 28% lower sIgA during URI. Due to infection incubation time being usually

shorter than one week, researchers postulated that the reduction in sIgA in the weeks prior to illness was a contributing factor to URS (Neville et al., 2008). Other published research does not reflect these findings; Stephenson et al. (2019) found no difference in relative deviation from individual median sIgA concentration regardless of whether URS were present, or when URS occurred within two weeks of sampling. Thus, previously the relationship between sIgA and URS has been less convincing, with only a few studies presenting an association (Fahlman & Engels, 2005; Gleeson et al., 1999; Mackinnon et al., 1993). Therefore the current findings help confirm the role of sIgA alongside URS; a decline in relative sIgA could contribute to risk of illness and so regular monitoring of this could benefit athletes and coaches.

Likewise, Cunniffe et al. (2010) reported lower sIgA concentration in rugby players who reported URS, than players who were symptom-free. However, there were no significant relationship between sIgA and incidence or severity of URS, which is similar to the current study. The current study found no association between sIgA concentration and incidence of URS episodes, symptom severity or duration. Equally, no relationship between sIgA and subsequent URS occurrence has been reported elsewhere (Leicht et al., 2012; Stephenson et al., 2019). Previously in swimmers, low concentrations of sIgA subclasses (IgA1 and IgA2), have been associated with increased incidence of URS (Gleeson et al., 1997). Within current research however, none of the swimmers were sIgA deficient or had a notable decline over the training period. Despite swimmers spending an average of 58% of the observed period below their average sIgA concentration, it could be argued that swimmers did not suffer with low enough sIgA concentration to have an impact on URS. That said, one swimmer spent 71% below their average sIgA and reported above average URS episodes for the duration of the study (total of 6). This may add to the notion that individual monitoring for sIgA, rather than group data is more impactful.

In regard to sIgA secretion rate however, no interaction was found for reported URS weeks vs. non-URS weeks for the swimmers. Previously, research has established sIgA secretion rate as a risk factor for URS in 75 American footballers (Fahlman & Engels, 2005). Likewise, Gleeson et al. (2012) found that participants suffering with URS had

significantly lower sIgA secretion rate and saliva flow rate, but not sIgA concentration, when compared to illness-free participants. However, these two studies contested findings from the current study. Overall, the lack of studies investigating the relationship between sIgA secretion and URS makes comparisons difficult. That said, a significant effect was found for sIgA between sexes, with females showing a significantly lower sIgA secretion rate than men. These findings are supported elsewhere (Gleeson et al., 2011), and could be explained by females having lower unstimulated saliva flow rates compared to men because of smaller salivary glands (Inoue et al., 2006).

4.4.3.1 Variability of sIgA

Mean absolute sIgA concentration was similar to that reported previously (Sari-Sarraf et al., 2008). However, making comparisons between group mean values of sIgA comes with difficulty due to the large variation presented. Differences in study design, sport population, level of fitness, saliva collection methods, and large between-subject variability of sIgA, could account for differences in mean sIgA concentration. The large between-subject variation of sIgA found (72%), has been seen elsewhere (Francis et al. 2005; Nehlsen-Cannarella et al., 2000; Neville et al., 2008). More specifically, Neville et al. (2008) reported 71% between-subject CV, providing a feasible explanation for the vast differences in findings for sIgA between literature. Together, these findings strongly indicate that sIgA levels are specific to each individual and strengthen the use of monitoring percentage change from an individual's 'healthy' normal. To support, previously Gleeson et al. (2017) highlighted the need to monitor changes in sIgA in individual athletes rather than cohort means.

The within-subject variation in the current study (33%), concurs with findings in elite rowing (Nehlsen-Cannarella et al., 2000), swimming (Francis et al., 2005), and sailing (Neville et al., 2008). However, this was a little lower than what has been reported previously. As an example, Neville et al (2008) reported mean within-subject CV as 48% in a group of 38 elite sailing athletes. It has been suggested that elite athletes have greater within-subject variability than those of a lower level of physical fitness and activity (Francis et al., 2005). The current athletes were national and international swimmers, with a few

having been selected for the Commonwealth Games 2018, so a high CV would have been expected. Alternatively, individual variation in sIgA may indicate that some athletes are more susceptible, or adaptable to training or stress than others (Francis et al., 2005; Neville et al., 2008). As an example, one swimmer's sIgA concentration was found to significantly differ between low and high training loads in the current study. Notably, this swimmer reported the highest symptom severity score (during a high training load week) over the whole study observation, for all swimmers. Therefore, the swimmer could be more susceptible to changes in mucosal immunity, 'at risk' of URS and arguably should be monitored more closely during changes in training load. Furthermore, as already identified, concentration of sIgA suffers from circadian variation and has significantly higher levels the morning (06:00-08:00 hours) (Dimitriou et al., 2002). Neville et al. (2008) took fasted samples at 07:45 hours, which may explain the higher within-subject variation found compared to the current study (samples were taken weekly on Wednesdays, at 12:00 hours), as sIgA becomes more stable from midday (Dimitriou et al., 2002; Li & Gleeson, 2004). Plus, fasted samples have been found to yield higher and more variable concentrations than non-fasting samples (Fahlman & Engels, 2005; Francis et al., 2005). These differences in saliva collection between just two studies, clearly shows why making comparisons between literature is nearly impossible.

Interestingly, individual analysis results showed that 10 out of 14 swimmers had above average sIgA concentration during and up to two weeks after competition, in week 16 and week 32. Findings could be because opposed to intense exercise, moderate exercise has been found to stimulate secretion of sIgA (Bosch et al., 2002). Therefore, sIgA has previously been observed during a taper period leading to competition in both adult (Gleeson et al., 1995) and adolescent swimmers (Papadopoulos et al., 2014); supporting current findings. Reductions in training load with taper, promoted an increase in sIgA levels in most swimmers leading into competition, adding support to other literature (Gleeson et al., 2000; Klentrou et al., 2002).

Despite some promising associations between sIgA and URS, the use of sIgA has been recently questioned due to the large variability shown; authors have suggested sIgA lacks

reproducibility, and sensitivity for the detection of illness susceptibility (Turner et al., 2021). Consequently, there is insufficient clear guidance in the biomarker's effectiveness in determining health surveillance and care of athletes. Other biological measures have been suggested that may provide a more sensitive indication of athlete training monitoring (Edmonds et al., 2015). However, in the current study, decreased sIgA concentration and drops in relative sIgA (%) was reported for weeks that swimmers reported URS. Thus, strengthening the use of sIgA as a biomarker in monitoring immune status in individual athletes. Perhaps it should be considered that instead of direct comparisons between literature, sIgA monitoring should be used as an in-house tool within athletic groups.

4.4.3.2 Link with Training Load

No relationship existed between sIgA and training load. Firstly, there was no difference in sIgA concentration between sprint and distance groups, suggesting that training volume had no impact. Secondly, no association was found between increased training loads on sIgA concentration or secretion rates. In agreement, no interaction was reported for Para-triathletes (Stephenson et al., 2019). More specifically, during a 14-week, periodised swim programme for Paralympic swimmers, no significant changes were observed for sIgA between different training phases (Edmonds et al., 2015). In contrast to current findings, some studies observing kayakers and American football players have shown that long-term strenuous training loads, led to a reduction of sIgA (Fahlman et al., 2005; Gleeson et al. 2000). Similarly, negative correlations were found for sIgA secretion rates and training load (Leicht et al., 2012), with 149 military recruits also reporting lower sIgA secretion during intense training (Scott et al., 2019). Perhaps the lack of significant change in sIgA between training load, was due to the fact that swimmers did not have a significant enough training load to promote a mucosal response, having lower training volume than reported elsewhere (Mujika et al., 2002; Pollock et al., 2019). Alternatively, the large between-variation that has already been discussed may have impacted group sIgA analysis, resulting in no significant findings.

4.4.4 Epstein Barr Virus

More than half (61%) of the swimmers in the current study, had previous infection with EBV. The level of EBV seropositivity in the current cohort of swimmers was lower than that of previous investigations of elite swimmers (79%) (Gleeson et al., 2002) and University athletes (84%) (He et al., 2013). However, a larger study conducted on 274 national-level young athletes from 10 different sports, also reported 60% of athletes to be EBV seropositive (Blume et al., 2018). These findings are important because recurrent infections and fatigue are often associated with EBV (Balfour et al., 2015). In addition, prior infection with EBV has been associated with increased URS risk in athletes (Gleeson et al., 2002). Gleeson et al. (2002) found no reported URS in any seronegative swimmers, and all episodes occurred within those who were seropositive. This was in contrast to current findings, where 40% of URS episodes were reported by EBV seronegative swimmers. The reason for the different result may be due to the fact only three swimmers were seronegative (Gleeson et al., 2002). In addition, duration of study may have impacted findings; observing athletes over 8-months would be more likely to have shown increased URS episodes in all athletes, regardless of serostatus, compared to 30 days (Gleeson et al., 2002).

Due to there being such limited research, there has been ongoing controversy on the impact that EBV serostatus has on URS. He et al., (2013b) examined a total of 267 healthy athletes, ranging from recreationally active to Olympic level, with an average training volume of 10 hours a week. No significant differences for number of URI episodes, symptom severity score or duration of episodes were found between EBV positive and negative serostatus. Furthermore, Blume et al. (2018) found that susceptibility to illness was not associated with EBV serostatus, which is similar to that presented in the current study. It should be considered however, that despite non-significance, EBV seropositive swimmers did report a higher number of URS episodes and missed training days. Results may not have been statistically significant due to the small sample size. *Post-hoc* power analysis suggested this was underpowered for detecting a significant interaction ($1-\beta = 0.613$; $\alpha = 0.05$; $n = 11$), which could explain why

the current research may not be comparable. Therefore, given the limited sample size, interpretation of these findings could only be speculative.

That said, there is ongoing debate within sport science research when conducted in elite athletes. Pre-study power calculations often suggest that high numbers of participants are needed to produce significantly powered findings. However, these participant recommendations are often unrealistic when recruiting elite athletes. As an example, no significance was found for symptom severity between the two seropositivity groups, *post-hoc* power analysis showed this to additionally be underpowered ($1-\beta = 0.362$; $\alpha = 0.05$; $n = 11$). However, the highest reported symptom severity score was by a seropositive EBV swimmer. Therefore, it could be argued that individual data and trends could potentially be more useful to coaches and researchers, when compared to group statistical data and *p* values.

To the investigator's knowledge, this was the first study in the UK, to examine EBV serostatus in elite national and international swimmers. It should be considered that increased URS episodes and more missed training days were presented in seropositive swimmers, and the limited sample size may account for lack of significant findings. Overall, evidence is inconclusive and further research is required to clarify the existence of a relationship between EBV serostatus, incidence of URS and sIgA. Perhaps considering viral reactivation with measurement of EBV-DNA in saliva, could be important for future studies. Increased viral EBV-DNA expression, has been detected in saliva before the appearance of URS (Gleeson et al., 2002; Yamauchi et al., 2011). However, determination of EBV-DNA within saliva was not monitored within the current study, and therefore cannot be further commented upon. It has been discussed that EBV could be controlled by altering training load to prevent sIgA suppression and EBV reactivation (Gleeson & Pyne, 2016), and may therefore be important in athlete monitoring and immune surveillance.

4.4.5 Sleep

4.4.5.1 Quantity

Night-time sleep data showed swimmers slept an average of 6 hours and 30 minutes each night, which is similar to previous research in swimmers (de Mello et al., 2020; Gudmundsdottir, 2020). Findings were below the NR of 7-9 hours and supported a recent review that found athletes were often unable to achieve ≥ 7 hours of total sleep time (Roberts et al., 2019). Sargent et al. (2014a) analysed two weeks (926 sleep periods) of habitual sleep in 70 elite athletes from seven different sports (including swimming), and found that average TST was 6 hours 30 minutes. Similar findings have been additionally found in military recruits (6 hours 18 minutes) (Bulmer et al., 2022). Specifically, a recent study observing 108 adolescent swimmers reported TST as 6 hours 14 minutes (Gudmundsdottir, 2020).

Risk factors for reduced sleep adequacy in athletes include high training loads (Dumortier et al., 2017; Hausswirth et al., 2014), early morning training (Sargent et al., 2014), travel, and competition (Erlacher et al., 2011; Juliff et al., 2015). Swimmers especially, are at more risk of poor sleep quantity due to routine early morning training sessions (Gudmundsdottir, 2020; Lastella et al., 2012; Sargent, Halson et al., 2014). Average sleep durations have been reported as low as 5 hours and 24 minutes for swimmers on nights prior to training (Sargent et al., 2014), which was not investigated in the current study. That said, amount of sleep can be heavily influenced by an athlete's training schedule (Surda et al., 2019). In the current study, both TTIB and TST was significantly less during moderate and high training load, compared to low, negatively impacting sleep quantity. Current findings were supported by previous literature which found intensified training significantly decreased TST for elite synchronised swimmers (Schaal et al., 2017), cyclists (Lastella et al., 2015), and gymnasts (Dumortier et al., 2017). Likewise to current findings, in a study conducted on 22 collegiate swimmers, longest sleep duration was found when training load was low (Astridge et al., 2021). In contrast, Robey et al. (2013) found no difference in TST between light and heavy training for soccer players (Robey et al., 2013). It has been reported however, athletes from individual sports report a lower sleep duration than those competing in team sports. Swimmers and triathletes had the

lowest TST (6 hours and 24 minutes), compared to other sports such as cycling, football, or rugby (Lastella et al., 2015), which may equate to the difference in findings.

Differing training loads additionally impacted on sleep adequacy during napping. Firstly, the average length of all awakening episodes was significantly less during moderate and high intensity training, compared to low. Secondly, swimmers had significantly increased latency for napping during high intensity training. These findings were unsurprising, as swimmers may have been feeling more fatigued during and when going to bed to nap. This was in agreement with previous research which found increased latency during intensified training (Schaal et al., 2017).

Interestingly, self-reported sleep data suggested that swimmers slept the NR of 7-9 hours per night significantly more during moderate and high intensity training loads. This may be due to swimmers feeling an increased requirement for rest and recovery during high training periods. Slow wave sleep (SWS), the most restorative sleep stage, has been shown significantly more during peak training than taper (Taylor et al., 1997); so perhaps intensified training increases the need for recovery. However, the swimmers' self-perceived sleep quantity is contradictory to data gathered from wrist-worn actigraphy. Using average quantitative data, the only time swimmers met the NR of 7-9 hours sleep was during low intensity training loads. This is not the first time that differences have been found between athlete sleep perception and objective measures. In a study conducted on 28 elite male cyclists, it was suggested that athletes were inaccurate when assessing their own sleep quality (Lastella et al., 2011).

4.4.5.2 Quality

Roberts et al. (2019) recently reviewed 54 studies and found that athletes were unable to achieve TST and sleep efficiency recommendations, which is further supported by the current study. It has been recommended that adults should achieve a sleep efficiency of at least 85% (Ohayon et al., 2016), below <85% was thought to be abnormal (Cohen et al., 2009). Swimmers in the current study did not meet this recommendation, averaging 75% sleep efficiency and was below this recommendation for all three training loads.

Findings were in agreement of a recent review which found athletes generally struggled to achieve $\geq 85\%$ sleep efficiency during training (Roberts et al., 2019). Despite having significantly more sleep during low training loads, average sleep efficiency was worse during this period (71%). Ohayon et al., (2016) suggested that a sleep efficiency of $\leq 74\%$, indicated bad sleep quality. Thus, swimmers in the current study had bad sleep quality at certain points during the study and findings were worse than what has been found elsewhere (de Mello et al., 2020; Gudmundsdottir, 2020). Previously, swimmers have shown average sleep efficiency below the threshold to be considered normal (de Mello et al., 2020; Lastella et al., 2015). Specifically, de Mello et al. (2020) reported an average sleep efficiency of 82% in 14 Olympic swimmers. However, current results show significantly worse sleep efficiency of studies elsewhere. Even though sleep efficiency decreases with age (Juliff et al., 2015; Ohayon et al., 2004), this was not a possible explanation for current findings. Swimmers were on average aged 20 years, which is younger than the other studies mentioned (aged 22-27 years). Alternatively, the disruptive effect of academic stress on sleep has been shown (Ahrberg et al., 2012), which may explain lower sleep efficiencies found for current swimmers. All swimmers were university students, so academic stress in addition to going out socially, may have influenced these findings.

As sleep efficiency is calculated by dividing TST by TTIB, it would make sense that this sleep parameter would be affected by increased latency (Sargent et al., 2014). Ohayon et al. (2016) suggested an appropriate indicator for good sleep included a sleep latency of ≤ 15 minutes, of which the current study average met. However, average reported sleep latency was mostly quicker than reported elsewhere (Lastella et al., 2015). Long durations of 35-40 minutes latency has been recorded for swimmers specifically (de Mello et al., 2020; Lastella et al., 2015). Similarly to current findings, Surda et al. (2019) examined 20 elite swimmers and reported an average latency of 18.3 minutes. However, authors reported a high sleep efficiency of 91%. With this in mind, it could be said that latency did not affect the sleep efficiency found for current swimmers.

Wakes after sleep onset could increase fragmentation of sleep, impacting sleep quality. Total WASO in the current study (79 minutes) was much higher than that shown in other studies. Looking at other studies in swimmers, average WASO ranged from 14.1- 64.7 minutes (de Mello et al., 2020; Gudmundsdottir, 2020; Lastella et al., 2015; Surda et al., 2019). Specifically, Surda et al. (2019) reported WASO duration of 13.1 minutes, suggesting that this may be the cause of decreased sleep efficiency in this cohort of swimmers. Furthermore, Leeder et al. (2012) found WASO to be similar to the current study, when examining a cohort of 47 Olympic athletes. They found that monitoring WASO with use of wrist-worn actigraphy may potentially be affected by those with larger amounts of movement (Sargent et al., 2016), something which has been found in athletes (Leeder et al., 2012). Overall, greater sleep fragmentation has been reported in athletes (Gupta et al., 2017), which could be the case here and therefore, could explain the high WASO and low sleep efficiency.

4.4.5.3 Leading into Competition

Another risk factor for poor sleep adequacy was competition (Erlacher et al., 2011; Juliff et al., 2015). Both quantity and quality of sleep appeared to be affected leading into competition; TTIB and TST, increased significantly from two weeks to one week before competition. These findings were in support of those by de Mello et al. (2020), who reported significantly more TST before competition, compared to training. A main review found that TST was reduced on the night of competition when compared with previous nights (Roberts et al., 2019). However, these findings cannot be compared to the current study as sleep was not monitored on the night before competition. Alongside this, a lower percentage of swimmers woke feeling fatigued in the week leading into competition, with “1-2 times” being the most reported (52%). Furthermore, sleep efficiency was higher leading into competition (81%) compared to mid-season and may be accountable to taper, and reduction in training load. These findings are in contrast to two large cohort studies by Erlacher et al. (2011) and Juliff et al. (2015), that found 64-65.8% of athletes experienced poor sleep leading into competition ($n = 915$).

That said, total number of awakenings significantly increased leading into competition. Moreover, latency increased and self-reported sleep score fell, leading into one week before competition, although these were not significant. Studies have reported the main issue being not being able to fall asleep leading into competition, and was found most for those competing in individual sports (Erlacher et al., 2011). Nervousness and thoughts about the competition were rated highest for causing these sleep problems (Juliff et al., 2015), which could have been the case in the current study, although this is speculative.

Overall, the fact that TST was below the recommendation of 7-9 hours is important because this can lead to impaired immune function (Besedovsky et al., 2019; Vgontzas et al., 2004), and increased risk of illness (Cohen et al., 2009; Orzech et al., 2014). Even though high WASO and potentially large amounts of movement could have affected sleep efficiency readings, these low results should not be ignored. Sleep inadequacy was paramount in the current study, with swimmers additionally reporting waking feeling fatigued significantly more during high training loads.

4.4.5.4 Effect on URS

Interestingly, visual trends could be seen between self-perceived sleep score and illness status. It has previously been suggested that sleep disturbances can lower immunity and increase chance of illness (Peake et al., 2017). Cohen et al. (2009) also found those with less than seven hours of sleep were 2.94 times more likely to develop a cold than those with eight hours or more. These trends complement that of unpublished work by Harrison (2020), who found runners were twice as likely to present with URI, if they reported poor perceived sleep quality. It was concluded that an individual's perception of sleep quality was the strongest predictor of URI. Lastly, no conclusions could be drawn on the effect of sleep on illness symptoms for the current swimmers. Further work is required to collect more data, which covers both URS and non-URS days.

4.4.6 Strengths, Limitations and Future Work

The current study successfully examined the relationship between training load, mucosal immunity, and sleep over an 8-month training period leading into major competition. Through use of an AIS adapted questionnaire used elsewhere (Fricker et al., 2005), self-perceived illness symptoms, training load and sleep was monitored via a 7-day recall method (Besedovsky et al., 2019). A strength of this was high compliance with questionnaires, plus no missing data gaps that have previously been highlighted elsewhere (Dubnov-Raz, Rinat, et al., 2015). A limitation of monitoring retrospectively for one week, was that these variables could be misjudged, over emphasised or forgotten. However, swimmers were encouraged by the researcher to note down what days they felt poorly in their mobile phones to prompt if needed. Training load monitoring was based on training volume and intensity, alongside RPE (Halson, 2014b). The method used within the current study involved gaining RPE of training intensity by questionnaires and use of a reliable and valid RPE scale (Lagally & Robertson, 2006). In addition to questionnaires, coach derived training load was used to ensure swimmers perceived it similarly, of which it was confirmed they did. Additionally, another strength of the study was the use of objective sleep measurement, in addition to self-reported measures of sleep. The use of wrist-worn actigraphy has been recommended for measures of sleep (Zinkhan et al., 2014) and validated against the gold standard PSG (Quante et al., 2018).

Variation in sIgA was found despite controlling for factors already identified (Chapter 2, page 24), that are known to influence concentration within-subjects. However, one of the strengths to the current study includes that samples were collected in standardised conditions (at a consistent time of day, same day each week, where athletes did not partake in morning training and had the longest rest time between sessions). Samples were taken regularly, every week over the whole duration of 8-months. Furthermore, recommendations of no food or drink being allowed 30 minutes prior to sampling, was followed to ensure samples were not contaminated (Granger et al., 2012). That said, despite being strictly controlled, within-subject variability of sIgA was still apparent which highlights the complex nature of mucosal immunity. Thus, other aspects not controlled in this investigation, could also play an important role in the complex relationship between

training and mucosal immunity. It should be considered that nutritional deficits, for example, vitamin D status, were not examined.

However, to the investigator's knowledge, this was the first study in the UK to examine EBV serostatus in elite national and international swimmers. Swimmers that were EBV seropositive, reported a higher incidence of URS episodes. Despite having significant power for other variables, a limitation of the current study would be the small sample size when grouping swimmers, either by training group, sex, or by EBV serostatus. As previously discussed however, individual data and trends could potentially be more useful to researchers in elite sport. Although findings for the effect of EBV serostatus on URS were underpowered, it could still be an important finding and requires further investigation. Future studies should also consider the use of EBV-DNA as a saliva measurement alongside sIgA to determine whether a relationship between these exists.

4.5 Conclusion

The current study presents a well-controlled, longitudinal observation of mucosal monitoring alongside risk factors associated with URS, in elite national and international swimmers. Findings strengthen the notion that increased training load presents increased number of URS episodes, severity, and duration of symptoms. Despite large within- and between-subject variation, absolute and relative sIgA was significantly lower on weeks where URS were reported, adding further support to the use of sIgA in athlete monitoring. Notably, swimmers regularly slept below the NR and suffered further significant falls in sleep duration, during increased training loads. Furthermore, swimmers suffered inadequate sleep efficiency throughout the entire study period, lower than that published in athletes previously. These findings should promote both the importance of sleep, especially in the avoidance of URS and the beneficial effect it could have on training and performance. In summary, highlighting the athletes at risk and identifying possible risk factors for URS, such as increased training loads and inadequate sleep, may allow for modifications in training or other protective strategies.

CHAPTER 5

Optimisation and Validation of an Enzyme Linked Immunosorbent Assay for Salivary IgA

5.1 Introduction

The enzyme-linked immunosorbent assay (ELISA) is considered the gold standard of immunoassays; providing a highly sensitive and precise method of quantifying many different specific antigens/analytes including proteins, peptides, nucleic acids, and hormones, in a complex sample (Sakamoto et al., 2018). The worldwide research and diagnostic impact of the ELISA has increased since the early 1970's (Engvall & Perlmann, 1971; Lequin, 2005). Now, the ELISA is one of the most extensively used laboratory techniques within research, healthcare, and industry. The underlying principle of ELISA is the specificity of interaction between antibody and antigen. Antibodies with a specific affinity for a particular antigen are used, so that the antibodies only bind to the antigen of interest, making it a powerful tool for measuring a specific analyte (Crowther, 2009). An enzyme conjugated antibody would detect the presence and quantity of specific antigen binding in complex mixtures, without having to separate them. For complex biological matrices especially, such as serum, plasma, blood, urine and saliva, this method is invaluable (Minic & Zivkovic, 2020). Therefore, initially the aim of the present chapter was to develop an in-house ELISA to examine sIgA from saliva samples gathered from swimmers in **Chapter 4**. Following development and optimisation, the assay was validated to ensure reliability for use.

5.1.1 Types of ELISA

5.1.1.1 Direct

The direct ELISA has been labelled the simplest form of ELISA, but is said to have limitations (Crowther, 2009). The direct method involves detection antibody binding directly to the target antigen. So, the antigen of interest is attached to the solid phase and

following a wash, enzyme-labelled antibodies are added. After incubation and sufficient washing to remove any non-bound material, a substrate system is added and colour develops for analysis. This is then done through use of the plate reader when an OD unit is given to quantify colour change. Advantages of the direct ELISA include eliminating cross-reactivity (due to not using a primary and secondary antibody) and speed at which it can be performed, compared to the in-direct method. However, this method has showed low sensitivity compared to others (Alhajj & Farhana, 2022). Furthermore, another major disadvantage includes not being able to use complex samples such as saliva. This is because many other proteins are present within, which could bind and interfere with the target analyte finding.

5.1.1.2 Indirect

The indirect ELISA method follows similar steps to the direct ELISA method mentioned above, however the detection antibody does not directly bind to the target antigen. Two antibodies are used; a primary detection antibody binds to the target antigen and the secondary enzyme-linked antibody must complement and bind to the primary (Alhajj & Farhana, 2022). The secondary detection antibody would be directly against the species in which the first antibody was from (anti-species), and so will bind. In addition, a blocking step should be employed to prevent the labelled antibody binding to the plate. The main advantage of the indirect method, when compared to direct, is that it has higher sensitivity as multiple labelled antibodies can bind to one primary. Additionally, it could be less expensive if making multiple assays for determination of different analytes, as the same labelled antibody can be used for different primary antibodies. Despite this, for the current research only one ELISA needs to be developed and so this did not apply. Plus, due to using two different antibodies, cross-reactivity would be higher; thus, it is important that the antibodies are carefully selected.

5.1.1.3 Sandwich

Unlike the two previously described methods, a capture antibody is coated to a solid phase. The term 'sandwich' ELISA refers to the target antigen being sandwiched between

two layers of antibody (capture antibody- antigen- detection antibody). Following incubation and washing, the remaining surface on each well is blocked to prevent non-specific binding. The antigen of interest would bind to the coating antibody, then an enzyme-labelled detection antibody would bind with the antigen. The major advantage of this method is that the capture antibody selectively binds the target antigen from a complex sample. Subsequent blocking and washing steps can remove unbound material that may interfere with the assay. The capture and detection antibody could be from different animals of the same species or from different species (Crowther, 2009). It should be stated however, that the capture and detection antibodies must recognise different epitopes (binding sites).

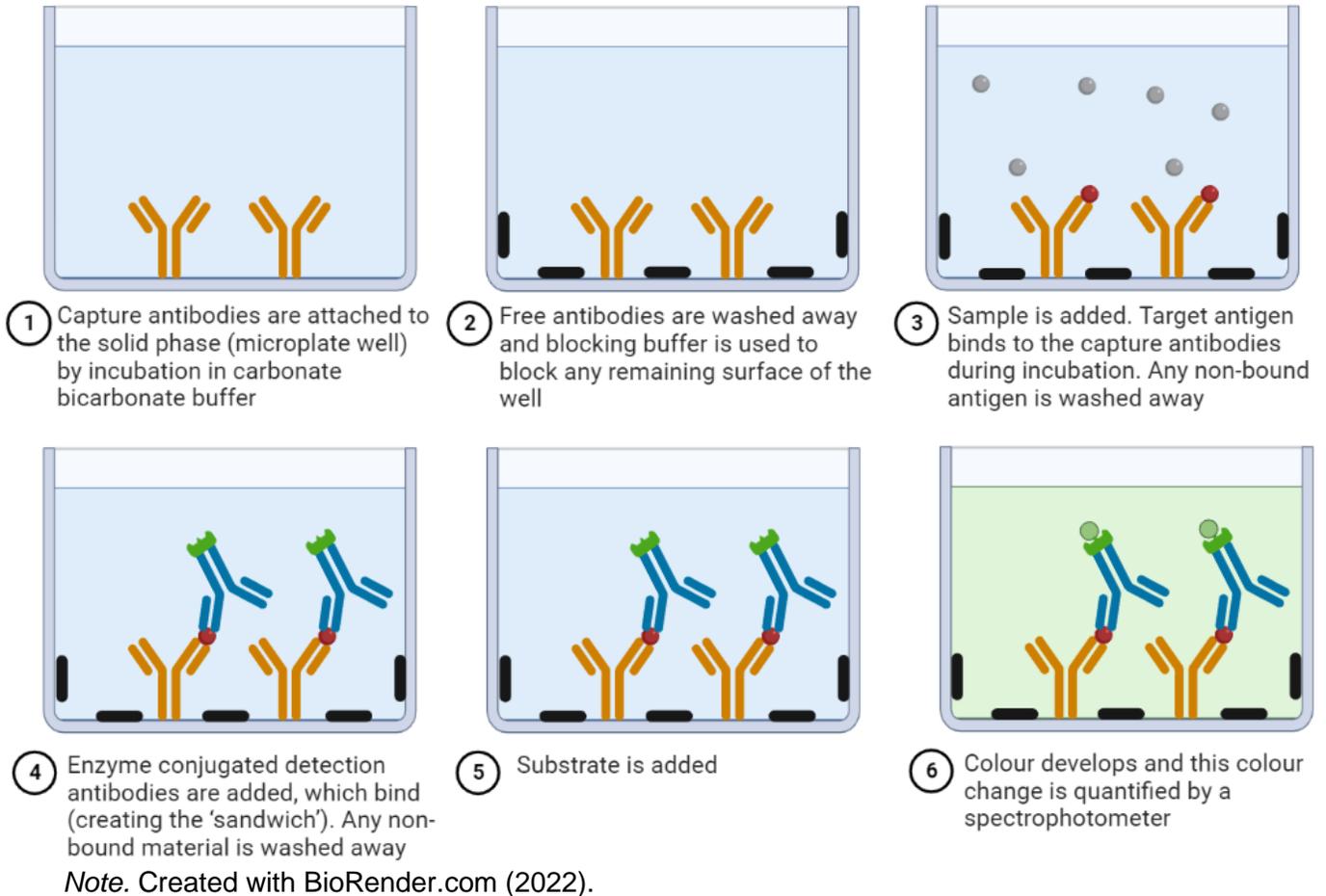
The sandwich ELISA is the most sensitive ELISA, compared to the other methods described. This method would enable the use of complex biological samples and yet would be selective and target the antigen of interest. Moreover, specificity would be higher as the antibodies used are against different epitopes of the target protein (Lugos, 2019; Osmekhina et al., 2010). Therefore, due to saliva being such a complex sample, the sandwich ELISA was chosen for the current study and the method steps can be seen in Figure 5.1.

5.1.1.4 Competitive

Competitive immunoassays are different to those described previously because the detecting antibodies (enzyme-labelled) are added with the sample simultaneously (Crowther, 2009). Here the sample is pre-incubated with labelled antibody and added to the wells, where capture antibodies are bound. Depending on the amount of antigen in the sample, more or less free antibodies will be available to bind the capture antibody. The more antigen there is in the sample, the weaker the signal and so presents a negative slope standard curve (SC), contrasting to the other ELISA methods. This method is most complex to develop in-house due to requirement of highly specific monoclonal antibodies, which are costly. That said, they are widely commercially available and were used in the current thesis for detection of sIgA (Chapter 3, page 54).

Figure 5.1

Chosen method; Sandwich ELISA, adapted from Crowther (2009)



5.1.2 Assay Optimisation

When developing an ELISA, optimal conditions must be established for entire protocol. Each stage of the ELISA should be experimented with in order to gain the most optimal conditions for an in-house assay.

5.1.2.1 Attachment of Capture Antibody on Solid Phase

The most used solid-phase for ELISA would be a 96-well microtiter plate made from polystyrene or polyvinyl chloride (Bio-Rad, 2017), as this surface has a high binding capacity for proteins (Engvall, 2010). Furthermore, it has been suggested, that when

using a spectrophotometer to assess colour development, flat-bottomed wells are recommended (Crowther, 2009). The process of coating the solid phase, would include the capture antibody being attached to the well surface by passive absorption. Most commonly, this is done by addition of the antibody into PBS or carbonate buffer (Minic & Zivkovic, 2020). The type of coating antibody used would be determined by the target analyte. Typical coating conditions involve adding 50-100 μL of coating buffer, containing antigen or antibody at a concentration of 1-10 $\mu\text{g}\cdot\text{mL}^{-1}$ (Bio-Rad, n.d.; Crowther, 2009).

5.1.2.2 Blocking

This crucial step ensures that free binding sites in each well become saturated with blocking buffer to prevent non-specific binding of other irrelevant proteins in the sample, plus this prevents binding of the labelled antibody to the plate (Minic & Zivkovic, 2020). Blocking agents commonly used include, Bovine Serum Albumin (BSA), non-fat dry milk and whole serum (Xiao & Isaacs, 2012). This would prevent the target protein from absorbing non-specifically to the plate surface, reducing overall background noise and increasing assay sensitivity. It has been advised that a greater amount of blocking buffer should be added to the well than coating buffer, to ensure that all attached coating antibody is covered.

5.1.2.3 Preparation of Standards and Samples

Here the specific recognition between antibody and antigen takes place; standards and samples are added to the wells, whereby the coating antibody recognises the specific analyte of interest in the sample and binds. Most often, standards and samples are diluted and this diluent should be as close to the sample matrix as possible (Minic & Zivkovic, 2020). Sample matrix is defined by the type of undiluted biological sample used within the assay e.g., saliva. It has been suggested that PBS with BSA acts as a successful diluent for serum (Minic & Zivkovic, 2020). However, if wanting to examine other matrices, alternative diluents such as PBS, BSA or Tween, should be considered. This is because the sample matrix may not be comparable to the chosen diluent. Therefore, spike and recovery experiments should be carried out to determine whether analyte detection is

affected by differences in standard diluent and biological sample matrices (Thermo Fisher Scientific, 2015). This can help find the right standard diluent, which works correctly with the chosen sample matrix. Thorough detail for these assessments and information on how they are carried out, are presented in Assay Validation (page 116).

5.1.2.4 Preparation of Detection Antibody

The detection antibody would need to recognise a different epitope on the target antigen than the capture antibody does, which would create the ELISA assay sandwich (capture antibody- antigen- detection antibody). The interaction between antibody and antigen are described by specificity and affinity. Specificity refers to whether an antibody binds solely to a unique epitope from a single antigen, or whether it may be cross-reactive. Then, affinity refers to the strength of bond between antibody and antigen (Minic & Zivkovic, 2020). As a result, it would be considered very important to carefully choose the correct detection antibody. There has been ongoing debate on the use of monoclonal antibodies versus polyclonal antibodies for use in ELISA (Bio-Rad, n.d.). Despite monoclonal antibodies having high specificity to a single epitope and consequent low cross-reactivity, polyclonal antibodies have higher affinity due to the recognition of multiple epitopes (Proteintech, 2017). Furthermore, polyclonal antibodies are less affected when labelled, compared to monoclonal antibodies. An enzyme-linked antibody can be useful due to additional detection of the antigen: antibody complex (Crowther, 2009); a conjugated enzyme label for colorimetric detection.

5.1.2.5 Choice of Substrate

With the addition of substrate, colour develops in proportion to the amount of bound analyte. The intensity of colour would give an indication of the amount of analyte present within the wells, which can be quantified using a spectrophotometer (Crowther, 2009). Substrates that work well with HRP include; TMB (3,3',5,5'-Tetramethylbenzidine), ABTS (2,2'-azino-di[3-ethylbenzthiazoline] sulfonate), and OPD (o-phenylenediamine) (Minic & Zivkovic, 2020). Elsewhere, ABTS has been praised as the ideal substrate for enzyme immunoassays with HRP as a marker enzyme. Samples react with ABTS quickly and

have reported reaching stability within 30 minutes, although temperature, exposure to light and time could affect this (Minic & Zivkovic, 2020).

5.1.2.6 Washing

Washing steps are crucial in order to separate bound and unbound reagents. This process includes emptying the wells and adding wash buffer, at least three times into each well. Typically, PBS (0.1 M, pH 7.4) is used, however sometimes detergents such as Tween 20 (0.05%) are added (Crowther, 2009). There are a few different wash methods, such as; dipping the whole plate in a large volume of buffer, using a nozzle wash bottle, multichannel pipet, or specialist plate washer.

5.1.2.7 Incubation Times

Incubation times between antigen and antibodies would depend on their distribution, time, temperature, and pH (Crowther, 2009). Coating time and temperature usually involve incubation at 37°C for 1-3 hours, overnight at 4°C, or a combination of the two (Crowther, 2009). Elsewhere, room temperature incubations have also been advised (Kohl & Ascoli, 2017). For the current research, plates were coated and incubated overnight in the fridge at 4°C. Significant incubation times are needed following the addition of standards, samples, and detection antibody in order to allow sufficient binding to occur.

5.1.2.8 Reagents handling

It must be ensured that the reagents are consistent and accessible (Lee et al., 2006). Things to consider would be; antibody instability and batch to batch variations. Proteins require refrigerated transport and storage, which cannot always be guaranteed (Sakamoto et al., 2018). From delivery, all orders should be dealt with and stored according to guidelines as quickly as possible. Batch to batch variations in reagents should be considered, as some require different starting concentrations. Furthermore, one solution for ELISA troubleshooting included avoiding the use of old buffers (R & D Systems, 2011).

5.1.3 Assay Validation

The quality of in-house ELISA methods vary widely, which has urged the need for more rigorous testing of assay performance (Andreasson et al., 2015). Previously, Lee et al., (2006) outlined several factors which helped create an assay to be “fit for purpose”. Specifically, method validation requires continuous reassessment of data and optimisation of the assay protocol (Lee et al., 2006). It is evident from the literature that during the development on an ELISA, completion of both optimisation and validation can happen alongside one another (Minic & Zivkovic, 2020). However, the next section will include comprehensive experiments outlined by Andreasson et al. (2015), to assess precision, accuracy, sensitivity, specificity, range, recovery, linearity, and robustness; these are to determine assay consistency and reproducibility.

5.1.3.1 Precision

Precision can be regarded as reproducibility of a measurement, and determines how close replicate measurements of the same sample are to each other or deviate from the mean (Minic & Zivkovic, 2020). There can be a statistical measure of variation in samples within the same assay (intra-assay) or from assay to assay (inter-assay); which can be reported by standard deviation but usually by coefficient of variation (CV%) (Andreasson et al., 2015). The degree to which duplicates differ can be expressed by calculating the standard deviation between replicates and converting to the CV% ((Standard Deviation ÷ Mean) X 100) (Schultheiss & Stanton, 2009).

Intra-assay validation assesses reproducibility between wells within one assay. Generally for an intra-assay variation assessment, you would cover a large measuring range (ISO, 1994), however if the concentration of standards cover a range that is relevant for the purpose, it has been suggested that two samples may be enough (Andreasson et al., 2015). Elsewhere it has been suggested assessing a minimum of three concentrations, run in duplicate (Minic & Zivkovic, 2020). Multiple sample replicates should be run on the same assay, and CV ≤10% is traditionally accepted. To assess inter-assay variation,

there should be multiple runs of a sample on different assays. This would be to determine reliability of data and whether it could be compared across different days or on different plates, and occasionally different laboratories (Minic & Zivkovic, 2020). Concentrations between assays could easily be influenced, perhaps from human error or repeated use of the same reagents, but should show a CV $\leq 15\%$.

5.1.3.2 Accuracy

Accuracy refers to the ability to measure the true value of the analyte, determined by the deviation from the actual nominal concentration (Minic & Zivkovic, 2020). Several experimental methods are used for this, including calibration standards and recovery (Andreasson et al., 2015). This could be assessed with samples spiked with known amounts of analyte, presenting the difference between the experimental value and the nominal value. Where possible, results should ideally be compared with values obtained from a 'gold standard' assay.

5.1.3.3 Sensitivity and Detection Limits

Strictly speaking, sensitivity refers to the ability of the assay to distinguish between sequential concentrations of analyte. Sensitivity can be assessed through the steepness of the SC; a steep curve has high sensitivity, whilst a flatter curve has low sensitivity. Although often, it can be often taken to mean limit of detection (LD); defined as the lowest level of detectable analyte, that can be distinguished from background.

5.1.3.4 Specificity

Specificity, sometimes referred to as selectivity (Andreasson et al., 2015), involves the target analyte being detected without cross-reactivity with additional related proteins. If the assay were to pick up additional analytes, results would have inaccurate quantification and have false positivity.

5.1.3.5 Detectable Range

A reportable assay range refers to the lower and upper limits of quantification (LLOQ and ULOQ retrospectively), and it is considered that within this range, results will be accurate and precise (Lee et al., 2006). This should be established, so that the range of concentrations for the assay to function reliably are known (Minic & Zivkovic, 2020). Firstly, the target range must be identified within the literature (93 - 974 $\mu\text{g}\cdot\text{mL}^{-1}$ by Salimetrics (2015)). The LLOQ is the lowest concentration that can be reliably measured on the linear part of the curve. Then the ULOQ is the highest concentration measured on the linear part of the curve, of which three SDs have been subtracted (Minic & Zivkovic, 2020).

5.1.3.6 Spike and Recovery

Spiking samples involves adding several known amounts of analyte to sample diluent or sample. With this assessment, it is explored whether percent recovery obtained from standard diluent can be compared to the sample matrix. It is recommended to try and match standard diluent as closely as possible to the sample matrix (Thermo Fisher Scientific, n.d.). Observed recovery of the spike between 80-120%, could be accepted. Recovery percentages that exceed this, would imply significant differences between the sample matrix and the standard diluent. If large matrix effects are shown, adjustment would include introducing dilutions of sample in the diluent, finding alternate diluents that are like the sample matrix, or using sample matrices that are free of detectable analyte.

5.1.3.7 Dilution of Linearity vs. Parallelism

Linearity of dilution assessment determines whether a spiked sample matrix with the target analyte above the ULOQ, could still be quantified following dilution. For example, examining whether a 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ sample be quantified accurately when diluted 1:2, on an assay that has a range of 12.5 – 700 $\mu\text{g}\cdot\text{mL}^{-1}$. The extent of spike response should be linear, with little or no deviation in concentration within the desired assay range, confirming a high degree of accuracy at varying dilutions. Similarly, to spike and recovery assessment, recovery would be accepted within 80-120%; outside of this suggests poor

linearity and could indicate an imbalance between the sample matrix and standard diluent (Thermo Fisher Scientific, 2010). Parallelism determines whether real samples containing high endogenous analyte provide the same degree of detection, following dilution. The key difference between dilution linearity, is the use of a real sample containing naturally high concentration of analyte. Parallelism between dilution curves can then be examined. However, samples identified to have high concentration of endogenous analyte would need to be gained for this to be examined.

5.1.3.8 Robustness

Robustness refers to the ability of the method to be unaffected by small variations. For in-house assays, robustness should be experimented as part of method optimisation and validation. As an example, buffer temperature, incubation temperature, incubation times and number of washes could all create deviations in performance (Minic & Zivkovic, 2020).

5.2 Methods and Materials

5.2.1 Materials

For the current assay, sterile, Polyvinyl chloride (VC), 96-well flat bottom microtiter plates were used. A polyclonal rabbit anti-human IgA (α -chain specific) antibody produced in rabbit (polyclonal), IgG fraction of antiserum, was used as the capture antibody in the current research, which was because it reacts specifically with human IgA when tested against purified IgA, IgG and IgM. A commercially available 0.05 M carbonate-bicarbonate buffer (pH 9.6) was used, and was reported as suitable for protein coating procedures for ELISA. Blocking buffer used was PBS, with 10% Bovine Serum Albumin (BSA). Standards were created using purified IgA from Human Colostrum, with a concentration of $2.3 \text{ mg}\cdot\text{mL}^{-1}$. As detection antibody, a F(ab')₂-Goat anti-Human IgA Secondary Antibody conjugated with horseradish peroxidase (HRP) was used, at a final concentration of $1 \text{ mg}\cdot\text{mL}^{-1}$. This secondary antibody was affinity-purified with well characterised specificity for human immunoglobulins, thus providing greater specificity and sensitivity. In addition, use of a polyclonal antibody offered financial benefits over using a primary and secondary enzyme-conjugated antibody, as well as being less effected when labelled with HRP. Finally, the wash used for current research was 0.01 M PBS, with 0.05% Tween 20 (pH 7.4). A summary of materials, plus more information can be seen in Table 5.1.

Table 5.1

Materials used for in-house ELISA for detection of sIgA

Item	Company	Order code	Link
Anti-human IgA (a-chain specific) antibody produced in rabbit (2mL)	Sigma	I9889-2ML	http://www.sigmaaldrich.com/catalog/product/sigma/i9889?lang=en&region=GB&cm_sp=Insite-_-prodRecCold_xorders-_-prodRecCold2-1
PBS with 10% Serum bovine albumin (500mL) dilute 10x =10L	Sigma	SRE0036-500ML	http://www.sigmaaldrich.com/catalog/product/sigma/sre0036?lang=en&region=GB
F(ab') ₂ -Goat anti-Human IgA Secondary Antibody, HRP (500µg)	ThermoFisher Scientific	A24458	https://www.thermofisher.com/antibody/product/Goat-anti-Human-IgA-Secondary-Antibody-Polyclonal/A24458?pluginName
Carbonate-Bicarbonate Buffer (100 capsules)	Sigma	C3041-100CAP	http://www.sigmaaldrich.com/catalog/product/sigma/c3041?lang=en&region=GB&cm_sp=Insite-_-prodRecCold_xviews-_-prodRecCold10-6
Purified Human IgA	Sigma	I2636-5MG	http://www.sigmaaldrich.com/catalog/product/sigma/i2636?lang=en&region=GB
Phosphate buffered saline tablets (100)	Sigma	P4417-100TAB	http://www.sigmaaldrich.com/catalog/product/sigma/p4417?lang=en&region=GB
Phosphate buffered saliva, pH 7.4, contains TWEEN® 20, dry powder	Sigma	P3563-10PAK	http://www.sigmaaldrich.com/catalog/product/sigma/p3563?lang=en&region=GB
ABTS tablets	Sigma	000000011204521001	http://www.sigmaaldrich.com/catalog/product/roche/abtsro?lang=en&region=GB
ABTS buffer	Sigma	000000011204530001	http://www.sigmaaldrich.com/catalog/product/roche/11204530001?lang=en&region=GB&cm_sp=Insite-_-prodRecCold_xorders-_-prodRecCold2-2
Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate	ThermoFisher Scientific	167008	https://www.thermofisher.com/order/catalog/product/167008?SID=srch-hj-167008

5.2.2 Assay Protocol

On the day before running the assay, Anti-Human IgA (α -chain specific) coating antibody, was prepared in carbonate-bicarbonate buffer (0.05 M, pH 9.6) at a 1:2000 dilution. A total of 100 μ L was added to each well of a 96-well microtiter plate. The plate was incubated for one hour at 23°C and then overnight at 4°C. The next day, any antibody that was unbound was then removed by washing the plate 3x with PBS, 0.05% Tween 20 (pH 7.4), using the blotting and multichannel pipet method. Well blocking was carried out with 150 μ L PBS, 10% BSA for 10 minutes at 23°C. Following another 3x plate wash, 100 μ L of standards, samples and controls were added into appropriate wells and incubated for 90 minutes at 23°C. Standards were prepared in PBS (Assay 1), with a range of 0-400 μ g·mL⁻¹. Note, following a matrix effect assessment, the standard curve was then prepared in 'IgA-blank' saliva, with a range of 0- 800 μ g·mL⁻¹ (Assay 2). Importantly, 3x washes were carried out to remove any unbound IgA. To complete the 'sandwich', 100 μ L of PBS with detection antibody at a 1:2000 dilution was added to each well and then incubated for 90 minutes at 23°C. After a final 6x wash, 100 μ L ABTS substrate solution was added to each well. The chosen ABTS solution had a light-green colour and should be analysed at 405nm. Following a \leq 20-minute incubation in the dark, absorbance was measured by a plate reader (405 nm, Multiskan™). This full protocol, with timings, can be seen in Appendix E.

5.2.3 Assay Optimisation

All assay optimisation experiments were carried out following the above protocol. Firstly, the optimal concentration of capture antibody was determined by attaching multiple dilutions to the solid-phase. Following an incubation with capture antibody, it has been suggested that the assay would be stable for use for up to 2 weeks (Martin et al., 2000), however for present research they were used the following day. In order to determine optimal coating antibody concentration, multiple dilutions of the stock anti-IgA capture antibody were examined. Each millilitre of antiserum contained 2.5-5.0 mg of specific antibody. Concentrations and dilutions of antibody analysed for the current research are presented in Table 5.2.

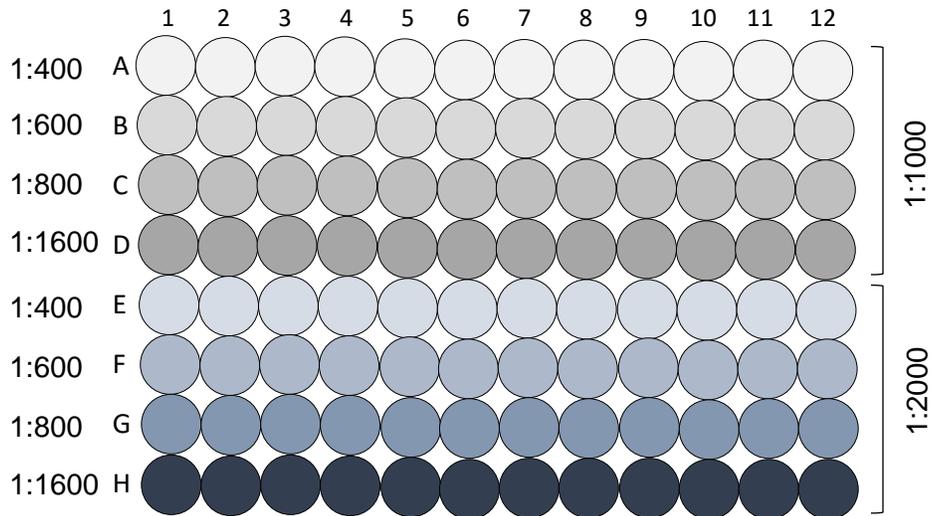
Table 5.2

Tested dilution factors of capture antibody, concentration range and dilution factors

Dilution Factor	Buffer (mL)	Anti-Human IgA (μL)	Concentration of anti-IgA ($\mu\text{g}\cdot\text{mL}^{-1}$)
1:50	19.6	400.0	50.00-100.00
1:100	19.8	200.0	25.00- 50
1:200	19.9	100.0	12.50-25.00
1:400	19.9	50.0	6.25-12.50
1:600	19.9	37.5	4.69- 9.37
1:800	19.9	25.0	3.12- 6.26
1:1000	19.9	20.0	2.50- 5.00
1:1600	19.9	12.5	1.56-3.13
1:2000	19.9	10.0	1.25- 2.50
1:10000	20.0	2.0	0.25- 0.50

Alongside this, differing dilutions of detection antibody were examined through use of checkerboard titration assays, an example plate layout is shown in Figure 5.2. Standard curves were made originally in PBS (Assay 1), and different ranges of SC were examined ($6.25\text{-}50\ \mu\text{g}\cdot\text{mL}^{-1}$, $12.5\text{-}400\ \mu\text{g}\cdot\text{mL}^{-1}$, $50\text{-}800\ \mu\text{g}\cdot\text{mL}^{-1}$). The development of the SC with titrations of coating and detection antibody, would help identify the range of the current assay. As previously mentioned, it is important to try to match the standard diluent as closely as possible to the matrix of the sample. To assess matrix effects, a full standard curve was run in both PBS and saliva, from recruited participants who were IgA deficient to determine differences. Throughout assay optimisation, the number of washes (3-6x) and length of incubation times were examined. For example, for the current assay, incubation times following the addition of substrate were examined at 10, 15 and 20 minutes following every assay to explore potential differences.

Figure 5.2

Assay template layout

Note. Each row represented a different coating dilution, either 1:400, 1:600, 1:800, 1:1600. Then detection antibody dilutions of 1:1000 and 1:2000, were used.

5.2.4 Assay Validation

To examine validity and reliability of assay performance, the assay protocol was additionally followed whilst examining the outcomes previously explained for assay validation. To assess precision, three different standard concentrations were used to determine intra-assay CV. Each concentration was tested by ten replicates, to produce statistically better results for calculation of the standard deviation and subsequent CV. Concentrations of 50, 100 and 200 $\mu\text{g}\cdot\text{mL}^{-1}$ were used; a CV $\leq 10\%$ was accepted. For the investigation of inter-assay variation for current research, the same saliva sample was used across 12 different assays and labelled as quality control (QC); a CV $\leq 15\%$ was accepted. Regarding spike and recovery assessments, known amounts of analyte IgA, were spiked into sample matrix (saliva) and standard diluent (PBS). This was to explore whether recovery obtained from PBS could be compared to the sample matrix, between 80-120% was accepted. Lastly, linearity of dilution experiments would be important if samples had sufficiently high endogenous concentrations of sIgA, however this was not the case for the current research.

5.2.5 Participant Recruitment

Six IgA deficient participants (n = 2 male; n = 4 female) were recruited via methods identified in Chapter 3 (page 53). Generally, serum IgA concentration of $<7 \text{ mg}\cdot\text{dL}^{-1}$ is considered for diagnosis of deficiency, since this is the lowest detectable limit established by most laboratories (Yel, 2010). Despite participants being clinically diagnosed with an IgA deficiency, most are tested by serum IgA concentration and therefore deficiency may not be transferable to the concentration found in saliva. Therefore, a commercially available ELISA was used to determine those with undetectable sIgA.

5.2.6 Sample Collection and Storage

Once informed consent was obtained, saliva sample pots were sent via post with instructions for saliva collection; further detail has been outlined previously in Chapter 3 (page 53). Two participants had undetectable IgA concentration in saliva, below $15 \mu\text{g}\cdot\text{mL}^{-1}$ and so were used as the 'IgA-blank' diluent for assay optimisation and validation (Assay 2).

5.2.7 Reagent Storage

Reconstitution and storage instructions provided with each reagent were followed, in order to guarantee performance. All buffer use by dates were checked and followed. To uphold good practice, most buffers for the current assay were made up the day before. If several assays were being run over several weeks, PBS, coating and wash buffers would be made and used for up to 2 weeks only before being re-made.

5.2.8 Analysis

Raw data files were examined and examined within Excel (Microsoft). All graphs were made using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). Using the Beer-Lambert Law, the relation of absorbance to the concentration of the analyte was plotted and the calibration curve was obtained. The absorbance value OD served as the basis to interpret sIgA concentration. Zero control was not deducted from other concentration points, so when plotting data, the background noise and baseline control could be examined.

5.3 Results

A total of 70 experiments were carried out for the development of the current in-house ELISA for detection of sIgA.

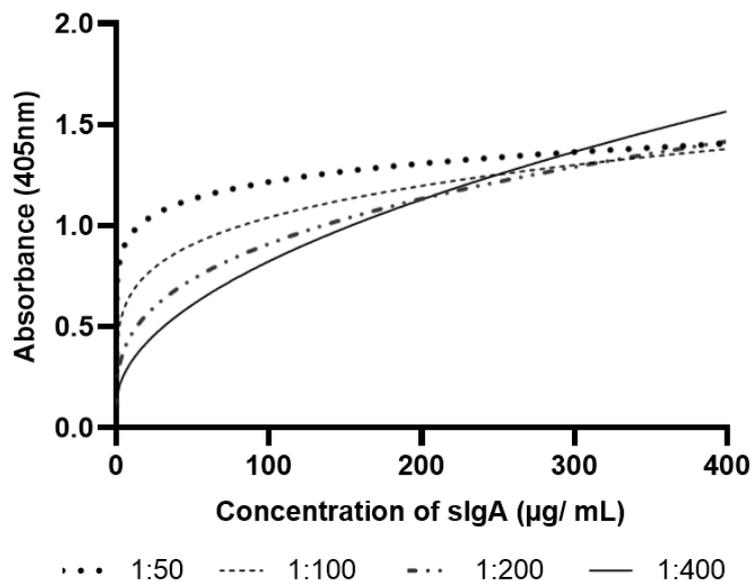
5.3.1 Assay 1

5.3.1.1 Determination of Capture Antibody

Standard curves were produced for four different coating dilutions in triplicate; 1:50, 1:100, 1:200 and 1:400. Findings presented in Figure 5.3; interpolated SC are presented so comparisons can be made between the four different coating dilutions. The lowest dilutions had flatter SCs and low sensitivity, compared to the highest dilution examined (1:400). In addition, absorbance OD values for the zero control were much higher showing large background noise. Therefore, the highest dilution of coating antibody was most effective (1:400) in showing defined differences between SC concentrations and high sensitivity. That said, this assay was unable to significantly detect a difference in lower concentrations of sIgA between $25\text{-}50\ \mu\text{g}\cdot\text{mL}^{-1}$, therefore lower concentrations of standard were examined too.

Figure 5.3

Standard curve ($25\text{-}50\ \mu\text{g}\cdot\text{mL}^{-1}$) for sIgA using four different dilutions of coating antibody

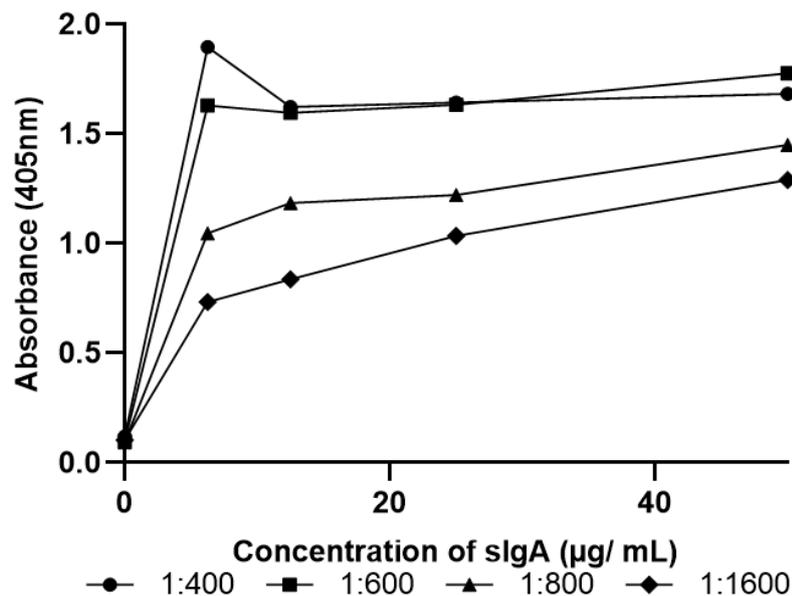


Note. Four SCs shown with coating dilutions 1:50, 1:100, 1:200, 1:400.

Next, higher coating dilutions were examined (1:400, 1:600, 1:800, and 1:1600), but a lower range of standards were used ($6.25\text{-}50\ \mu\text{g}\cdot\text{mL}^{-1}$) to examine sensitivity at lower concentrations. Interpolation of the SC could not be achieved for coating dilutions 1:400 and 1:600, due to OD values being irregular. Flat curves can be seen from these concentrations, showing an inability to determine differences between small amounts of sIgA concentration. Thus, results showed higher dilutions (1:800 and 1:1600) of coating antibody were most effective in producing high sensitivity at the lower end of the standard range (Figure 5.4). An example of a completed assay examining five SCs with different capture antibody dilutions, can be seen in Figure 5.5.

Figure 5.4

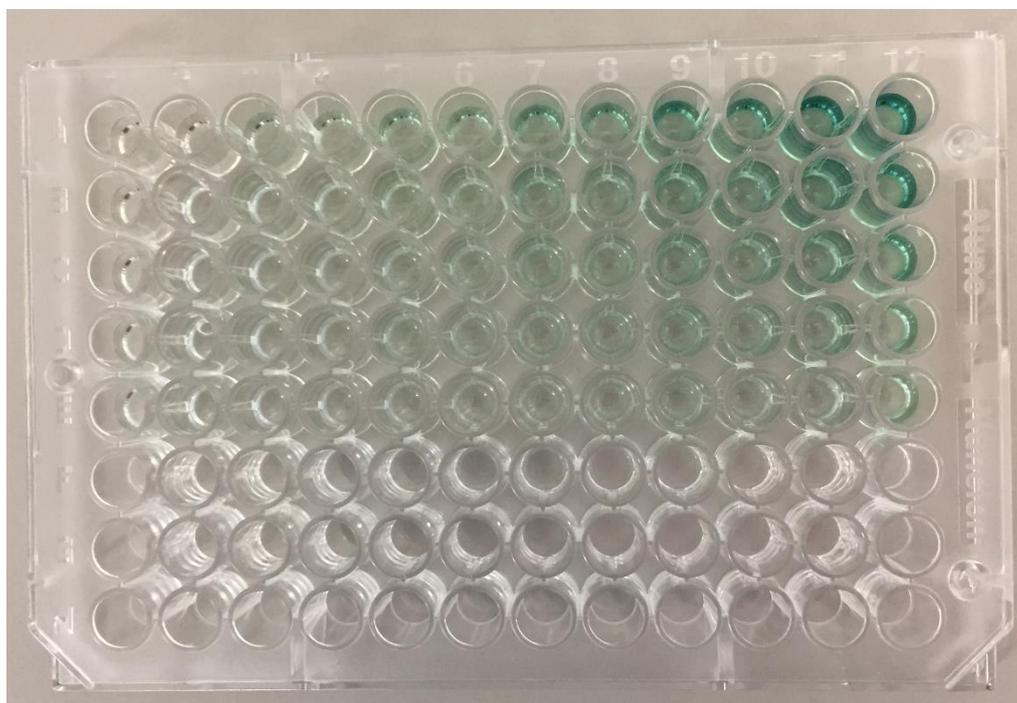
Low range standard curve ($6.25\text{-}50\ \mu\text{g}\cdot\text{mL}^{-1}$) for sIgA using four different dilutions of coating antibody



Note. Four SCs shown with coating dilutions 1:400, 1:600, 1:800, 1:1600.

Figure 5.5

Image of completed assay, examining five coating antibody dilutions



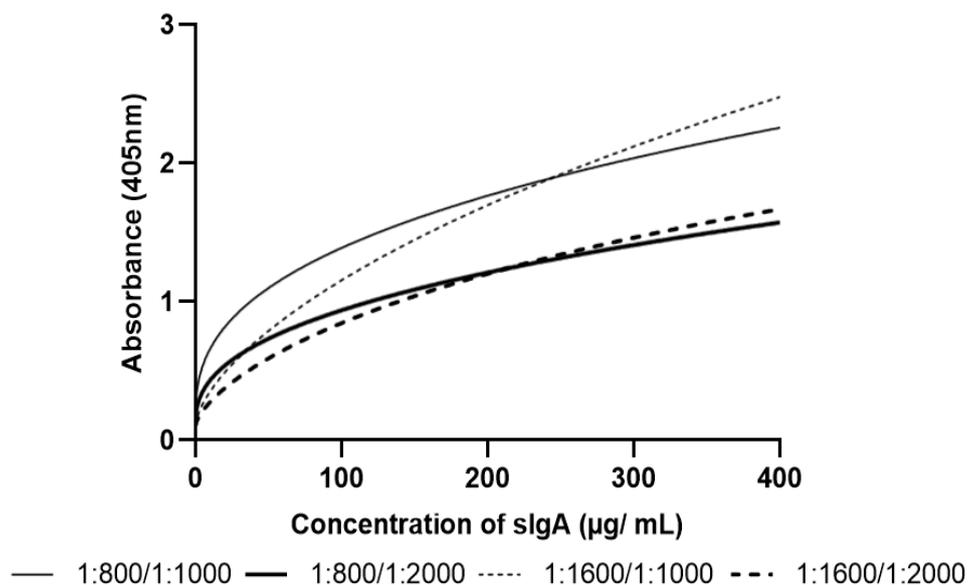
Note. Five SCs run in duplicate, examining five different coating antibody dilutions.

5.3.1.2 Determination of Conjugated Detection Antibody

Determination of coating antibody and conjugated detection antibody were examined together through use of methods previously identified, checkerboard titration (Figure 5.2). Coating dilutions 1:800 and 1:1600 were performed alongside two different detection antibody dilutions (1:1000 and 1:2000). This assay was run with a large range SC, 12.5-400 $\mu\text{g}\cdot\text{mL}^{-1}$. Interpolated SCs can be seen in Figure 5.6; successful SC results were presented for coating concentration 1:800 and 1:600. These showed clear definition between concentrations on the SC, which were separated by at least 0.2 OD units. When examining conjugate antibody dilutions; 1:1000 caused OD to exceed 2 and therefore was considered unreliable for use. It was debatable whether a differentiation could be made between 12.5 and 25 $\mu\text{g}/\text{mL}$, however arguably this low range of IgA detection would not be needed (norm ranges 93 – 974 $\mu\text{g}\cdot\text{mL}^{-1}$ (Salimetrics, 2015)). The accepted conditions producing the most optimal SC were as follows; 1:800 coating dilution with a conjugated antibody dilution of 1:2000 (Figure 5.6).

Figure 5.6

Standard curve (12.5-400 $\mu\text{g}\cdot\text{mL}^{-1}$) for sIgA following checkerboard titration assay



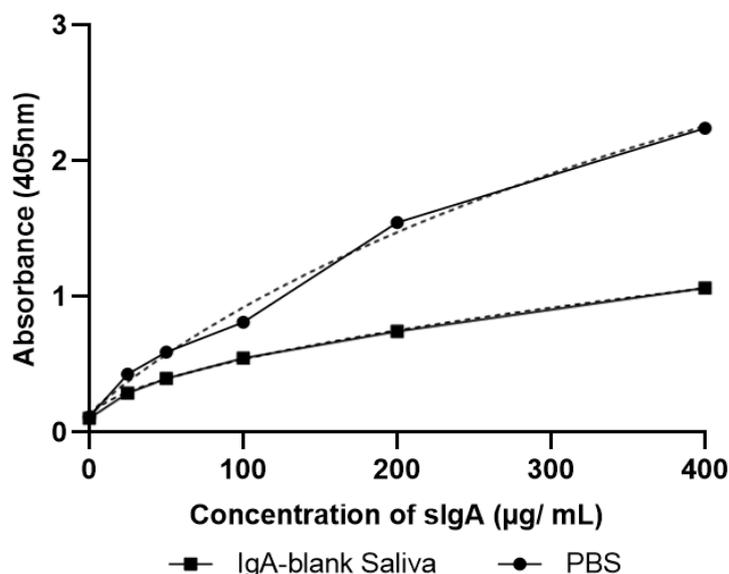
Note. Four SCs shown with coating dilutions 1:800 and 1:1600, and conjugated antibody dilutions 1:1000 and 1:2000. Accepted conditions were 1:800 coating dilution with a conjugated antibody dilution of 1:2000.

5.3.1.3 Assessing Matrix Effects

Results showed that an SC in saliva could not be compared to an SC made in PBS (Figure 5.7) and therefore was to be further examined, through spike and recovery assessment.

Figure 5.7

Standard curve for sIgA in PBS and IgA-blank saliva



Note. Standard curves made in both IgA-blank saliva and PBS, with 1:800 coating dilution and 1:2000 conjugated detection antibody dilution

5.3.1.4 Spike and Recovery

Three different quantities of spike were prepared in IgA-deficient saliva samples and PBS. Results can be seen in Table 5.3, showing large matrix effects. More specifically, for low concentration spikes (50 and 100 $\mu\text{g}\cdot\text{mL}^{-1}$) in PBS, recovery was $\leq 50\%$ (half that of the expected concentration). Findings showed that standards produced in PBS would not be comparable to saliva, the target matrix. Importantly, the IgA-blank saliva collected from deficient participants showed recovery values of 95-113%, which was within the acceptable range. Following this, it was clear that use of PBS was not transferable to saliva due to matrix effects. Therefore, all above experiments were repeated and assay optimisation was completed with standards in the sample matrix, defined as Assay 2.

Table 5.3

Spike and recovery percentages from two diluents

Sample	Spike ($\mu\text{g}\cdot\text{mL}^{-1}$)	Observed ($\mu\text{g}\cdot\text{mL}^{-1}$)	Recovery %
PBS	50	25	49
	100	50	50
	200	134	67
Saliva (undiluted)	50	56	112
	100	95	95
	200	227	113

Note. Spike and recovery (%) obtained from saliva, compared to both PBS and a different 'IgA-blank' saliva.

5.3.2 Assay 25.3.2.1 Optimisation of capture and conjugated detection antibody

Due to the creation of a new standard curve using 'IgA-blank' saliva as diluent, elements of the assay development had to be repeated; checkerboard titration assays to assess coating and conjugated antibody dilutions. In order to see whether the cost per assay could be lower, another checkerboard titration assay was completed using an even higher dilution of both capture antibody and conjugated detection antibody.

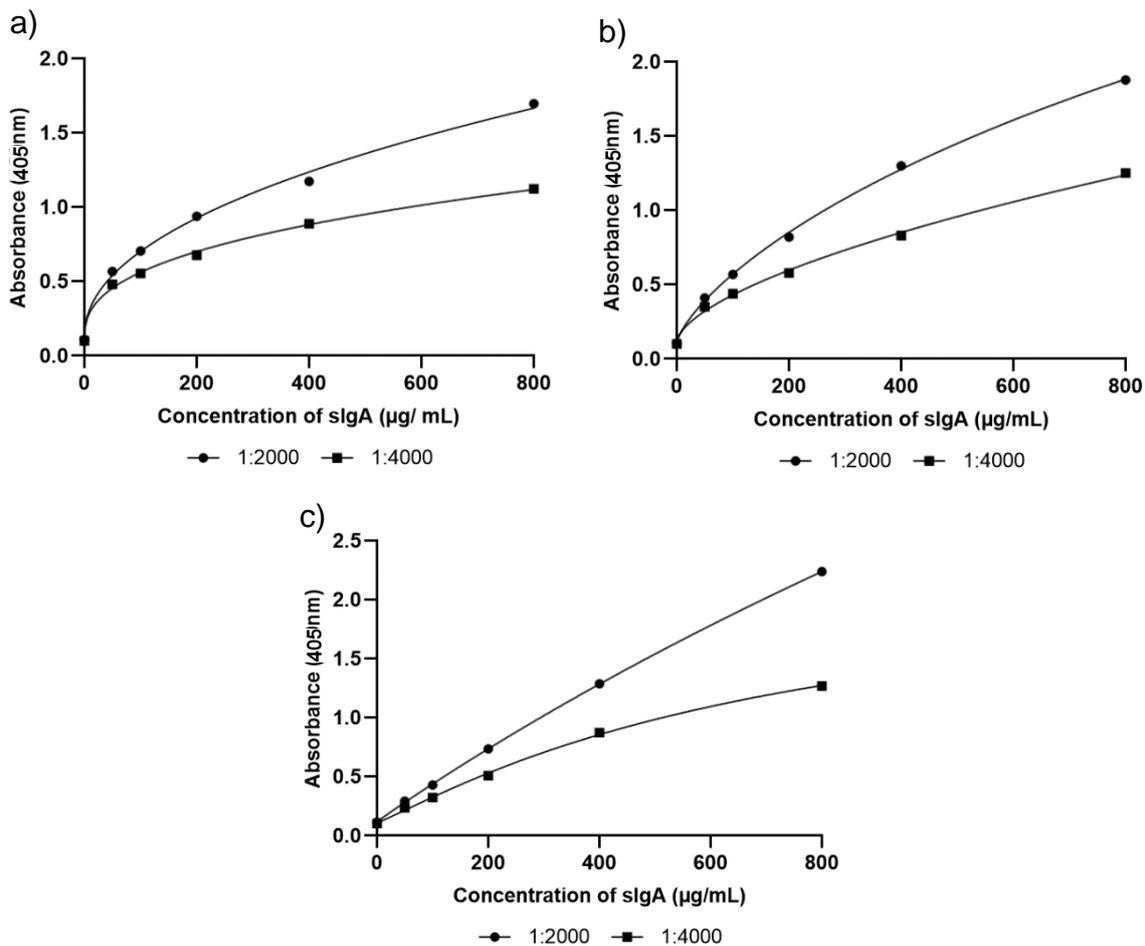
Results showed that for all three coating antibody conditions, there was less discrimination between sequential sIgA concentrations for the 1:4000 dilution of conjugated detection antibody; suggesting concentration was not enough to initiate a steady OD increase. Therefore, the 1:4000 dilution of detection antibody offered less sensitivity, than the alternative 1:2000 dilution for all three SCs and was not explored further. The 1:1000 coating dilution had the lowest detection limit, showing high sensitivity for the lower concentration range, however sensitivity decreased with increased sIgA concentrations. Lower sIgA limits were not clinically relevant for the current research requirements.

Overall, the 1:2000 and 1:10000 coating dilutions provided the greatest discrimination between sequential concentrations of sIgA. Both efficiently detected a range of 100-400 $\mu\text{g}\cdot\text{mL}^{-1}$. Despite being the most linear, the 1:10000 assay showed OD values that exceeded 2. It was therefore considered that concentrations above 400 $\mu\text{g}\cdot\text{mL}^{-1}$ could

not be deemed accurate. Therefore the 1:2000 dilution coating antibody, with 1:2000 detection antibody was accepted for use (Figure 5.8c). This condition was most optimal showing the largest OD separation between concentrations of the desired range (100-800 $\mu\text{g}\cdot\text{mL}^{-1}$). This assay reported a background noise OD value of ≤ 0.09 for the zero control.

Figure 5.8

Interpolated standard curves of sIgA following checkerboard titration assay



Note. Graphs show interpolated standard curves for both concentrations of conjugated antibody, 1:2000 and 1:4000. The three graphs show differing coating dilutions of a) 1:1000, b) 1:2000 and c) 1:10000.

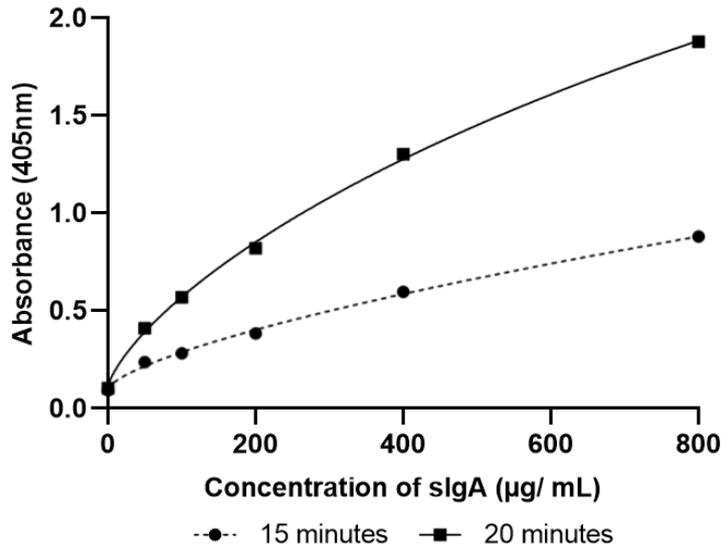
5.3.2.2 Incubation Times

Following the addition of substrate, quantification with use of the plate reader was completed every 5 minutes; 15 minutes was the minimum requirement for acceptable OD values. Therefore, moving forward every assay was analysed at 15 and 20 minutes (Figure 5.9). Further definition between sequential concentrations was always

achieved following incubation with substrate for 20 minutes and so was used for all analyses.

Figure 5.9

Standard curves for sIgA shown with two different substrate incubation times



Note. Standard curves showing the differences between 15- and 20-minute incubations with substrate, before quantification with a plate reader.

5.3.2.3 Precision

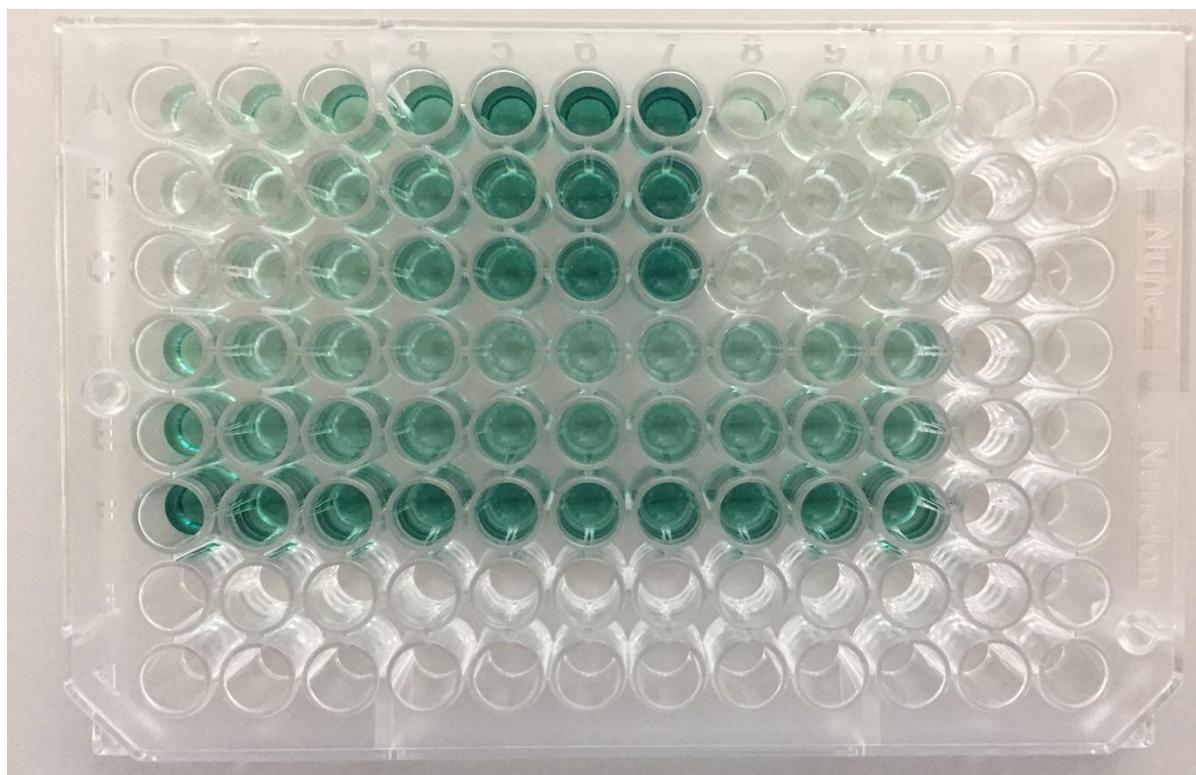
Following experimentation of three concentrations tested with ten replicates (Figure 5.10), results for intra-assay variability are shown in Table 5.4. Findings showed a CV of 10-11%. Following another experiment, 20 natural saliva samples were measured in duplicate. It could be argued that this is a more accurate representation of intra-assay variation due to the likelihood of duplication being used throughout testing. Average CV for this experiment was 12%. Through use of a QC saliva sample in 10 individual assays, inter-assay CV was reported as 45%. This variation was very high and was outside of acceptable guidelines.

Table 5.4

Precision assay, assessing intra-assay variability

Sample ($\mu\text{g}\cdot\text{mL}^{-1}$)	Mean ($\mu\text{g}\cdot\text{mL}^{-1}$)	Standard Deviation (SD)	CV %
50	58	6	11
100	97	11	11
200	196	19	10

Figure 5.10

Image of completed precision assay

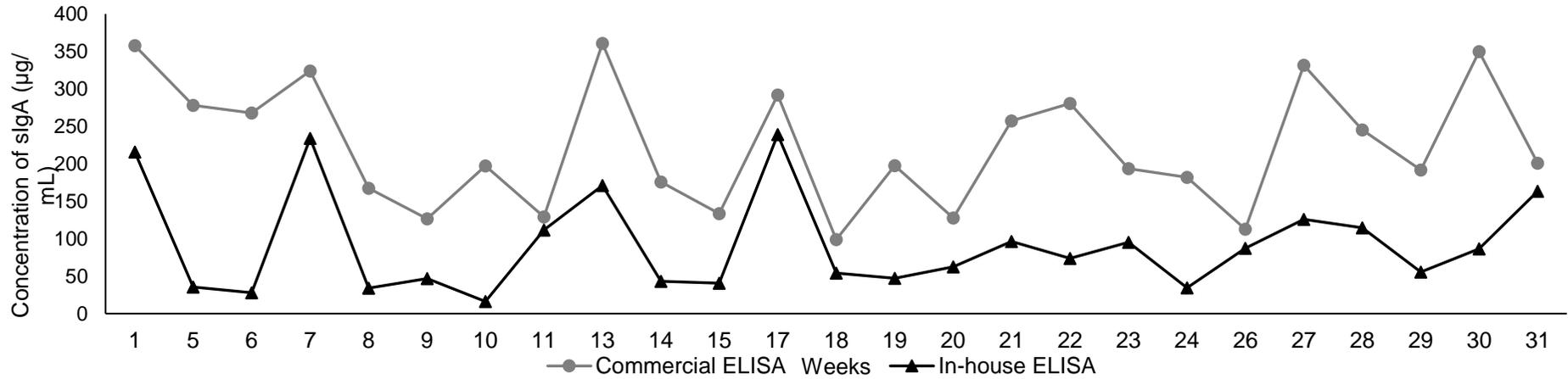
Note. Seven-point SC was run in triplicate, with 10 replicates of three different concentrations to examine precision.

5.3.2.4 Accuracy

When compared to a commercial sIgA assay, it was found that the in-house ELISA was less successful in detecting low concentrations of sIgA. For example, the in-house assay was only able to detect two of the sIgA deficient participants, those above $70 \mu\text{g}\cdot\text{mL}^{-1}$. However due to the required detection range for the current thesis, this was not seen as a limitation. Despite sIgA concentration from the in-house assay being lower than that of the commercial assay (Figure 5.11), Spearman correlation analysis presented a significant correlation between the two assays ($r_{s(25)} = .479$, $p = .015$). Variation from the average IgA concentration plotted over the 8-month season, with comparison of commercial and in-house ELISA can also be seen (Figure 5.12).

Figure 5.11

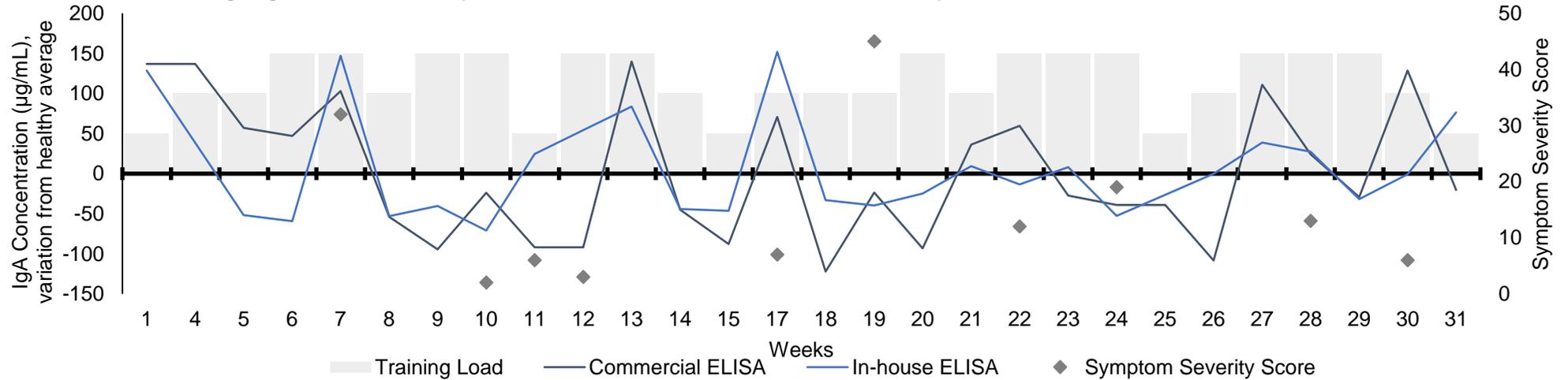
Comparison of commercial and in-house ELISA



Note. Salivary IgA concentration plotted over 31 weeks from one swimmer from Chapter 4, via both the commercial and in-house ELISA (n = 1). Data was significantly correlated.

Figure 5.12

Variation from the average IgA concentration plotted over the 8-month season, with comparison of commercial and in-house ELISA



Note. Variation from the average sIgA concentration plotted over 31 weeks from Chapter 4, via both the commercial and in-house ELISA (n = 1).

5.4 Discussion

Overall, the current research was successful in development of an in-house ELISA for detection of IgA in saliva. The use of checkerboard titrations led to the development of an effective assay, with a successful SC showing clear differentiation between sequential sIgA concentrations. Midway through development, the SC had to be developed within 'IgA-blank' saliva due to large matrix effects. Despite the difficulty of participant recruitment, sufficient range was gathered for Assay 2, meeting the required limits of detection ($100\text{-}800\ \mu\text{g}\cdot\text{mL}^{-1}$). Determination of IgA concentration at lower concentrations ($50\text{-}100\ \mu\text{g}\cdot\text{mL}^{-1}$) was limited and could not be trusted. But due to the detection range requirement for **Chapter 4**, this was not seen as a limitation. Moreover, the current research did well in optimising an in-house ELISA for detection of IgA in saliva. Precision assessments showed intra-assay CV of 10-12%, which could be accepted. Despite this, inter-assay CV was high suggesting that assay to assay comparisons could not be made. That said, when observing data alongside that of a commercial assay, findings were significantly correlated showing similar trends between the two assays.

5.4.1 Creation of the Standard Curve

Determination of coating and conjugated antibodies for use on the assay was performed by checkerboard titrations, a common method used in ELISA development. Following each experiment, the SC was examined for clear differentiation between points (≥ 0.2 OD value). If there was poor discrimination between points (low or flat curve), it should be considered that more detection antibody or HRP would be needed (R & D Systems, 2011). These findings were shown in Figure 5.7c, 1:10000 coating antibody dilution and 1:4000 detection antibody dilution. Even between the two different detection antibody (and HRP) dilutions (1:2000 and 1:4000), a flatter curve was seen for the SC that had less detection antibody. Furthermore, if the SC shows poor discrimination between concentrations, another possible solution includes increasing the substrate incubation time (R & D Systems, 2011).

5.4.1.1 Incubations

Incubation of coating antibody, standards, samples, and detection antibody have been mostly established in the literature and were used for the current assay. Once the plate was coated with antibody, the plate would be given one hour at room temperature (Kohl & Ascoli, 2018), followed by overnight incubation in the fridge at 4°C. Sample and detection antibody incubations should be performed at room temperature (R & D Systems, 2011). Thus, all incubations were completed at the recommended 90 minutes at room temperature for the current in-house ELISA. To ensure no environmental conditions varied and affected the results, an incubator was purchased. So then, all room temperature incubations were completed at a constant temperature of 21°C in the incubator.

It has been suggested that sensitivity could be increased by longer incubation times at room temperature (R & D Systems, 2011). As sensitivity for lower sIgA concentrations for Assay 2 was questionable, this could have been something to explore further. Despite this, when examining most commercial IgA ELISAs, incubations were less than 90 minutes. Furthermore, it has been reported that the higher range of the calibration curve may flatten and become unstable, limiting the assay range if incubation time is too long; however, this was not found to be the case for the current assay. It has been reported that some substrates require a longer incubation time in order to get higher absorbance and better distinction between concentrations. Typically, incubation time for substrates range from 10 to 30 minutes (R & D Systems, 2011), so this was continually examined throughout both assay development and optimisation. Every time an assay was run, results would be quantified using the plate reader (405nm) \leq 20 minutes, examining the optimal time and robustness for each assay. Results showed that a 20-minute substrate incubation was best at ensuring the SC had optimal height (Figure 5.10).

5.4.1.2 Range of Detection

During the creation of the standard curve for Assay 1, where PBS was used as diluent, lower concentrations of sIgA were examined to detect the sensitivity of assay range. A concentration as low as 6.25 $\mu\text{g}\cdot\text{mL}^{-1}$ was observed with multiple coating and conjugate dilutions (Figure 5.4), to determine optimal assay condition for LLOQ. It was

identified that a higher dilution of coating antibody was more appropriate for increased sensitivity, thus a 1:2000 dilution of coating antibody was used. Following the change in diluent to 'IgA-blank' saliva for Assay 2 and the consideration of the required range, 50 $\mu\text{g}/\text{mL}$ was used as the lowest concentration for standards. The use of the calibration curve beyond the LLOQ should be undertaken with caution (Lee et al., 2006). Results presented in Figure 5.7, showed little differentiation at the lower end of the calibration curve, between 50 and 100 $\mu\text{g}\cdot\text{mL}^{-1}$, for all conditions (conjugated detection antibody, 1:2000 and 1:4000, and three differing coating antibody dilutions of 1:1000, 1:2000 and 1:10000). Whilst the 1:1000 coating dilution had the lowest detection limit, showing the best sensitivity for lower concentration range, sensitivity decreased as sIgA concentrations increased. Therefore, it was decided that anything under 50 $\mu\text{g}\cdot\text{mL}^{-1}$ concentration, should be seen with caution.

To eliminate this issue, paired monoclonal antibodies could instead be used to ensure the assay was more sensitive at lower concentrations. Unlike polyclonal (used within the current in-house assay), monoclonal antibodies do not limit the available epitopes. As previously mentioned (page 114), polyclonal detection antibodies have lower affinity and may suffer from reduced binding site/ epitope availability (because the antigen has already bound to that site of the capture antibody). Use of monoclonal antibodies are highly specific and create very reproducible assays, however these are very costly. Moreover, it was discussed that the requirements of the assay would not require detection between 50 and 100 $\mu\text{g}\cdot\text{mL}^{-1}$. The required use of the current assay was to determine sIgA concentration change over time (8-months) as stated in Chapter 4. Therefore, the 1:2000 dilution of coating antibody was accepted and used for assay optimisation assessments.

5.4.2 Assessing Matrix Effects

Due to the difficulty of finding suitable blank matrices, many biomarker immunoassays use standards to calibrate, prepared in a substitute matrix which is different from the test sample matrix (Lee et al., 2006). Thus, Assay 1 standards were first made in PBS. However, when examining the comparison between SC made in two different diluents, large differences were seen. Following spike and recovery analysis, recovery ranged from 49-67%, which was far less than requirements. Most optimum recovery should

be no less than 100, however results between 80-120% are considered acceptable (Andreasson et al., 2015). Therefore, the current in-house ELISA showed large matrix effects, suggesting that PBS could not be used as an effective diluent.

Obtaining a matrix as close as possible to that of the study samples is crucial (Lee et al., 2006). One strategy discussed to overcome matrix effects, was to introduce different ratios of sample diluent into the sample matrix, which could present improvements to percentage recovery. For example, if a neat biological sample such as saliva has been used, it could be retested upon dilution in standard diluent PBS (1:2 for example). Serum and plasma samples would need at least a 2-fold dilution in diluent to overcome matrix effects (R & D Systems, 2011). Alternatively, another possible adjustment for improving recovery is to use a standard diluent where composition more closely matches the final sample matrix. It has been suggested that by obtaining blank matrices from multiple individuals and spiking with a known concentration of biomarker, would eliminate matrix effects (Lee et al., 2006). Therefore, IgA deficient participants were recruited from around the UK to provide saliva samples, so that IgA-blank saliva could be used to develop Assay 2. Results showed 95-113% recovery for saliva, compared to 49-67% recovery presented in PBS. This was a significant finding as it meant that the IgA-blank, deficient sample could be used as diluent for all in-house ELISAs.

5.4.3 Precision

Following optimisation of Assay 2, methods of validation were carried out and precision was examined. Firstly, the in-house ELISA showed an intra-assay variation of 10-12%. Literature on accepted CV differs between authors, with some stating $\leq 15\%$ and others $\leq 10\%$ (Thermo Fisher Scientific, n.d.). Despite findings not being perfect and presenting between these recommendations, this variation was acceptable by the criteria stated above. These findings were seen as a strength of the current work. Within this work, regular QC was carried out by researcher to ensure reduced human error and to ensure pipette accuracy. To keep CV% low, many rules were followed such as; using fresh pipette tips, disposing of excess reagents, using an incubator, using an assay lid to avoid drafts and to minimise temperature variations,

and significant washing. Therefore, it was considered that no more could be done to ensure low intra-assay variation.

Possible causes of poor inter-assay variation and assay reproducibility include insufficient washing, variations in protocol or incubation temperature, improper calculation of standard curve dilutions, and buffer contamination (R & D Systems, 2011). Each of these factors were examined individually, and results for the current assay were assessed. Firstly, washes were increased from three times to six, which had no effect. There were no variations in protocol or miscalculated standard curve dilutions as these were all double checked. Fresh buffer was made up for each assay following these findings, of which also made no changes to variation between assays. The current assay followed suggested incubations from other literature, including incubation length and temperature. Plus, incubation temperatures were kept the same throughout, as an incubator was purchased. As previously mentioned, length of incubations was examined and seemed to be successful for the current assay. It should be considered that room temperature incubations were carried out, and therefore a possible cause could have been that higher temperatures, such as 37°C were not explored (Kohl & Ascoli, 2018). The cause of the high inter-assay variability shown (45%) is unknown to the current researcher; however, perhaps higher incubation temperatures should be considered.

Arguably the current assay lacked repeatability and would not be comparable from one to the next. Results showed an inability to measure the true value of the analyte, with large deviation from the actual nominal concentration achieved by the commercial ELISA (Figure 5.11). Instability of samples was considered for a possible explanation of findings as there are practical limitations to stability of long term storage (Lee et al., 2006). Despite this, Salimetrics (2015) noted that sIgA samples can be stored at -80°C for several years, most showing little to no degradation even after four years and so would not be the case here. That said, in-house ELISA sIgA data was significantly correlated with that of a commercially available assay. Furthermore, positive findings were found for inter-assay CV (Table 5.4), presenting within acceptable range. It should be considered that perhaps all samples could be tested on one assay, as trends of sIgA over time could be successfully gathered.

5.4.4 Strengths, Limitations and Future work

One strength of the current in-house assay optimisation was the low background identified. High background is ≥ 0.2 and many factors could contribute to this; insufficient washing, too much HRP, insufficient blocking reagent, or too long incubation times (R & D Systems, 2011). When looking at the used substrate safety data sheet specifically, colourless, sIgA negative wells should have a background of <0.16 . During assay optimisation and validation assessments, the background noise for negative wells was lower than what was mentioned above ($OD \leq 0.09$). These findings were lower than limit guidelines and was not a concern, suggesting there was sufficient washing, no non-specific binding and effective blocking within the current research.

It was identified that if money and time was not an issue, paired monoclonal antibodies could instead be used to ensure the assay was more sensitive at lower concentrations. However, when weighing this against the requirements for the current research, it was decided that this was unnecessary. Therefore, another strength of the study was that the range of detection met the requirements of the current samples. It was unnecessary to dilute samples to get them within working range, therefore linearity of dilution assessment did not need to be carried out. Furthermore, a main strength of the current in-house ELISA was found following spike and recovery assessment. Due to finding large matrix effects, salivary IgA deficient participants were successfully recruited from around the UK. Then, successful recovery % was found when examining spike and recovery of saliva, to enable the creation of a SC in diluent that matched samples.

Following assay optimisation, validation was successful in showing inter-assay CV to be within acceptable guidelines. Furthermore, in-house ELISA sIgA data was significantly correlated with a commercially available assay, suggesting valid and reliable use if all samples could be used on one assay. These two findings were major strengths of the current work. The large intra-assay CV was the most profound limitation of the current chapter and should not be ignored. Despite consideration of washing, correct calculations of dilutions and no buffer contamination, it was unclear why variation was so high. However, with consideration of the required use of the

assay, it was thought to be a successful chapter of work. In view of the acceptable intra-assay variation, comparable data to that of a commercial assay and spike and recovery results, there is clearly a basis for future development. Perhaps increases in incubation temperatures or use of monoclonal antibodies should be considered for future work, in reducing the large intra-assay CV and lowering the detection limit.

5.5 Conclusion

To conclude, development of an in-house ELISA was achieved through methods such as checkerboard titrations, standard curve establishment and regular assessment of washing, incubations, sensitivity, and detectable range. Assay optimisation was approached by conducting spike and recovery, plus assessing precision, accuracy, and robustness. Spike and recovery assessment revealed recovery % that was within acceptable guidelines, following the use of sIgA-blank saliva as diluent. Successful recruitment of sIgA deficient participants, made this part of the assay optimisation achievable. Intra-assay variation was within acceptable guidelines, showing accurate results within one plate. Furthermore, when observing data alongside that of a commercial assay, findings were significantly correlated showing similar trends. Despite these findings, the current researcher struggled to eliminate the high inter-assay CV% and due to being heavily labour intensive and expensive, further work was not undertaken. In an ideal world without time and financial constraints, there are enough positive findings within the current work for someone to build upon.

CHAPTER 6

Effect of Vitamin D Supplementation on Markers of Immune Function and Upper Respiratory Illness in Athletes; a Meta-analysis and Systematic Review

6.1 Introduction

Upper Respiratory Illness (URI) is frequently reported by athletes, especially for those who train predominantly indoors (Constantini et al., 2010; Farrokhyar et al., 2015). On analysis of 70 elite athletes in Australia (35° N), it was reported that there were no obvious underlying deficiencies in immune or inflammatory markers that were likely to contribute to the onset of symptoms consistent with URI, with the exception of 25(OH)D concentration (Cox et al., 2008). As previously described in Chapter 2 (page 44), vitamin D plays an important role for immune function (He et al., 2016), as VDRs have been discovered in almost all immune cells (Baeke et al., 2010). Serum 25(OH)D concentrations have been correlated with incidence of URI (Berry et al., 2011; He et al., 2013; Laaksi et al., 2010). Advantageous 25(OH)D levels have been defined for health at around 75 nmol·L⁻¹ (Bischoff-Ferrari, 2008), with concentrations of >95 nmol·L⁻¹ being associated with a two-fold decrease in incidence of URI (Sabetta et al., 2010). On the other hand, low concentrations of 25(OH)D have been associated with increased incidence of URI, longer duration of illness and increased severity of symptoms for athletes (Cox et al., 2008; He et al., 2013). This highlights the importance of examining 25(OH)D levels in athletes and the impact it may have on illness.

Due to primary production of vitamin D being from UVB sunlight exposure (Farrokhyar et al., 2015; Walsh, 2019), significantly lower 25(OH)D concentrations have been reported in winter (Lin et al., 2021; Scullion et al., 2018), for those that live at higher latitudes (>37° North or South) (Barcal et al., 2016), and for those that train predominately indoors (Constantini et al., 2010; Halliday et al., 2011). Specifically, studies conducted in the UK (55° N) have reported up to 65% of athletes to have insufficient 25(OH)D concentrations in winter (Close et al., 2013; He et al., 2013).

Interestingly, a study conducted in Israel (latitude 31°N) found prevalence of vitamin D insufficiency to be 73% for athletes and dancers (Constantini et al., 2010). Despite being at a latitude <37 degrees North, insufficiency was vast with a higher proportion from indoor sports compared to outdoor (80% vs 48%) (Constantini et al., 2010). This is thought to be due to significantly restricted vitamin D synthesis, increasing risk of 25(OH)D deficiency and consequent risk of illness (Halliday et al., 2011).

Therefore, vitamin D supplementation has received a lot of attention in sports nutrition (Owens et al., 2018), due its promising anti-inflammatory effects on illness (Walsh, 2019). The most recent meta-analysis included 46 studies from the general population (75,541 participants), and found that vitamin D supplementation (400–1000 IU for up to 12 months) reduced risk of URTI specifically, and gave some protection against illness (Jolliffe et al., 2021). Protective effects from supplementation against illness have been reported to be even stronger in those with baseline deficiency (Martineau et al., 2019). Given the global, high prevalence of insufficiency in athletes already identified (Chapter 2, page 46) (Farrokhyar et al., 2017), it would come as no surprise that regular supplementation has been advised by sport governing bodies. These findings likely encouraged the Australian Institute of Sport (AIS) to create a sport supplement framework, outlining the importance of 2000 IU vitamin D supplementation for athletes at most risk of deficiency, to restore baseline status (AIS, 2021).

Despite these findings, few studies have explored the effect of vitamin D supplementation on URI in athletes. Published RCTs on vitamin D supplementation for prevention of URI, and the subsequent effect on immune markers in athletes, have produced heterogeneous results. For example, Jung et al. (2018) found vitamin D supplementation to have a significant effect on frequency of URI, whereas others did not (Da Boit et al., 2015; Dubnov-Raz et al., 2015). Regarding mucosal immunity, He et al. (2016) reported significantly increased SIgA and cathelicidin secretion rates following vitamin D supplementation. Whereas large cohort studies in military recruits, have found no changes in mucosal immunity (Harrison et al., 2021). These varying findings mean that a definite conclusion on the effects of vitamin D supplementation on illness and immune markers cannot be gathered for athletes, providing further justification for the current meta-analysis.

More commonly, vitamin D has been associated with maintaining bone health and optimising muscular function in athletes (Close et al., 2013; Owens et al., 2018). Studies, in addition to several meta-analyses, have been conducted in athletes and observed the following: the prevalence of vitamin D insufficiencies, the effect of Vitamin D supplementation on physical performance, and predominantly the effect on muscular strength and injury (Farrokhyar et al., 2017, 2015; Zhang et al., 2019). But few studies have explored the association between both supplementation and 25(OH)D levels, with URI in athletes. In addition, despite a large-scale meta-analysis being conducted in the general population (Jolliffe et al., 2021), no meta-analysis has investigated this effect in the athletic population. Therefore, there is no clear consensus whether supplementation of vitamin D could act as a preventative strategy for illness and improve immune markers for the athletic population. This is important because lower 25(OH)D levels have been reported in trained athletes, compared to sedentary controls (Jerome et al., 2017). Therefore, this will be explored in the current meta-analysis and systematic review, which to the author's knowledge is the first to do so.

6.1.1 Aims

The primary aim of this study was to systematically review existing literature and analyse existing data to determine whether vitamin D supplementation improves markers of immune function in athletes including but not limited to; salivary IgA, TNF- α , and anti-microbial peptides (cathelicidin, lactoferrin).

The secondary aims were to:

- a) to determine whether vitamin D supplementation has a preventative effect on upper respiratory symptoms in athletes.
- b) to determine whether the effect of vitamin D supplementation on upper respiratory symptoms is different for athletes who are vitamin D deficient and non- deficient (baseline vitamin D status).
- c) to explore the effect of differing doses and type of vitamin D on upper respiratory symptoms in athletes (vitamin D dose amount, frequency of vitamin D dose, duration of supplementation).
- d) Examine the association of vitamin D (25(OH)D) serum levels with upper respiratory symptoms (number of symptom days, severity of symptoms), in athletes.
- e) Examine the association between vitamin D (25(OH)D) serum levels and markers of immune function in athletes including: salivary IgA, TNF- α , and anti-microbial peptides (cathelicidin, lactoferrin).

6.2 Methods

6.2.1 Search Strategy and Eligibility

The current systematic review and meta-analysis was pre-registered with PROSPERO, the international prospective register of systematic review CRD42022308162

(https://www.crd.york.ac.uk/prospERO/display_record.php?ID=CRD42022308162).

The review was conducted using a predefined protocol, the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement (Moher et al., 2010).

The PICO approach was used to create the search terms, which can be seen below:

- Population (P) was defined as adolescent (aged 10-17) and adult (aged 18+) athletes, male and female of any race/ethnicity. Participants could be recreational up to elite level athletes.
- Intervention (I) was oral supplementation of vitamin D, at any dose or duration.
- Comparison (C) was a placebo or no intervention.
- Outcomes (O) examined included upper respiratory symptoms, or biological markers of immunity.

The search string used was: `athlet* OR sport* OR swim* OR compet* OR endurance OR recreational OR elite OR trained AND "vitamin D" OR ergocalciferol OR cholecalciferol OR "25(OH)D" OR calcidiol OR calcifediol OR "25-hydroxyvitamin" OR "25-hydroxycholecalciferol" AND immune OR "upper respiratory" OR "respiratory symptoms" OR "respiratory tract infection" OR URI OR URTI OR URS`. Using the keywords identified, an online literature search was performed on the databases PubMed and Scopus. Search criteria included paper titles and abstracts, that had been published in the English language from inception until March 2022.

Inclusion criteria included: Studies conducted with athletes, randomised, double-blind, trials of supplementation with either vitamin D3 [cholecalciferol], vitamin D2 [ergocalciferol] or 25-hydroxyvitamin D (25(OH)D) [calcidiol] administered at any dose with any frequency, of any duration, with a placebo control group. Observational studies with trials of supplementation, similarly to above, were included. Studies were also required to have incorporated some measure of: immune function, mucosal or

innate immunity, upper respiratory symptoms, on athletic participants. This included upper respiratory infection diagnosed by a clinician, or self-reported upper respiratory symptoms such as on an illness scale, and impact on all immune function and biomarkers. Inclusion included athletes from recreational up to elite level athletes; a recreational athlete was considered to be a “physically active individual that participates in sport at an amateur, masters or club level, with the primary goal to be physically fit” (Wilson-Barnes et al., 2020). Exclusion criteria included studies that measured any other parameters, such as muscular power or performance effects, and did not include any measure of immune function or URI.

Two independent reviewers (L.B. and T.D.) completed the initial screening process using the predefined screening process, and search results were independently reviewed and comparisons were made to avoid discrepancies.

6.2.2 Data Extraction

Data was extracted from the eligible studies into a form created using Excel (Microsoft Corporation, New Mexico, USA) software. Data involving athlete information was collected as mean, standard deviation (SD) and sample size. Such data included; age, sex, pre and post 25(OH)D status, sport, vitamin D supplementation dosage and duration, immune markers (including but not limited to IgA, Cathelicidin, TNF- α), and URI incidence, symptom severity and duration. Where a response could not be derived, a well-established and validated (Drevon et al., 2016) web-based tool was used to extract data from figures (Rohatgi, 2020). Vitamin D intake was reported in either international units (IU) or micrograms (μg); however, for consistency all data was converted to IU for the current review. It was intended to include vitamin D supplementation dose as a moderator for meta-regression analysis, to observe the influence different doses had on immune outcomes.

As previously discussed in Chapter 2, included studies tended to define URI differently, some as diagnosed URTI, while some reported findings on undiagnosed symptoms. Regardless of definition or diagnosis, the participating athletes suffered URS and it was therefore important to determine whether vitamin D could positively

influence this. The current analysis will present results in accordance to how the primary author reported findings, and so may use different terminology throughout.

6.2.3 Risk of Bias

Eligible RCTs were assessed for risk of bias, via the Cochrane Risk of Bias 2 (RoB2) assessment tool (Sterne et al., 2016). The Cochrane tool was used to determine any bias due to; randomisation (D1), deviations from intended intervention (D2), missing data (D3), outcome measure (D4), and selection of reported result (D5). Through this analysis, each article was scored as either low, moderate or high risk.

The pre-registration for the current review specified using the Risk of Bias in Non-randomized Studies of Interventions (ROBINS-I) (Sterne et al., 2016). However, due to the nature of the studies included, only one case study was assessed using this tool. This tool was used because it was a case study, and involved answering 'yes', 'probably yes', 'probably no', 'no' and 'no information' to a series of questions within seven domains. The domains included: bias; due to confounding (D1), in selection of participants into the study (D2), in classification of interventions (D3), due to deviations from intended interventions (D4), due to missing data (D5), in measurement of outcomes (D6), and in selection of the reported result (D7). If none of the answers for each domain suggested a potential problem, then risk of bias could be judged as low. If this however was not the case, then potential for bias could exist (Sterne et al., 2016).

It was decided that eligible observational studies would be assessed for risk of bias, via the well-validated Appraisal tool for Cross-Sectional Studies (AXIS) (Downes et al., 2016). This critical appraisal involves answering either "yes", "no", or "don't know" to a selection of questions designed to assess the quality of the eligible articles. Of the 20 AXIS questions, seven assessed reporting quality (question 1, 4, 10, 11, 12, 16, 18), seven related to study design (2, 3, 5, 8, 17, 19, 20), and six to possible biases (items: 6, 7, 9, 13, 14 and 15). Following previous research (Dean et al., 2022), AXIS quality scores were classified for each study according to the number of "Yes" responses for the 20 questions; poor quality <50%, fair quality 50-69%, good quality 70%-79%, strong quality >80%. The quality of the included articles was assessed independently by two investigators (L.B. and D.G.), then an interrater reliability analysis using the Kappa statistic was performed to determine consistency amongst

rating. Kappa interpretation is as follows: <0 poor, 0.0-0.2 slight, 0.21-0.40 fair, 0.41-0.61 moderate, 0.61-0.80 substantial, 0.81-1.00 almost perfect (Landis & Koch, 1977).

6.2.4 Statistical Analysis

The statistical database package used for this meta-analysis was Comprehensive Meta-Analysis V3 software (Borenstein, 2013). When data was unavailable in articles, the corresponding authors were contacted (two authors for the current study).

Hedge's g was calculated using the mean, standard deviation and sample sizes of the intervention and control groups at the end-of-trial (post-supplementation). When multiple time points were available for assessment in studies, post-treatment values were favoured. Where studies did not provide these data, Hedge's g was calculated using sample sizes and a t -value or sample sizes and an independent groups p value. Hedge's g based on random effects was used to calculate the effect sizes. Following Cohen's convention, an effect size of 0.2 was considered small, 0.5 as moderate, and 0.8 as large (Cohen, 1988).

Heterogeneity was assessed using the I^2 statistic and for interpretation Cochrane guidance was followed (Higgins et al., 2019), where I^2 values of 0%-40% identified as might not be important; 30-60% as may represent moderate heterogeneity; 50-90% may represent substantial heterogeneity; 75%-100% representing considerable heterogeneity.

Subgroup analyses were proposed to be conducted within the pre-registration, for categorical moderator variables and meta-regression analyses for continuous moderator variables. Publication bias would be assessed by observing funnel plots to test for any asymmetry. Test statistics such as Duval and Tweedie's trim and fill method (Duval & Tweedie, 2000), could be used to infer the potential of there being small-study bias within the literature. Despite this, the small number of studies included in the current review, meant that these analyses were not feasible.

6.3 Results

6.3.1 Eligibility and Selection Assessment

Using the PRISMA flowchart (Figure 6.1), 208 publications were identified and assessed for eligibility; of these, 18 studies met the inclusion criteria and were sought for retrieval. This process was completed by the main researcher (L.B.) independently, and checked by a secondary researcher (T.D.). Six studies were excluded after reviewing the full text; three for having no immune measure (Bauer et al., 2019; Krzywanski et al., 2016; Vitale et al., 2018), one was a review (He et al., 2016), one for not meeting the review aim (Cox et al., 2008), and one was a duplication of another accepted, with the same study population (Mayan et al., 2015). Review articles were cross referenced to identify additional articles which could be considered for the current analysis; two articles were added during the search update, establishing 14 papers for analysis.

Of the 14 papers (748 athletes), seven RCTs (249 athletes) could be included in the current meta- analysis and are detailed in Table 6.1. All RCTs supplemented a daily dose of vitamin D₃ either through an oral tablet or buccal spray, with a blinded placebo group. It should be mentioned that two studies (one RCT, one observational) did not supplement vitamin D₃ solely; Omega-3 fish oil, whey protein (Da Boit et al., 2015) and quercetin (Ranchordas et al., 2016) alongside vitamin D₃. The remaining seven observational studies were reviewed and assessed for risk of bias. Additionally, these observational studies included within the review can be seen in Table 6.2.

Figure 6.1

PRISMA flowchart

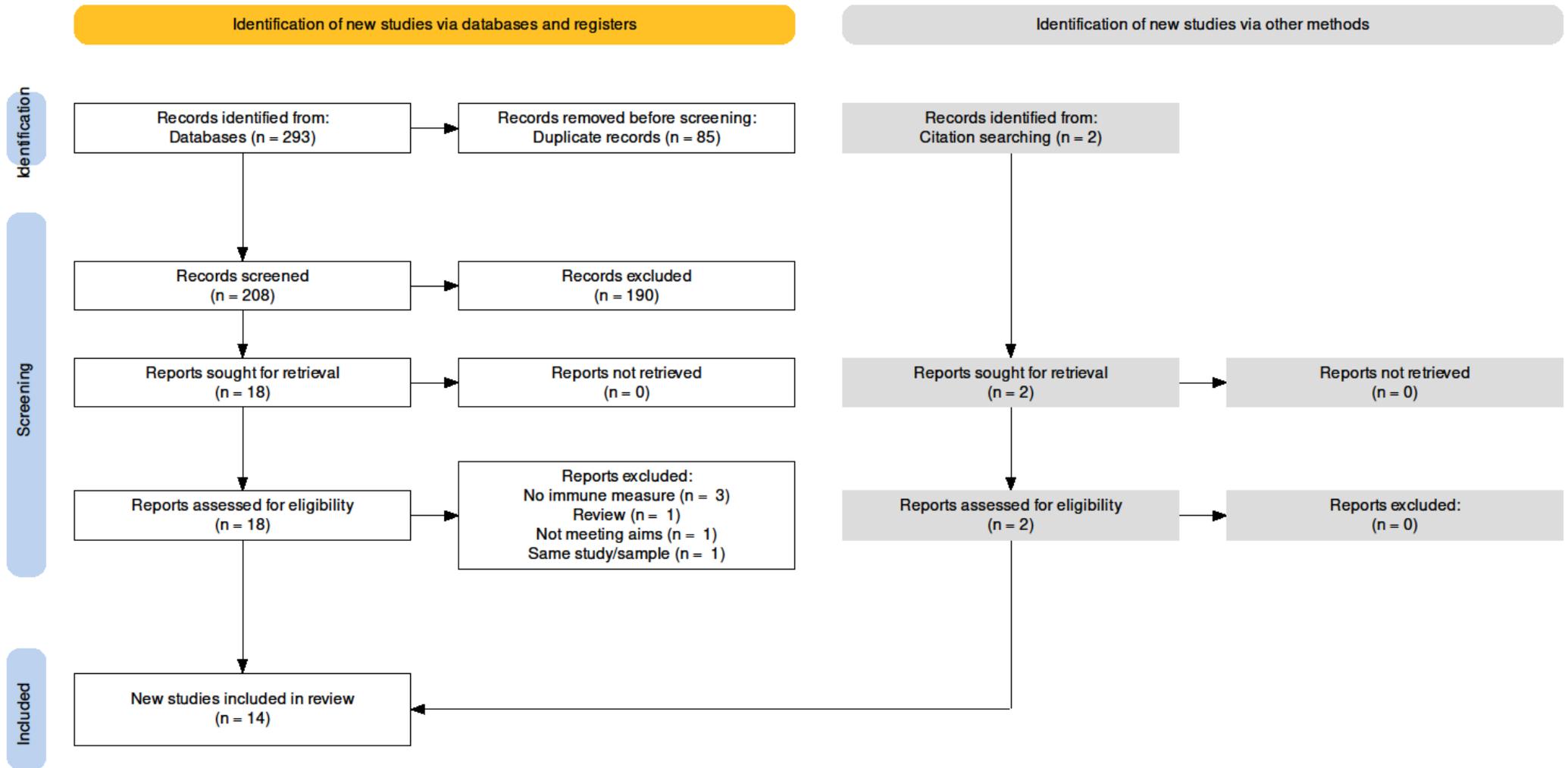


Table 6.1

Characteristics of RCT studies included within the meta- analysis

Study	Age (years)	Sample size	Male (%)	Mean baseline 25(OH)D concentration (nmol/L)	Daily Dose of Vitamin D3 for intervention group (IU)	Length of intervention (weeks)	Control	Sport	Location/ Latitude
Lewis et al., 2013	19 ± 1	32	59	58 ± 15.2	4000	26 (autumn-spring)	Placebo	Swimming and diving (NCAA Division I collegiate)	South-eastern United States (latitude 38°N)
Da Boit et al., 2015	28 ± 5	36	67	N/A	400	16 (spring-summer)	Placebo	Recreational sport (≥3 hours of moderate/high intensity exercise per week)	Scotland, UK (latitude 56°N)
Dubnov-Raz et al., 2015	15 ± 2	55	64	24.4 ± 4.8	2000	12 (winter)	Placebo	Swimmers (competitive)	Israel (latitude 31°N)
He et al., 2016	21 ± 2	39	100	55.8	5000	14 (winter)	Placebo	Rugby, volleyball, swimming, triathlon, cycling and racquet sports (regular training)	Loughborough, UK (latitude 53°N)
Todd et al., 2017	20 ± 2	42	N/A	45	3000	22 (winter)	Placebo	Gaelic football	Ireland (latitude 55°N)
Jung et al., 2018	20 ± 2	25	100	31.5 ± 1.8	5000	4 (winter)	Placebo	Taekwondo (collegiate)	Jeju Island in South Korea (latitude 33°N)
Pastuszek-Lewandoska et al., 2020	41 ± 7	20	100	N/A	10000	2	Placebo	Ultra-marathon running (amateur)	Poland (latitude 51°N)

Note. Numerical data presented as mean ± SD where applicable.

Table 6.2

Characteristics of observational studies, plus one case study intervention

Study	Age (years)	Sample size	Male (%)	Length of Study (weeks)	Supplement	Control	Sport	Location / Latitude
Larson-Meyer et al., 2012	19 - 45	19	47	<1	None	N/A	Running	Baton Rouge, LA, United States (latitude 30°N)
He et al., 2013	21 ± 3 years	239	71	16 (winter)	None	N/A	Running, cycling, swimming, triathlon, team games and racquet sports	Loughborough (latitude 53°N)
Barcal et al., 2016	21 ± 2	16	100	32 (autumn-spring)	None	N/A	Wrestling	Wyoming (latitude 43°N)
Ranchordas et al., 2016	25	1	100	12	Single oral dose of 100000IU D3, after 4 weeks, 20000IU each week.	N/A	Premier league soccer	United Kingdom (latitude 52°N)
Scullion et al., 2018	23 ± 3	53	74	24 (summer/winter)	None	N/A	Rugby and rowing	New Zealand (latitude 40°N)
Fikratkerimov et al., 2019	22	100	100	52	None	Control group	Freestyle and Greco-Roman wrestling	Uzbekistan (latitude 41°N)
Umarov et al., 2019	19 - 24	70	0	72	None	Matched sex and age control group	Synchronised swimming or competitive swimming	Uzbekistan (latitude 41°N)

Note. Numerical data presented as mean ± SD where applicable.

Despite seven RCTs meeting the inclusion criteria, not all RCTs could be used for each outcome and random effects model of the meta-analysis. This was due to the included studies having different study designs and measuring different immune markers and illness outcomes. As an example, three RCTs out of the seven examined sIgA as an immune biomarker and could be used for that analysis (Da Boit et al., 2015; He et al., 2016; Jung et al., 2018); details of this can be seen in Table 6.3. This was the case for each marker of immune function or URI, as enough studies must have examined the same outcome for the meta-analysis to be carried out.

Table 6.3

RCT studies used for each random effects model

Outcome	Study	Number of Participants
sIgA	Da Boit et al., 2015	36
	He et al., 2016	39
	Jung et al., 2018 (not included for secretion rate)	25
	TOTAL	100
TNF- α	Todd et al., 2017	42
	Pastuszek- Lewandoska et al., 2020	20
	TOTAL	62
Duration of URI	Da Boit et al., 2015	36
	Dubnov-Raz et al., 2015	55
	TOTAL	91
Symptom severity	Da Boit et al., 2015	36
	Dubnov-Raz et al., 2015	55
	Jung et al., 2018	25
	TOTAL	116

Note. RCTs used for each random effects model, for outcomes of IgA concentration, TNF- α , duration of URI and symptom severity, plus number of participants included ($n = 116$)

6.3.2 Primary Outcome - Vitamin D and markers of immune function

Using a random effects model, supplementation vs. placebo effect size change for athletes who received vitamin D supplementation was calculated. This was used to determine whether supplementation improved markers of immune function, specifically IgA. A moderate correlation of 0.5 was assumed between supplementation and placebo groups. The analysis revealed no effect between supplementation and placebo groups for sIgA concentration (Hedges $g = 0.02$ [95%CI -0.36 to 0.40]) (Figure 6.2) or sIgA secretion (Hedges $g = 0.11$ [95%CI -0.34 to 0.55]) (Figure 6.3). Heterogeneity was low ($I^2 = 0\%$) suggesting little variability in the outcomes. Additionally, no effect was found between supplementation and placebo groups post-supplement analysis of TNF- α (Hedges $g = 0.29$ [95%CI -0.79 to 1.38]; Figure 6.4), with heterogeneity being considerable ($I^2 = 76\%$).

The association between vitamin D status, plus effects of vitamin D supplementation on immune markers was also examined (Table 6.4). The main immune function markers analysed were sIgA, cathelicidin, and TNF- α .

Figure 6.2

IgA concentration, intervention vs. placebo (n = 100)

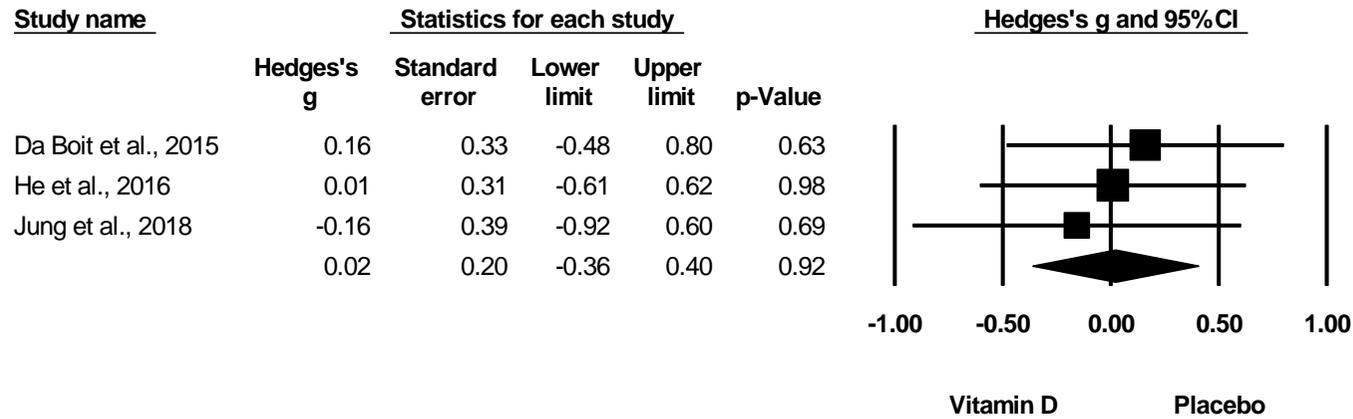


Figure 6.3

IgA secretion rate, intervention vs. placebo (n = 75)

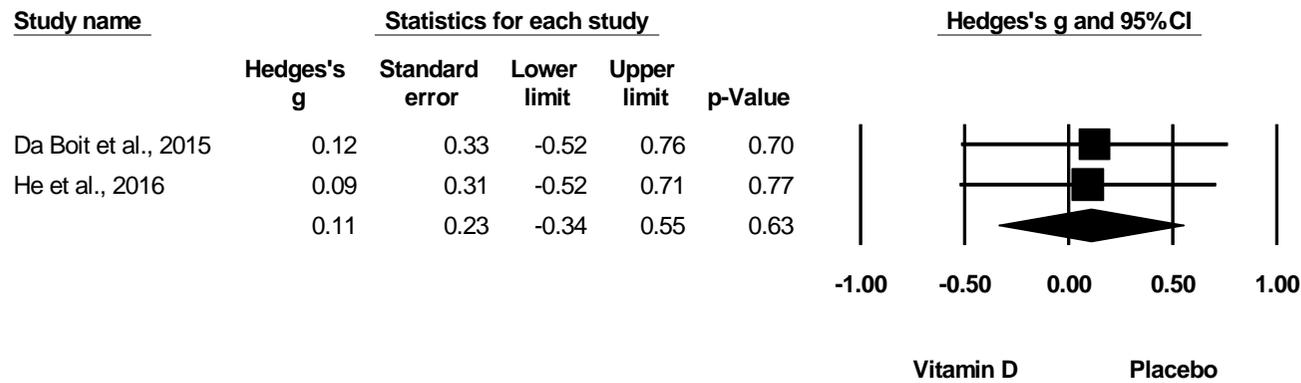


Figure 6.4

TNF- α , supplementation vs. placebo (n = 62)

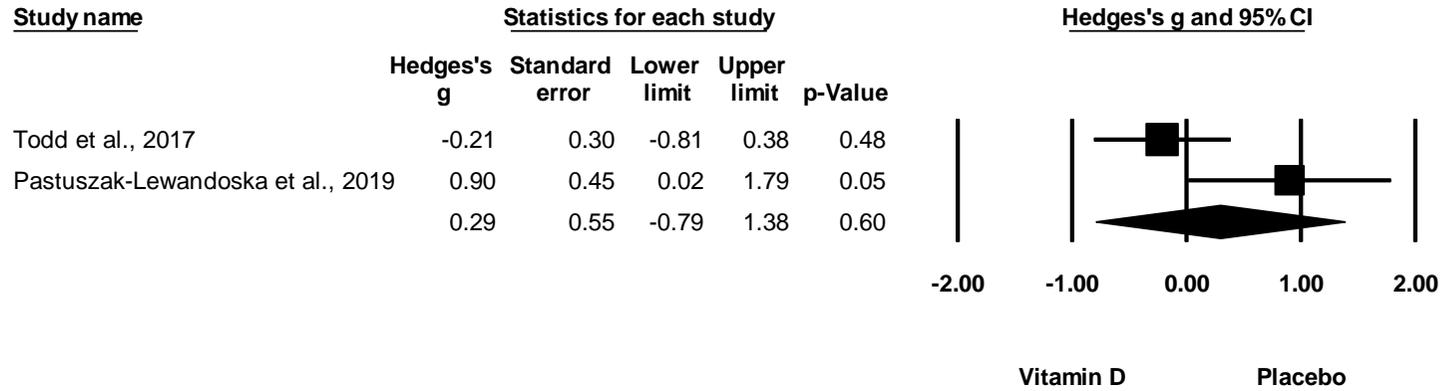


Table 6.4

Study results examining association between vitamin D and immune markers

Study	Adequacy of serum 25(OH)D level at baseline	Salivary IgA (plus other salivary variables)	Cathelicidin	TNF- α (plus other cytokines and inflammatory markers)
Lewis et al., 2013 (RCT- supplement)	Sufficient = 100%			No difference in inflammatory cytokine measures by time or between supplement groups. No correlation between 25(OH)D and inflammatory markers (TNF- α , IL-6, IL1- β).
Da Boit et al, 2015 (RCT - supplement)	Not reported	No difference in SIgA concentration between groups.		
He et al., 2016 (RCT - supplement)		No difference in AMPs concentrations. SIgA secretion rate increased over time in supplement group only ($p = 0.026$). Salivary lysozyme secretion rate increased for both groups ($P = 0.002$). No change in lactoferrin secretion rate.	Plasma cathelicidin concentration increased in both groups, but higher percentage change of cathelicidin in D3 group ($p = 0.025$).	
Todd et al., 2017 (RCT - supplement)	Sufficient = 29% (three footballers had 25(OH)D concentration >75 nmol/L). Insufficient = 50%. Deficient = 21%		Vitamin D3 supplementation had no effect on cathelicidin concentration.	No effect from D3 supplementation on WBC, lymphocyte, neutrophil, CRP, IL-8, TNF- α concentrations.
Jung et al., 2018 (RCT - supplement)	Insufficient = 100%	No difference in SIgA or lactoferrin concentration between groups. There was a significant time effects, for both groups.		

Study	Adequacy of serum 25(OH)D level at baseline	Salivary IgA (plus other salivary variables)	Cathelicidin	TNF- α (plus other cytokines and inflammatory markers)
Pastuszek-Lewandoska et al., 2020 (RCT - supplement)	Not reported			Significantly increased TNF- α with D3 supplementation ($p = 0.009$). Significant decrease in IL1- β ($p = 0.04$).
Larson-Meyer et al., 2012 (Observational)	Sufficient = 47%, Insufficient = 42%, Deficient = 11%,			Significant inverse correlation with 25(OH)D and TNF- α , but not with other cytokines, IL-4 and IL-10.
He et al., 2013 (Observational)	Optimal = 5%, Adequate = 57%, Inadequate = 30%, Deficient = 8%.	SIgA concentration was lower in the deficient group compared to optimal. No difference in Lactoferrin, Lysozyme or Amylase. Significantly higher SIgA secretion rate in 'optimal' vitamin D status group ($p = 0.018$), than other groups.	Plasma cathelicidin was significantly higher in vitamin D 'sufficient' group than 'deficient' ($P = 0.023$). Positive correlation between 25(OH)D and cathelicidin ($p = 0.036$).	No relationship with 25(OH)D on total leukocytes, neutrophils, lymphocytes or monocytes. TNF- α , IL-1 β and IL-6 were significantly lower in 'deficient' group compared with 'sufficient' ($p < 0.005$).
Barcal et al., 2016 (Observational)	Optimal = 0%, Sufficient = 26%, Insufficient = 63%, Deficient = 11%.			No significant difference in IL-6, IL-10 and TNF- α concentration. Neither 25(OH)D or vitamin D category (sufficient, insufficient, and deficient) correlated with IL-6, IL-10, or TNF- α at any time.
Ranchordas et al., 2016 (Case study intervention - supplement)	Sufficient = 100%	SIgA concentration increased from 98 mg-dL ⁻¹ at baseline to 150 mg-dL ⁻¹ at Week 12, after supplementation.		

Study	Adequacy of serum 25(OH)D level at baseline	Salivary IgA (plus other salivary variables) Cathelicidin	TNF- α (plus other cytokines and inflammatory markers)
Scullion et al., 2018 (Observational)	Insufficient = 2%, Sufficient = 98%.	Serum 25(OH)D (< 0.001) and SIgA ($p = 0.028$) differed significantly between summer and winter. No significant correlation between 25(OH)D and SIgA.	
Fikratkerimov et al., 2019 (Observational)	Sufficient = 10% (control 30%), Insufficient = 80% (control 60%), Deficient = 10% (control 10%).		Vitamin D deficient and insufficient athletes had significantly higher TNF- α , than control group ($p < 0.05$).
Umarov et al., 2019 (Observational)	Sufficient = 10% (control 30%), Insufficient = 90% (control 63%), Deficient = 10% (control 6%).		Increased TNF- α , IL-4 and IL-6 in athletes with vitamin D insufficiency in athletes.

6.3.3 Secondary Outcome - Vitamin D and upper respiratory illness

Secondary-outcome analyses included investigating the potential association between vitamin D level with upper respiratory symptoms (number of symptom days, severity of symptoms) and whether vitamin D supplementation could have a preventative effect on URI and symptoms in athletes. Using a random effects model, supplementation of vitamin D had no effect on reducing duration of symptoms (Hedges $g = 0.46$ [95%CI - 0.05 to 0.96]; Figure 6.5), with low heterogeneity ($I^2 = 0\%$). That said, this did have a moderate effect size. Following analysis of supplementation vs. placebo, supplementation showed no significant reduction in symptom severity score (Hedges $g = 1.0$ [95%CI -0.70 to 2.70]; Figure 6.6). This had a large effect size and heterogeneity was considerable ($I^2 = 90\%$). No other secondary outcomes were suitable for analysis because an insufficient number of studies were found that reported the required immune outcomes. In addition, it was intended to include vitamin D supplementation dose as a moderator, but inadequate studies provided relevant data for this analysis.

The association between 25(OH)D levels and vitamin D supplementation on upper respiratory illness parameters was also examined (Table 6.5). Overall, most studies found no significance between vitamin D supplementation and URI frequency, duration or severity. Saying this, on further investigation, a few studies did report correlations between lowered 25(OH)D concentration and increased URI duration and severity.

Figure 6.5

URI Duration, intervention vs. placebo (n = 91)

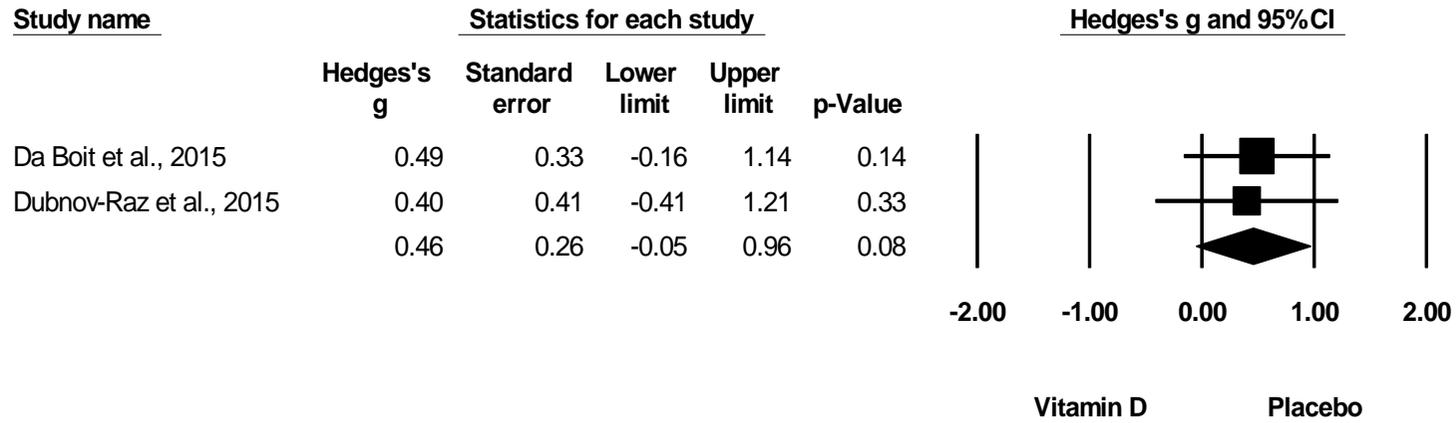


Figure 6.6

Symptom Severity Score, intervention vs. placebo (n = 116)

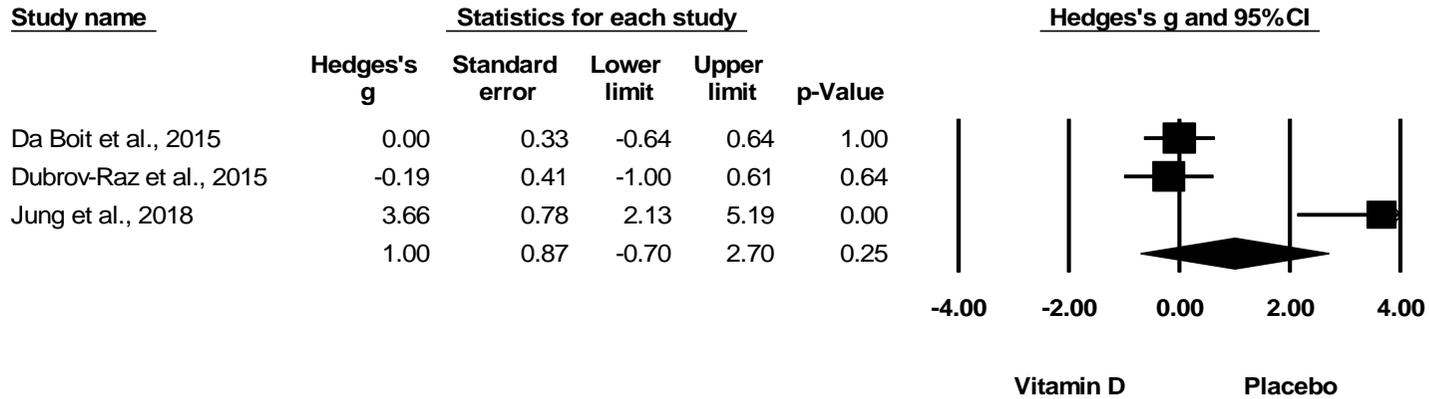


Table 6.5

Study results examining association between vitamin D and URI

Study	Adequacy of serum 25(OH)D level at baseline	Frequency	Duration (number of symptom days)	Severity of symptoms
Lewis et al., 2013 (RCT)	Sufficient = 100%	One athlete reported one illness		
Da Boit et al, 2015 (RCT)		No differences in the number of URI episodes between groups.	No differences in URI episode duration between groups. Total number of symptom days was significantly lower in the supplement group vs. control group ($p < 0.05$).	No significant differences in the URI episode severity.
Dubnov-Raz et al., 2015 (RCT)	Insufficient = 100%	No difference in URI frequency (total of 11 participants from each group reported URI symptoms).	No difference in URI duration between study groups. A significant negative correlation was found between change in serum 25(OH)D concentration and URI duration in the placebo group ($p < .001$).	No difference in URI severity between groups. Decreases in serum 25(OH)D concentrations were associated with more severe URI episodes for the placebo group ($p = .043$).
Jung et al, 2018 (RCT)	Insufficient = 100%			Significantly lower total URTI symptom score with D3 supplementation ($p = 0.011$)
He et al., 2013 (Observational)	Optimal = 5%, Adequate = 57%, Inadequate = 30%, Deficient = 8%.	'Optimal' group experienced one or more URTI episodes significantly less than 'deficient' ($P = 0.039$)	'Deficient' group experienced significantly higher number of symptom days, than 'Optimal' group ($p = 0.040$). URTI episodes were longer with lower vitamin D status.	Symptom-severity scores were significantly higher in 'deficient' group ($p = 0.013$).
Barcal et al., 2016 (Observational)	Optimal = 0%, Sufficient = 26%, Insufficient = 63%, Deficient = 11%	Neither 25(OH)D status or vitamin D category (sufficient, insufficient, and deficient) was associated with number of illnesses.		

Chapter 6. Vitamin D Meta-analysis and Systematic Review

Study	Adequacy of serum 25(OH)D level at baseline	Frequency	Duration (number of symptom days)	Severity of symptoms
Ranchordas et al., 2016 (Case study intervention)	Sufficient = 100%			URTI symptom score declined significantly over the 12-week supplementation.
Scullion et al., 2018 (Observational)	Insufficient = 2%, Sufficient = 98%.	No association between serum 25(OH)D concentrations and URI occurrence.		Significant difference between URI duration between summer and winter ($p = 0.044$). No correlation between serum 25(OH)D concentrations, SIgA and URI duration.
Fikratkerimov et al., 2019 (Observational)	Sufficient = 10% (control 30%), Insufficient = 80% (control 60%), Deficient = 10% (control 10%).	URTI were observed more frequently in winter-spring period for both athletes and controls. 'More than 5 episodes' of URTI were reported by athletes only.		
Umarov et al., 2019 (Observational)	Sufficient = 10% (control 30%), Insufficient = 90% (control 63%), Deficient = 10% (control 6%).	URTI were observed more frequently in winter-spring period for both groups.		

6.3.4 Risk of Bias Assessment

Findings from Cochranes RoB2 tool for the RCTs, showed five studies to have 'some concerns' and two studies to have 'high' risk of bias, (Figure 6.7.1 and 6.7.2). All seven RCT's had low bias in domains 1- 4. All RCTs were reported to have used a randomisation process (D1). As an example, Dubnov-Raz et al. (2015) reported randomising sexes separately; athletes were randomised in pairs using a computer software, to ensure equal number of males and females in each group. Furthermore, all studies clearly stated that the vitamin D supplements were virtually identical to the placebo given (D2). Four studies suffered from participant drop-out over 10% (D3), which meant that not all data was available; however, these were not excluded in analysis and did not affect the results. Lastly, methods of the included studies meant that assessors were not aware of the intervention received (D4) and therefore had low risk of bias.

Bias in selection of the reported results (D5) showed to be the main area for study improvement, in the RoB analysis. Five studies had 'some concerns' due to the fact there was no pre-registered protocol, so results could not be compared with a pre-specified study aim. Two studies had 'high' risk of bias and this was because there were differences between the pre-registered protocol and the protocol carried out. Examples of this included reporting secondary outcome findings as their primary, and reporting findings that weren't even listed as an aim in pre-registration. Regarding RCTs, both researchers (L.B. and D.G.) carried out RoB2 independently and were in 100% agreement (Kappa = 1.000, $p < 0.05$).

Results from ROBINS-I tool for risk of bias, showed that the one case study included within the observational studies showed overall 'serious' risk of bias. This was due to 'serious risk' of bias in two domains. This was recorded in bias in measurement of outcomes (D6), due to the fact supplementation was not blinded for the participant, and in bias in selection of the reported result (D7). Both researchers carried out ROBINS-I independently, and were in 100% agreement with these results.

Findings from the AXIS tool showed average score for all included observational studies (n = 6) was within the 'good quality' range; two studies were rated as 'strong quality' (33%), three studies as 'good quality' (50%) and one as 'fair quality' (17%). The breakdown of number of papers meeting each AXIS question, can be seen in Appendix F. It can be seen that all studies scored positively on regarding appropriate measurement of outcome variables and appropriate conclusions justified by the results. However, two main areas for study quality improvement were highlighted by the AXIS assessment; sample size justification and non-responders. The two authors (L.B. and D.G.) independently rated each study, and these two sets of ratings had a moderate strength of agreement (Kappa = 0.538, $p < 0.05$).

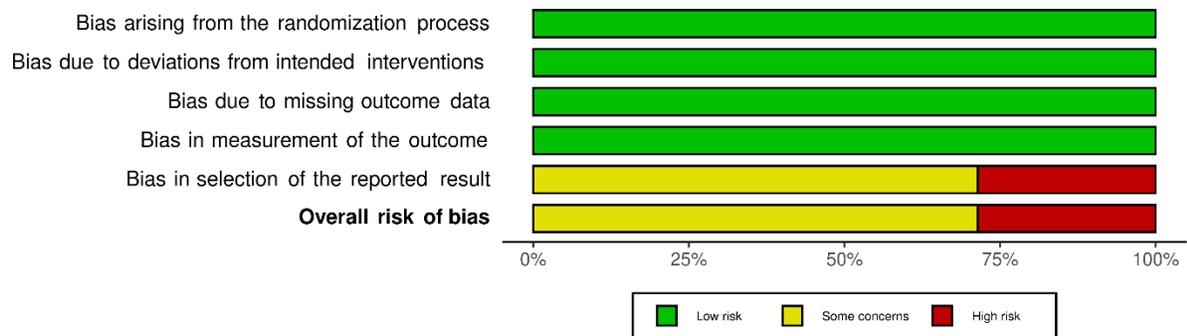
Figure 6.7.1

RoB2 output, traffic light plot (McGuinness & Higgins, 2021)



Figure 6.7.2

RoB2 output, summary plot (McGuinness & Higgins, 2021)



6.4 Discussion

As far as the authors are aware, the current preregistered meta-analysis was the first to examine the efficacy of vitamin D supplementation for athletes, on improving markers of immune function and the relationship with URS. The current review identified 293 publications; however, only 14 studies met the inclusion criteria; seven RCTs and seven observational studies. Due to differences in study design and measurement of different outcomes for URS, not all RCTs could be used for each analysis. The primary outcomes included a random effects meta-analysis of supplementation vs. placebo that showed no effect for sIgA concentration, sIgA secretion or for TNF- α . Furthermore, no effects were found for secondary outcomes, between illness duration and symptom severity scores.

6.4.1 Vitamin D and Immune Markers

6.4.1.1 IgA

The current meta-analysis found no effect between groups for concentration of sIgA (Figure 6.2). No RCT included in the meta-analysis, found any significant effect on sIgA concentration following vitamin D supplementation in athletes. It should be mentioned that individually, the three studies were insufficiently powered, needing 55 participants to detect an effect. But, the overall meta-analysis for sIgA was significantly powered to detect a present effect, of which it did not. Studies showed low heterogeneity ($I^2 = 0\%$), nor suffered from publication bias, and therefore it could be said with confidence that a larger study would show no different outcome. Despite Jung et al. (2018) reporting increased 25(OH)D concentration, no change in sIgA was found following vitamin D supplementation. When examining alternate population groups, this was also found to be the case. One study conducted in military personnel found that vitamin D supplementation did not influence the innate mucosal AMPs, specifically sIgA (Harrison et al., 2021). Current findings and the agreement between literature offers validity to this conclusion, suggesting that vitamin D supplementation would not have an effect on IgA concentration.

Overall, the relationship between vitamin D status and sIgA concentration was not noteworthy. Although significantly lower sIgA concentrations have been reported for 25(OH)D 'deficient' ($12-30 \text{ nmol}\cdot\text{L}^{-1}$) athletes, compared to 'optimal' (He et al., 2013), this

has only been reported once. More recently, no significant associations have been found between 25(OH)D levels and sIgA concentrations (He et al., 2016; Scullion et al., 2018). Therefore, this offers justification for the lack of significant findings for supplementation on sIgA concentration, in the current analysis.

That said, concentration of sIgA is not the only valid way of measuring this biomarker; sIgA secretion has been commonly reported. The current meta-analysis additionally found no effect between groups for sIgA secretion rate (Figure 6.3). This was because one RCT included within this analysis reported no change in sIgA secretion rate following supplementation of vitamin D (Da Boit et al., 2015). It should be considered that researchers did not measure 25(OH)D concentrations at baseline or post-intervention, and therefore had no way of knowing the potential effect of supplementation. In addition, a significantly lower dose of vitamin D (400 IU daily) was given compared to other studies within the current review, and therefore findings may not be comparable (Da Boit et al., 2015). In contrast, He et al. (He et al., 2016) was the first research group to report increased secretion of sIgA, following a daily supplement of 5000 IU vitamin D. These findings were supported by research conducted in military recruits, following 12 weeks of vitamin D supplementation (1000 IU) (Scott et al., 2019). It should be mentioned however, sIgA secretion significantly increased at 7 weeks and not 14 weeks following supplementation (He et al., 2016); this may have influenced analysis findings as only end point post-supplementation data was used. Findings were in agreement elsewhere, which found significance at 4 weeks only and had no change at any other time points (Scott et al., 2019), which perhaps was due to a lower supplement dose (1000 IU).

Another possible reason for this initial elevated sIgA secretion rates could be explained by increased saliva flow rates, which have been found to be positively associated with 25(OH)D concentration (He et al., 2013). In addition, sex differences in saliva flow rate have been identified; due to smaller salivary glands, females have lower unstimulated saliva flow rates compared to men (Inoue et al., 2006). Both studies with identified associations for vitamin D supplementation and sIgA secretion rate recruited either all (He et al., 2013), or a greater proportion of males (Scott et al., 2019). Thus, the

combination of high 25(OH)D concentrations initially increasing saliva flow rate, and greater male population could be considered possible explanations for findings. Overall, the lack of well-designed research examining the effect of supplementation on sIgA secretion, highlights the need for more exploratory research to be conducted.

6.4.1.2 Cathelicidin

One research group reported that plasma cathelicidin (sometimes referred to as LL-37) concentration significantly increased with supplementation of vitamin D (He et al., 2016). Previously, associations between 25(OH)D status and cathelicidin concentration have been reported (Bhan et al., 2011; Gombart et al., 2005; He et al., 2013). However, only one research group in athletes has presented this relationship between supplementation and cathelicidin, and so reliability should be questioned. Firstly, cathelicidin increased significantly in both supplementation and placebo groups (He et al., 2016). As already established (Chapter 2, page 6), cathelicidin plays an important role in the first line of defence and has shown to increase following pathogen exposure (Bucki et al., 2010). Thus, a plausible explanation for the observed increase in concentration for both groups could be due to higher incidence of illness. In addition, researchers reported percentage change of cathelicidin; which has been criticised elsewhere as being statistically inefficient, as it does not correct for any imbalances between groups at baseline (Vickers, 2001). Consequently, it would be questionable whether supplementation caused the increase in plasma cathelicidin. Outside this research group, vitamin D supplementation has shown no effect on plasma cathelicidin concentration in females with 25(OH)D deficiency (Das et al., 2014), or male footballers (Todd et al., 2017). One RCT included within this review, Todd et al. (2017) found no change in cathelicidin, however it was significantly underpowered for cathelicidin analysis ($1-\beta = 13\%$).

Moreover, cathelicidin can also be examined within saliva. He et al. (2016) reported increased cathelicidin secretion rate in saliva samples, which was only seen in the vitamin D supplementation group. It was proposed that elevated cathelicidin secretion could be due to the influence of 25(OH)D in the human oral epithelial cells (He et al., 2016a; He et al., 2016b). These increases in secretion however, could also be because of increased saliva

flow rate (He et al., 2013). Another study conducted in military personnel found that vitamin D supplementation did not influence the innate mucosal AMPs, specifically cathelicidin (Harrison et al., 2021).

6.4.1.3 TNF- α

Vitamin D did not significantly influence other markers of immune function, including pro-inflammatory cytokines such as TNF- α . A small effect was found between groups in post-supplement analysis of TNF- α ; however, heterogeneity was considerable. Pastuszek-Lewandoska et al. (2020) reported significantly higher TNF- α 12 hours following an ultra-endurance marathon, and so was likely to be reflective of the inflammation from acute exercise. These results could have skewed data for the meta-analysis, as others showed no effect on inflammatory markers (Lewis et al., 2013; Todd et al., 2017). Todd et al. (2017) found no impact on measures of inflammation, including cytokines and AMP concentrations in footballers. This was comparable to research in swimmers whom found no effect for TNF- α , IL-6, and IL-1 β concentrations following supplementation of vitamin D (Lewis et al., 2013). A review proposed that supplementation may have a more significant effect for those who have elevated baseline markers of inflammation, with low baseline 25(OH)D concentrations ($<45 \text{ nmol}\cdot\text{L}^{-1}$), or compromised immune systems (Cannell et al., 2015). Therefore, it could be considered that any effects on inflammatory cytokines would be more likely to occur, if these were the case. Athletes included were 25(OH)D sufficient (Lewis et al., 2013) and baseline markers of inflammation were within normal ranges (Todd et al., 2017); providing potential rationale to why no changes were observed for the RCTs in the current review.

Following these findings, it was deemed important to examine the effect of low baseline 25(OH)D status on pro-inflammatory cytokines. Observational research in the general population found that those that were 25(OH)D insufficient, had significantly greater concentration of circulating pro-inflammatory cytokines (TNF- α , IL-2, IL-1 β) (Barker et al., 2013). This has also been supported in the athletic population, where a significant inverse correlation was found for TNF- α and 25(OH)D (Larson-Meyer & Willis, 2010). This is because vitamin D reduces inflammation by decreasing production of pro-inflammatory

cytokines, such as TNF- α (Liu et al., 2006; Yagüe et al., 2020). In contrast, He et al. (2013) reported lower TNF- α concentrations for 25(OH)D 'deficient' athletes and authors could not provide rationale for this discrepancy. Also, one study reported no differences in TNF- α between 25(OH)D status groups (Barcal et al., 2016). However, a remarked limitation was that cytokine data collection began eight weeks after training commencement, which would alter the true picture of 25(OH)D status influence (Barcal et al., 2016).

6.4.2 Vitamin D and Illness

6.4.2.1 Duration of URI

The current meta-analyses suggested that vitamin D supplementation would not act as a useful intervention to reduce duration of URI and associated symptoms. Independently, RCTs lacked significance on URI duration between groups (Da Boit et al., 2015; Dubnov-Raz et al., 2015). In contrast to this, a recent study conducted in military recruits reported 36% fewer days of illness following vitamin D supplementation, compared to placebo (Harrison et al., 2021). These findings are comparable to the current review, due to still being within a population with high training load. A reason for the lack of significant findings in the current review may have been that population size for the individual studies was too small to have an effect. Harrison et al. (2021) included 250 military recruits and was significantly powered, compared to the 91 athletes combined for this analysis. Equally, poor compliance with illness questionnaires suggested that the whole picture may not have been clear. For example, Dubnov-Raz et al. (2015) reported 33 out of 55 participants had missing URI data.

In addition to URTI duration, number of days where symptoms were mild but were not classified as URTI, have also been reported and showed promising results (Da Boit et al., 2015). Researchers found where participants were not classified with URTI, but suffered with URS, number of days were significantly reduced with supplementation. Despite this, athletes were given not only vitamin D, but with protein and fish oil too. The benefits of protein (Witard et al., 2014) and fish oil (Mendivil, 2021) on immune function have been

established. Consequently, it cannot be established what caused the reduction in days of symptoms observed, and should be considered when making comparisons. Overall, RCTs included in the analysis for URI duration could be described as unreliable for use, because of low participant numbers and incomparable supplements. Perhaps more well-structured, powered studies are required to assess the effect of supplementation on reducing duration of symptoms and URI for athletes.

Another consideration was the suggestion that reporting only the p -value for analysis would not provide adequate insight into results and that effect size helps readers understand the magnitude of differences found (Sullivan & Feinn, 2012). With that, despite being non-significant, it could be considered that this result was 'trending toward' significance, with a moderate effect size. However, this would need to be caveated with the limitation of only having two studies within the analysis. With so few studies generally, conclusions are somewhat limited and need further examination.

6.4.2.2 Symptom Severity

Findings showed no effect in reducing symptom severity score for vitamin D supplementation groups versus placebo. Two RCTs found no relationship between supplementation and symptom severity scores (Da Boit et al., 2015; Dubnov-Raz et al., 2015), which have been previously criticised. Only one RCT, Jung et al. (2018), reported significantly reduced symptom severity with vitamin D supplementation. Similarly, research in military recruits, reported 15% lower URI severity following supplementation, which has been supported elsewhere in the same population group (Laaksi et al., 2010). With these positive findings within a similar population, explanations for why no significant response was found were explored.

This analysis produced a large effect size with no significance, perhaps again due to small participant numbers already discussed. Despite recruiting 25 athletes, low motivation was reported as only 17 returned the illness questionnaires (Jung et al., 2018). Which could provide sufficient rationale to the large effect size found. Furthermore, the analysis produced considerable heterogeneity ($I^2 = 90\%$). High variation in the random effects

model could be explained by the differences in study design and ways the outcome was measure. For example, studies used different illness questionnaires, the Wisconsin Upper Respiratory Symptom Survey (WURSS) and an illness log validated elsewhere (Fricker et al., 2005); and so quantitative URI data may not have been comparable. So far, the use of included studies for symptom severity analysis could be incomparable due to differences in study design, and could explain the current findings.

6.4.2.3 Frequency of URI

Overall, more studies showed that vitamin D supplementation had no effect on frequency of URI occurrence; RCTs reported no difference in the number of URI episodes between groups (Da Boit et al., 2015; Dubnov-Raz et al., 2015). In contrast however, studies conducted in the general population do not support these findings. Previous large scale meta-analyses conducted in the general population have reported vitamin D supplementation to have a protective effect against illness, reducing the risk of experiencing URI (Jolliffe et al., 2021; Martineau et al., 2019). Only one RCT in the current review was in agreement with these findings; Jung et al. (2018) reported significantly lower total URI symptoms with supplementation of vitamin D. Observational studies additionally reported no association between 25(OH)D with number of illnesses (Barcal et al., 2016; Scullion et al., 2018). This again, is in contrast to studies in other populations who found significant associations with incidence of URI (Sabetta et al., 2010). In addition to the limitations of the individual studies already identified, deeper analysis is needed to identify why no protective effects for illness were found in the current review.

6.4.3 Considerations

One explanation for the apparent lack of association between vitamin D supplementation and illness within the current review, could be the difference in baseline 25(OH)D status. Martineau et al. (2019) found that protective effects from vitamin D supplementation against illness were stronger in those with baseline deficiency. To support, a larger effect size was found in reducing URI duration following supplementation, for participants with

serum 25(OH)D $<50 \text{ nmol}\cdot\text{L}^{-1}$ (Harrison et al., 2021). In one example, no association of URI with 25(OH)D status was found in elite rugby players and rowers (Scullion et al., 2018). However, Scullion et al. (2018) reported a very low percentage of participants to have vitamin D insufficiency (28.3%) and this may explain the lack of findings for risk of URI. For the current meta-analysis, RCTs found no difference in duration of URI; while one study did not report 25(OH)D concentration (Da Boit et al., 2015), the other reported baseline 25(OH)D concentrations of $61 \text{ nmol}\cdot\text{L}^{-1}$ (Dubnov-Raz et al., 2015). Therefore, lack of significance could be due to higher 25(OH)D concentrations above the proposed sufficient threshold ($\geq 50 \text{ nmol}\cdot\text{L}^{-1}$), suggesting that supplementation may have more impact on illness for those with low serum 25(OH)D status.

Furthermore, the majority of RCTs within the current review, reported a mixture of 25(OH)D levels for athletes; sufficient, insufficient and deficient. As previously identified in Chapter 2 (page 46), definitions of vitamin D insufficiency vary to some extent. Dubnov-Raz (2015) reported swimmers to have baseline 25(OH)D insufficiency, however, they had a mean concentration of $61 \text{ nmol}\cdot\text{L}^{-1}$. This means concentration was above the sufficient definition identified earlier, $\geq 50 \text{ nmol}\cdot\text{L}^{-1}$ (Institute of Medicine, 2011), which could be misleading. Only one RCT examined an athlete population who were all 25(OH)D deficient, and upon review, this study had some of the most promising associations for URI following supplementation (Jung et al., 2018). Differences in baseline 25(OH)D status, could justify why no effects were found in the current meta-analysis. This additionally raises the question of whether blanket supplementation of vitamin D for all athletes is necessary.

It has been said that 'blanket' supplementation for entire teams and athletes is common (Owens et al., 2018). However, it has been proposed that there is no additional benefit of providing vitamin D supplementation to those who are already sufficient, showing more advantages for illness in those with baseline deficiency (Martineau et al., 2017; Owens et al., 2018); therefore, the requirement for athletic 'blanket' supplementation has been questioned. So, supplementation should be employed for each athlete independently, taking into consideration length of sunlight exposure, whether the athlete has a darker

skin pigmentation, and availability of serum 25(OH)D testing (Owens et al., 2018). These findings highlight the need for individual athlete testing to identify those with 25(OH)D deficiencies. It should be considered however, that not all athletes have access to methods of determining serum 25(OH)D levels due to being costly; whereas vitamin D supplements are widely available and affordable. Toxicity levels (10,000 IU) already described, should be considered (EFSA, 2012). However, given the high prevalence of vitamin D deficiency (Public Health England, 2019), blanket supplementation poses very few disadvantages and explains the 400 IU supplement recommendation from the NHS.

There is no clear consensus on optimal vitamin D supplement dosage for immune health (He et al., 2016). Included RCTs with study protocols ≥ 12 weeks all tested daily dosages equal to and more than the UK recommendation (400-5000 IU). Despite 400 IU being recommended by the UK government (Public Health England, 2019), other government recommendations suggest 600 IU (Walsh, 2019). Due to guidance being based upon skeletal health benefits, some have proposed increasing the recommendation to at least 1000 IU to benefit immune health (Harvard, 2022). In support, a recent meta-analysis found that vitamin D supplementation ≤ 1000 IU reduced risk of URTI and gave some protection against illness (Jolliffe et al., 2021). Researchers suggested that optimal vitamin D dosing were daily standard doses of 400–1000 IU, for up to 12 months. It was found that this would provide greater protective effects against illness compared to high bolus doses of vitamin D, due to potentially causing harmful effects on its own metabolism (Jolliffe et al., 2021). However, suggestions of 1500-2000 IU daily vitamin D supplementation has been advised to consistently raise 25(OH)D >75 nmol·L⁻¹ (Holick et al., 2011), which was found to be beneficial in reducing risk of illness (Bischoff-Ferrari, 2008; He et al., 2013; Sabetta et al., 2010). In contrast however, Owens et al. (2017) suggested that there would be little point in elevating 25(OH)D concentration beyond the guideline for sufficiency (>75 nmol·L⁻¹). Therefore, more work is needed to establish the most optimal vitamin D dose for illness benefits.

Overall, if serum testing is unavailable, a daily supplement of 1000 IU vitamin D should be advised for athletic supplementation during winter months and for those with darker

skin pigmentation. The beneficial effect of 400–1000 IU daily vitamin D supplementation is clear, but more work is needed to establish the most optimal dosage. But it should be considered that deficient athletes may require a higher dose to restore status, so if serum deficiencies are found, the athlete may benefit from short term 2000 IU vitamin D supplementation (as highlighted by sport governing bodies) (AIS, 2021; Owens et al., 2018).

6.4.4 Strengths, Limitations and Future Work

A strength of the current analysis, would be that the quality of outcome is substantially improved when small increases in sample size are made, via a meta-analysis, compared to the primary sample size (Koetse et al., 2011). Viewing collated study findings together would be far more effective in reducing variance than primary study sample size, which arguably is crucial for studies in athletes with commonly small sample sizes.

However, the main limitation was the lack of studies which met the inclusion criteria for the current meta-analysis and review. Preregistered secondary aims included; determining whether the effect of vitamin D supplementation on upper respiratory symptoms is different for athletes who are vitamin D deficient and non-deficient, and to explore the effect of different doses and type of vitamin D on upper respiratory symptoms in athletes (amount, frequency of vitamin D dose, duration of supplementation). Sadly, these aims could not be tested due to low number of studies. It had also been discussed to use meta-regression analyses for continuous moderator variables, such as exploring the different vitamin D supplementation dosages. Furthermore, one of the best ways to assess heterogeneity is through moderator analyses; however again, the small number of studies included in the current review, meant that this was not feasible. Although no definitive minimum number of studies is required for meta-regression, we followed the general recommendations of at least 6 to 10 studies for a continuous variable (Fu et al., 2008; Higgins et al., 2019) and for a categorical subgroup variable, a minimum of 4 studies per group (Fu et al., 2008). On reflection, studies conducted in military recruits should have been included within the pre-registration. This not only would have increased

number of studies, but also participant numbers; consequently, conclusions drawn in the current review on supplementation and illness, may have been different.

The other limitation largely reflects the limitations of the original studies. Aside from there being few studies, the RCTs used for the meta-analysis included small participant numbers (average = 36). Undoubtedly, it could be assumed that these existing studies were significantly underpowered, which would likely increase the effect size, and heterogeneity. In addition to this, even though seven RCTs met the inclusion criteria, analysis was difficult due to differences in study design. Available studies lacked consistency in classification of URI (some reporting diagnosed infection and others reporting symptoms), with different illness recording methods which make comparisons of quantification difficult. Furthermore, the studies did not all investigate similar URI outcomes or immune markers, making comparisons between RCTs challenging. To avoid this, future studies need to have larger participant numbers and clearly outlined methods which are comparable to other studies. Lastly, quality of two RCTs included within the meta-analysis had high risk of bias. Both studies showed high risk in selection of the reported result (D5), where they did not follow the original pre-registration. This increased bias as author's conducted further analyses and reported only reported significant findings, ignoring primary outcomes which were listed within the registration (Dubnov-Raz et al., 2015; Todd et al., 2017). Future research should clearly identify primary and secondary aims within pre-registration, to avoid reporting only significant findings.

6.5 Conclusion

To conclude, the current meta-analysis did not show a notable change in immune markers following vitamin D supplementation; no effect for salivary IgA or TNF- α was observed. A main finding was that there was no effect from vitamin D supplementation on sIgA concentration and secretion; lack of significant findings for immune markers has been supported elsewhere (Harrison et al., 2021). Upon further review however, vitamin D supplementation may be beneficial in increasing cathelicidin secretion rate; more research is needed to explore this further. Furthermore, no effect was found for illness parameters with supplementation of vitamin D in athletes. This was in contrast with other literature and findings could be due to limitations of the studies themselves, such as; small participant numbers, missing data and differences in study design and outcomes. Moreover, supplementation may have a more significant effect for those who have low baseline 25(OH)D concentrations ($<45 \text{ nmol}\cdot\text{L}^{-1}$), compromised immune systems or elevated baseline markers of inflammation (Cannell et al., 2015; Harrison et al., 2021). However, only one RCT in the current analysis had participants with low baseline 25(OH)D concentrations. It should therefore be considered that the reasons above may not reflect the full picture on illness parameters.

High prevalence of vitamin D insufficiency in athletes, plus associated risk factors, has been identified (Farrokhyar et al., 2017). As those who are serum 25(OH)D deficient could more likely benefit from vitamin D supplementation, the importance of individual athlete serum testing was highlighted. However, it was considered that when testing is not possible, 1000 IU daily vitamin D supplementation could be recommended in winter, for those than train predominately indoors, and have a darker skin pigmentation. On review of promising findings found for reducing the duration and severity of URS, vitamin D supplementation may be of use for the athletic population for maintenance of training adherence. That said, results remain conflicting and there is a clear requirement for more well-designed and significantly powered, RCT studies. Overall, more work is needed to confirm vitamin D supplementation effectiveness on immune function and URI in athletes.

CHAPTER 7

General Discussion

This chapter contains an overview of thesis aims and development over time, plus the main findings for each chapter included. Following this, contributions to knowledge for general themes in relation to other literature will be discussed, alongside the relevance and practical implications of these findings. Finally, thesis strengths and limitations, and direction for future work will be addressed.

The opening study (**Chapter 4**) of the current thesis, aimed to monitor mucosal immunity and identify risk factors associated with URS in elite national and international swimmers (Chapter 4, Table 4.1). To do so, EBV serostatus, sIgA, URS, and sleep parameters were monitored at certain time points over 8-months leading into major competition. The initial idea for this study came from Gleeson and Pyne (2016), who highlighted the need for further athletic monitoring of mucosal immunity and identification of illness risk factors, in order to consequently enable modification of training and other illness preventative strategies. The basis for **Chapter 5** was developed from work done in **Chapter 4**, due to the requirement of an ELISA for detection of sIgA in elite swimmers. Therefore, an in-house ELISA was developed and optimised, using validation and optimisation methods. Finally, due to the observed increase in incidence, duration and severity of URS following high training loads in **Chapter 4**, proposed research for the final study included the use of an illness preventative intervention during high training loads in elite swimmers, such as Vitamin D supplementation. However, due to COVID-19 restrictions, this type of research was unable to be conducted. Therefore, the decision was made to instead analyse existing literature and conduct a meta-analysis and systematic review (**Chapter 6**). The aim of the meta-analysis and systematic review was to investigate the effect of vitamin D supplementation on immune markers and URI in an athletic cohort.

7.1 Summary of Main Findings

7.1.1 Chapter 4

- i. This study identified that individual monitoring was useful in identifying associated risk factors of URS in elite swimmers. A main finding of this study was that high training loads were associated with significantly increased URS episodes, severity, and duration of symptoms.
- ii. Despite no association between sIgA and incidence or severity of URS, sIgA was significantly lower when swimmers presented with URS, than when no symptoms were present.
- iii. Sleep inadequacy was paramount in elite swimmers, with low sleep efficiency (75%) presented throughout the 8-month observation. Furthermore, elite swimmers regularly slept below the NR (6 hours 30 minutes), with further significant reductions in sleep duration during high training loads.
- iv. Epstein Barr Virus prevalence was 61% in the current cohort, however serostatus did not appear to be an effective immune marker for URS, as no association was found with URS episodes or severity of symptoms. To the investigator's knowledge, this was the first study to examine this in British elite swimmers.
- v. Fluctuations in body mass were not correlated with incidence or severity of URS in female elite swimmers.

7.1.2 Chapter 5

- i. The development of an in-house ELISA for determination of sIgA underwent successful optimisation, showing an efficient SC with good range (100-800 µg/mL) and sensitivity between concentration points.
- ii. Spike and recovery assessment confirmed large matrix effects between the chosen diluent and saliva samples, therefore IgA-blank saliva was successfully gained from deficient participants throughout the UK. Using saliva as diluent for standards, recovery (95-113%) was within accepted guidelines.

- iii. Precision experiments found successful intra-assay CV of 10-12%, which was within acceptable guidelines.
- iv. In-house ELISA sIgA data was significantly correlated with a commercially available assay, showing similar trends over time.
- v. Methods and protocols used within this novel ELISA development and optimisation chapter could be used as a basis for future work, especially to reduce the inter-assay variation.

7.1.3 Chapter 6

- i. A main finding from the meta-analysis included that vitamin D supplementation had no effect on immune markers, sIgA and TNF- α , in athletic cohorts.
- ii. Additionally, vitamin D supplementation had no positive influence on illness parameters, showing no effect on duration and severity of symptoms for URI in athletes.
- iii. Investigation of findings highlighted the fundamental gap in literature surrounding the influence of vitamin D on immune markers and URI within the athletic population; therefore, more well-designed and sufficiently powered studies are required.

7.2 Contribution to Knowledge

7.2.1 Risk Factors for URI in Athletes

In **Chapter 4**, significantly more URS episodes were reported during high training loads. The majority of research has found this to be the case, with most URI occurring during heaviest training periods (Spence et al., 2007; Rama et al., 2013). Similar to the current work, Rama et al. (2013) monitored 19 elite swimmers for 7 months in winter, reporting that 67% of URS occurred around high training loads. Moreover, findings have repeatedly been shown in swimmers following high intensity training bouts (Fricker et al., 1999; Gleeson et al., 2000; Spence et al., 2007). Therefore, findings were consistent with previous literature, strengthening the relationship for incidence of URS in relation to

training load. In addition to this, the current study found significantly longer duration of URS and higher severity of symptoms during high training loads. Previously it has been suggested that in order to become a successful elite athlete, the individual must have an immune system capable of fighting infection during prolonged periods of intense training (Mårtensson et al., 2014). While this may be true, it should be considered that small reductions in training load may promote more success for those with weaker immune systems. The importance of avoiding missed training days has previously been highlighted (Raysmith & Drew, 2016), plus current findings showed no significant difference in number of URS episodes between low and moderate training. One practical application of this may be that coaches could reduce training load (low to moderate, for example) for athletes at increased illness risk, in order to prevent further symptoms. It could be argued that for those at heightened risk of URS, small reductions in training load would outweigh the negative impact that increased URS episodes and missed training could have. Consequently, this could lead to the potential for increased performance and athletic success for that individual.

Previously, inadequate sleep and sleep disturbances have been identified as a risk factor for increased illness (Peake et al., 2017). Cohen et al. (2009) highlighted the impact of reduced sleep duration on illness; those with < 7 hours of sleep were 2.94 times more likely to develop a cold. This was significant because similar to other research in swimmers (de Mello et al., 2020; Gudmundsdottir, 2020), sleep duration in the current cohort (**Chapter 4**) was 6 hours and 30 minutes. Findings were below the NR of 7-9 hours and supported a recent review that found athletes were often unable to achieve ≥ 7 hours of total sleep time (Roberts et al., 2019). One study observing swimmers specifically, reported TST as 6 hours 14 minutes (Gudmundsdottir, 2020), which was less than that reported here. That said, amount of sleep can be heavily influenced by an athlete's training schedule (Surda et al., 2019). In the current study, TST was significantly less during moderate and high training load, compared to low. Current findings were supported by previous literature which found intensified training significantly decreased TST, negatively effecting overall sleep duration (Dumortier et al., 2017; Lastella et al., 2015; Schaal et al., 2017). Likewise, in a study conducted on 22 collegiate swimmers, longest

sleep duration was found when training load was low (Astridge et al., 2021). In addition to reduced sleep duration, poor sleep quality was also shown.

Swimmers in the current study had low sleep efficiency at all three timepoints throughout the 8-month study observation. Findings were in agreement of a recent review which found athletes generally struggled to achieve $\geq 85\%$ sleep efficiency during training (Roberts et al., 2019). Previously, swimmers have shown average sleep efficiency below the threshold to be considered normal (de Mello et al., 2020; Lastella et al., 2015). However, in the study presented in **Chapter 4**, swimmers had the lowest sleep efficiency recorded, in comparison to other literature (Gupta et al., 2017). Specifically, Ohayon et al., (2016) suggested that a sleep efficiency of $\leq 74\%$ indicated bad sleep quality, of which was found in this study. Greater sleep fragmentation has been reported in athletes (Gupta et al., 2017), so perhaps the high WASO found in this cohort could have affected sleep efficiency. That said, there would be no way to determine this so the disruptive effect of academic stress on sleep (Ahrberg et al., 2012) was considered as they were university students (**Chapter 4**). Regardless of causal effect, the need for sleep hygiene education was highlighted. Teaching sleep hygiene methods such as, afternoon napping, avoiding television, computers and phones before sleeping and avoiding caffeine 4-5 hours before sleeping, were effective methods to improve sleep quantity and quality for Olympic athletes (de Mello et al., 2020). Practical application should include sleep monitoring to identify those with inadequacy, then sleep hygiene education or training management from coaching staff (by changing scheduled training times for example), could help in improving sleep adequacy and subsequent risk of URI in athletes.

It has been suggested that previous infection with EBV may contribute as a risk factor for URI and associated symptoms (Gleeson et al., 2002). In the current study, 61% of swimmers had previous infection with EBV (**Chapter 4**), which is important as there is limited research on prevalence of EBV seropositivity in elite British swimmers. That said, current findings suggest that EBV status did not appear to determine whether an individual was at increased risk of URS, with no impact on incidence, duration, or severity of URS between the two groups. Other studies have presented support for this finding

(Blume et al., 2018; He et al., 2013). In contrast however, other studies have found a significant association between serostatus of EBV and incidence of illness and infection (Balfour et al., 2015; Gleeson et al., 2002); therefore, more work is needed to confirm whether this relationship exists. In addition to those listed above, nutritional deficiencies could also increase risk of URI in athletes.

Research has shown a negative association between incidence of URI and 25(OH)D level (Berry et al., 2011; Sabetta et al., 2010). Military recruits (Laaksi et al., 2010), and athletes (Cox et al., 2008; Halliday et al., 2011; He et al., 2013; He et al., 2016) that were vitamin D deficient had significantly longer duration and increased severity of URS. On the other hand, beneficial 25(OH)D levels for reducing risk of illness have been defined ≥ 75 nmol/L (Bischoff-Ferrari, 2008; He et al., 2013; Sabetta et al., 2010). This may be because vitamin D plays a vital role in regulation of AMPs, such as cathelicidin and β -defensin (Liu et al., 2006), and can activate T and B lymphocytes (Bermon et al., 2017; He et al., 2016). Despite not testing for vitamin D deficiency in **Chapter 4**, the longitudinal observation went throughout winter months (September to April), which is when significantly lower 25(OH)D concentrations have been reported (Lin et al., 2021; Scullion et al., 2018). Therefore, this could have been a contributing factor for the high number of URS episodes presented for the swimmers. Thus, **Chapter 6** investigated the effect of vitamin D supplementation on immune markers and URI risk in athletes.

7.2.2 Effect of Vitamin D Supplementation on sIgA and URI

Due to the identification of several risk factors of URS in **Chapter 4**, **Chapter 6** examined the effect of vitamin D supplementation on immune markers and URI in athletes, via a meta-analysis. **Chapter 6** did not show a notable change in immune markers following vitamin D supplementation. Specifically, no change in sIgA concentration or secretion rate was found following vitamin D supplementation, which has been supported elsewhere (Harrison et al., 2021). Individually, RCTs included were insufficiently powered, but the meta-analysis was significantly powered to detect an effect, of which it did not. Therefore, the relationship between vitamin D status and sIgA concentration was not noteworthy and it could be said with confidence that a larger study would show no different outcome.

Upon further review however, He et al. (2016) was the first research group to report increased secretion of sIgA, following a daily supplement of 5000 IU vitamin D. It should be considered however, supplementation dose was above the government guidelines for tolerable upper intake (4000 IU) (EFSA, 2012). Increased sIgA secretion was additionally found in military recruits following supplementation of 1000 IU (Scott et al., 2019), however increased saliva flow rates (associated with increased 25(OH)D levels) and sex differences may have influenced the findings in both these studies. Elsewhere, no change in sIgA secretion rates have been shown following supplementation of vitamin D (Da Boit et al., 2015). Taken as a whole, very few studies have examined the effect of supplementation on sIgA secretion. However, given the findings for sIgA concentration, it would seem unlikely that a relationship exists.

In **Chapter 4**, sIgA had no interaction with incidence, duration, or severity of URS, yet swimmers regularly self-reported URS episodes. Therefore, the effect of vitamin D supplementation on URI and illness parameters was also examined. No effect was found for incidence, duration or severity of URI following supplementation of vitamin D in athletes (**Chapter 6**). This was in contrast with other literature and overall, it was considered that lack of significant findings was due to limitations of the studies themselves such as; small participant numbers, missing data and differences in study design and outcomes. Within the general population, Sabetta et al. (2010) reported significant associations between supplementation of vitamin D and incidence of URI (Sabetta et al., 2010). Since then, large scale meta-analyses have been conducted and reported that vitamin D supplementation has a protective effect against illness, reducing the risk of experiencing URI (Jolliffe et al., 2021; Martineau et al., 2019). Furthermore, 36% fewer days of illness were recorded following vitamin D supplementation in military recruits (Harrison et al., 2021). However, only one RCT in **Chapter 6** was in agreement with these findings; Jung and colleagues reported significantly less URS and reduced symptom severity with supplementation of vitamin D (Jung et al., 2018). Due to the positive findings found elsewhere in large cohort studies, it was considered that baseline 25(OH)D status may have affected findings for the current analysis.

It has been suggested that supplementation may have a more significant effect for those who have low baseline 25(OH)D concentrations ($<45 \text{ nmol}\cdot\text{L}^{-1}$) (Cannell et al., 2015; Harrison et al., 2021). Therefore, a practical application would include the importance of individual serum testing. However, findings from the meta-analysis in **Chapter 6**, could not comment upon this as only one RCT reported 25(OH)D deficiencies within their athletic cohort. Alternatively, it could be considered that this study had some of the most promising findings due to the 5000 IU supplementation dose of vitamin D (Jung et al., 2018), which was above the tolerable upper limit of 4000 IU (EFSA, 2012). Although there is no clear consensus on optimal vitamin D supplement dosage for immune health (He et al., 2016), current recommendations have been criticised due to potentially not being enough to provide non-skeletal health benefits. Therefore, some have proposed increasing the recommendation to at least 1000 IU (Harvard, 2022), and others 1500-2000 IU (Holick et al., 2011). Therefore, when testing is not possible, daily 1000 IU vitamin D supplementation should be considered in winter, for those than train predominately indoors, and have a darker skin pigmentation. Overall, vitamin D supplementation may be of use for the athletic population for maintenance of training adherence during winter months, especially if 25(OH)D levels are insufficient. Despite the requirement of further research, this would provide a positive impact on training routines for an athlete and would outweigh the very few negatives to vitamin D supplementation.

7.2.3 Individual Athlete Monitoring vs. Group Mean Data

Firstly, comparisons of group mean values of sIgA comes with difficulty due to the large variation presented. The large between-subject variation of sIgA found in **Chapter 4** (72%), was presented elsewhere (Francis et al. 2005; Nehlsen-Cannarella et al., 2000; Neville et al., 2008). This provides a feasible explanation for the vast differences in findings for sIgA between literature, so many have questioned the validity of sIgA as an effective marker of mucosal monitoring. Differences are accountable to study design, athletic population, level of fitness, saliva collection methods, and large between-subject variability of sIgA. Overall, differences in saliva collection between studies, clearly shows why making group comparisons between literature to be near impossible. Which could contribute to explain findings in **Chapter 6**, regarding the lack of effect from vitamin D

supplementation on this mucosal marker. Salivary IgA concentration additionally suffers from large within-subject variation and was found to be the case within the first study (**Chapter 4**). Sources of heterogeneity include environmental factors, dietary intake, alcohol or caffeine, stress, and illness status (Francis et al., 2005; Gleeson, 2000; Salimetrics, 2015). Even though circadian variations (Dimitriou et al., 2002; Li & Gleeson, 2004), timing of food and drink intake (Granger et al., 2012), and post-exercise effects (Gleeson et al., 2004) on sIgA were controlled for in **Chapter 4**, within-subject variation was 33%. It should be considered that these findings in variability were lower than what has previously been reported (Neville et al., 2008), which could have been due to the strict control of affecting variables. The need to monitor changes in sIgA in individual athletes through use of relative sIgA, reporting percentage change, rather than cohort means has been highlighted (Gleeson et al., 2017).

Previously, the association between sIgA and URI has been well documented (Fahlman & Engels, 2005; Gleeson et al., 1999; Neville et al., 2008), however has also been refuted (Leicht et al., 2012; Stephenson et al., 2019). Differences in findings could again be due to the large individual variation of sIgA, indicating that some athletes are more susceptible to illness than others (Francis et al., 2005; Neville et al., 2008). **Chapter 4** showed that sIgA concentration was significantly lower during weeks with reported URS, compared to weeks where swimmers reported no URS; adding strength to the use of sIgA as a mucosal biomarker alongside URI for individual monitoring. Visual trends were seen with relative sIgA (**Chapter 4**) decreasing in the weeks before a URS episode, which has been supported elsewhere (Neville et al., 2008). These findings suggested that one threshold concentration should not represent all athletes and instead, change from an individual's healthy baseline could be a more promising determining factor of illness to consider. To support, one swimmer in **Chapter 4** spent 71% below their sIgA average and reported a total of 6 (above average) URS episodes. Additionally, while there was no association found for incidence of URS and symptom severity or duration with group data, 13 out of 14 swimmers spent $\geq 50\%$ of time below their average sIgA over the whole 8-month season. Arguably, this data should be used by coaches to determine when training load could be reduced for example.

It has been suggested that individual monitoring may also identify athletes that are more adaptable to training or stress than others (Francis et al., 2005; Neville et al., 2008). As an example, identification of a swimmer who has reported the highest symptom severity score (during a high training load week) over the whole study period. It should be questioned whether this swimmer could be more susceptible to changes in mucosal immunity, 'at risk' of URS and arguably should be monitored closely during increased training load. As previously mentioned, identification of at-risk athletes could promote training management to improve sleep quality, increase sIgA concentration and reduce subsequent URS. These findings further support the use of individual monitoring, exploring individual trends over time in relation to other risk factors such as 25(OH)D level or EBV serostatus.

Despite findings showing no effect of EBV serostatus on incidence, duration or severity of URS in **Chapter 4**, results were underpowered due to small sample size and did not assist the ability to find significance. Participant recommendations from pre-study power calculations are often unrealistic when recruiting elite athletes. As previously discussed, individual data and trends could be more useful to coaches and researchers, when compared to group statistical data and *p* values. Despite lacking significance, EBV seropositive swimmers reported 25 missed training days compared to those who were negative (9 missed training days). Therefore it should be considered that past infection of EBV may interact alongside other factors to put an athlete at higher risk of illness, which is why in-depth individual analysis is preferred over group mean data. Arguably, these were still highly important findings as it could be suggested to coaches to slightly reduce training load (high to moderate) for those at increased risk, or those that suffer frequent URS in order to prevent further symptoms. Ultimately, factors such as differences in sex (Gleeson et al., 2011), immune or nutritional deficiencies (Bermon et al., 2017; Reid et al., 2004), or other factors identified within this thesis, may act together to influence increased risk of illness for an individual. Therefore, the complexity of the immune system and nature of URI, highlights the need for monitoring individual change rather than cohort means and median analysis.

7.3 Strengths and Limitations of Thesis

One strength of the first study (**Chapter 4**) was that to the investigator's knowledge, it was the first in the UK to examine EBV serostatus in elite national and international swimmers. These findings not only add strength to the high prevalence of past EBV infection for athletes, but they also suggest no relationship with URS. Due to lack of research and contrasting findings, the results from **Chapter 4** add to this gap in knowledge. Another strength of **Chapter 4** includes the fact that samples were collected in standardised conditions, at a consistent time of day, on the same day each week, where athletes did not partake in morning training and had the longest rest time between sessions. Samples were taken consistently every week over the whole duration of 8-months, collecting up to 32 samples for each athlete, which was significantly more than other longitudinal studies (Fahlman & Engels, 2005; Francis et al., 2005; Gleeson et al., 1995; Stephenson et al., 2019). With fewer samples taken, there would be less likelihood of detecting real change and would account for previous inconsistent findings (Hopkins, 2000); and therefore was a strength of the current work. Furthermore, circadian variations, timing of food and drink intake, fasted and post-exercise effects were considered and controlled. This could explain why less within-subject variation was found, compared to previous work (Neville et al., 2008). An additional key strength was the use of objective sleep measurement, in addition to self-reported measures of sleep. This is because the use of wrist-worn actigraphy has been recommended for measures of sleep (Zinkhan et al., 2014) and validated against the gold standard PSG (Quante et al., 2018). Overall, very few longitudinal studies have monitored as many variables (training load, sIgA concentration and secretion, EBV serostatus, weekly self-perceived URS, sleep and training load, and sleep parameters) in athletes, as in **Chapter 4**; which can be seen as a key strength of the current thesis.

However, the small sample sizes found in **Chapter 4**, plus that of the included RCTs in **Chapter 6**, could be regarded as a limitation. That said, this is a common issue when research is targeting specific groups of elite athletes. Regarding **Chapter 4**, there are few elite national and international swimmers to recruit and was similar to that elsewhere (de Mello et al., 2020; Lastella et al., 2012; Rama et al., 2013). Sample size and length of

study was also considered for cost implications, which was why the in-house ELISA was so important to develop in **Chapter 5**. Nevertheless, larger sample sizes of athletes would of course enable more appropriate and reliable conclusions. With this, a strength of **Chapter 6**, was that the quality of outcomes were substantially improved through use of a meta-analysis, due to increased sample size compared to the primary independent study (Koetse et al., 2011). Collated study findings would be far more effective in reducing variance together than primary study sample size, which is arguably crucial for studies in athletes with commonly small sample sizes.

Most importantly, the current meta-analysis (**Chapter 6**), was novel and the first to examine the effect of vitamin D supplementation on immune markers and URI in athletes. Unfortunately however, some aims could not be met due to low number of RCTs available investigating the effect of vitamin D supplementation on URI. This meant that some analyses were not feasible, and other non-significant findings suffered from large effect sizes and heterogeneity. It has been considered that studies conducted in military recruits could have been included within the pre-registration. This would have increased number of studies and participant numbers; consequently, conclusions drawn in the current review on supplementation and illness, may have been different. Despite being a limitation of the current thesis, it does however provide a clear gap in the literature for future studies.

Finally, there were several strengths to take away from the work completed in **Chapter 5**. The current thesis showed successful development of an in-house ELISA, demonstrating method validation and analytical technical skills. Acceptable sensitivity, spike and recovery, precision and intra-assay variation were achieved. Large matrix effects meant that the diluent of standards did not closely match that of the target sample. However, this hurdle was overcome through recruitment of sIgA deficient individuals throughout the UK. Use of this IgA-blank saliva for the SC, meant that the current ELISA could be developed and optimised in-house for determination of sIgA. Perhaps the largest strength, was that the in-house ELISA data was significantly correlated with a commercially available assay, suggesting that it could detect similar changes and

patterns of sIgA. Despite unsuccessful elimination of high inter-assay %CV, overall development was positive and provides a basis for future work.

7.4 Future work

Regarding immune monitoring, further research is needed to investigate sIgA and secretion rates over time, but with the analysis being conducted on an individual-to-individual basis. The focus of research has previously been on comparisons between literature, but due to the large between-subject variation, group mean comparisons should be taken with caution in the literature. Plus, saliva collection should be strictly controlled to reduce factors that affect sIgA. Measurement of sIgA should be used to identify trends, reductions from baseline or averages, to determine risk of URS and associated risk factors in an individual. Importantly, successful outcomes from the ELISA development for sIgA in **Chapter 5** would be useful as a basis for future researchers. Perhaps some consideration should be given to the development of point of care sIgA analysis (Gleeson et al., 2017). This new method could provide faster results, within minutes of sample collection. Specifically, the IPRO LFD has shown to be reliable when compared to the gold standard ELISA (Coad et al., 2015) and therefore, more research is needed to examine this method of sIgA monitoring in athletes. Overall, too few studies have looked into individual data and sIgA relationships between factors, so more work is needed.

Furthermore, consideration of viral reactivation with measurement of EBV-DNA in saliva, could be more important for future studies than determination of serostatus alone. Increased viral EBV-DNA expression has been detected in saliva before the appearance of URS (Gleeson et al., 2002; Yamauchi et al., 2011). Therefore, it has been suggested that EBV could be controlled by altering training load to prevent IgA suppression and EBV reactivation (Gleeson & Pyne, 2016), and may therefore be important in athlete monitoring and immune surveillance. Despite one study suggesting no relationship between EBV-DNA and URS (Perkins & Davison, 2021), further research is needed to investigate the use of EBV-DNA as a marker of immune function and the relationship with URI and associated symptoms.

Further research is needed to explore the relationship between sleep and illness. Longer durations of wrist-worn actigraphy should be used (compared to 7 days used in the current study), alongside self-reported sleep quality and symptoms of URI. This would enable a clearer picture of the effect of sleep inadequacy paramount in athletes and the effect it has on illness. Despite not monitoring psychological stressors in **Chapter 4**, financial hardship and studying could have influenced some findings as they were University students and should be considered for future research. Lastly, future longitudinal work on individual athletic monitoring could observe other factors that could influence immune function, such as menstrual cycle stages for female athletes, diet, or vitamin D status, especially if monitoring during winter months.

Overall, the risk of widespread vitamin D insufficiency in athletes is clear, and more work is needed to be done to confirm supplementation effectiveness and optimal dosage for URI in athletes. Despite positive findings within the general population, results in athletic populations regarding the effect of vitamin D supplementation on URI incidence, duration and severity remain conflicting. **Chapter 6** demonstrated an evident gap in literature, with a requirement for more well-designed and significantly powered RCTs. Future studies should ensure to measure URI outcomes or immune markers, similar to that conducted by other researchers to enable comparisons to be made. Overall, studies need larger participant numbers and clearly outlined methods which are comparable to other studies.

7.5 Conclusion

To conclude, the current thesis presented a well-controlled, longitudinal observation of mucosal immunity and risk factors associated with URI in elite national and international swimmers. Findings strengthen the notion that increased training loads are associated with higher number of URS episodes, with increased duration and severity of symptoms. Absolute and relative sIgA was significantly lower on weeks where URS were reported, adding strength to the use of this biomarker alongside self-reported symptoms. Despite the risk of increased illness with inadequate sleep, elite swimmers suffered from paramount sleep inadequacy. These findings promote the importance of sleep, and the beneficial effects on performance and URS. Overall, highlighting athletes at risk and identifying possible risk factors for URS, such as increased training loads and inadequate sleep, may allow for modifications in training or other illness preventative strategies within a group of elite athletes. Vitamin D supplementation did not appear to be an effective illness preventative strategy, showing no notable changes in sIgA or TNF- α , or illness parameters such as URI duration or severity of symptoms. These findings were in contrast with large cohort studies in the general population, so may be attributable to; the small number of studies included, small participant numbers, and differences in study design. Furthermore, supplementation may have a more pronounced effect for those who have low baseline 25(OH)D concentrations ($<45 \text{ nmol}\cdot\text{L}^{-1}$); therefore, the importance of individual athlete serum testing was highlighted and should be the focus for future longitudinal studies conducted throughout winter. Findings remain conflicting, thus more work is needed to confirm the effectiveness of vitamin D supplementation on immune function and URI in athletes.

CHAPTER 8

References

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CHAPTER 9

Appendices

Appendix A

Instructions sent to IgA deficient participants (Chapter 5) for saliva sampling

UH Protocol Number: aLMS/PGR/UH/02940(3)

Thank you again, for consenting to participate in my study.

In this envelope, you'll find the following;

- Health Screen (Please return in the package)
- 50ml saliva sample pot (x4)
- Biohazard labelled bag with cotton wool
- Ice packs
- Royal Mail leaflet

Participant Instructions

1. Please place the ice packs in the freezer for at least 48 hours.
2. Once the ice pack has been frozen for at least 48 hours, be ready to provide your sample.

The sample must be taken in the **afternoon (pm)**, on a day where you have completed **no exercise**. Please leave at least **1 hour after eating**, so that no food can contaminate the sample. **Water ONLY** can be drunk **up to 30 minutes before** providing the sample, no mouth rinsing is required. The sample requested is a passive drool. When ready, tilt the head forwards and dribble into the saliva test tube. It is asked that you do not try and 'cough up' saliva, it should collect in the mouth naturally when the head is tilted forward and then can be drooled into the tube. Please aim to fill the tube with **10ml of saliva** (lines are indicated on the sample pot). Then repeat these steps. Please note, I'd like you to provide all samples together; they can be done one after the other so please allow some time!

3. Complete the health screen.
4. Provide your samples and place saliva sample pots in the biohazard labelled bag, with the cotton wool (for padding) and seal.
5. Freeze the saliva samples in the biohazard bag, **overnight**.
6. Next day, place both the frozen ice packs, saliva sample pots (in the sealed biohazard bag) and health screen, into the thermal envelope. The sample pots should sit in the middle/on top of the 2 ice packs.
7. Send back to me immediately after sealing the thermal envelope via your local post office. If possible, do this in the afternoon so that samples have less time to defrost before getting back to me. However, you must ensure the parcel gets to the post office before 5pm, so that it will be delivered next day. The full return cost of the envelope has been paid, with the prepaid special delivery stamps.
8. Please **DO NOT** send your samples on a Friday, Saturday or Sunday as I do not work over the weekend and will not be there to collect your samples.

Please note, I have enclosed a Royal Mail leaflet stating that human saliva (biological material) is a restricted (but not prohibited) item to be sent. The relevant protocol for transport of biohazardous material has been met and ethically approved. This leaflet can be used at the Post Office, if for any reason they question sending your saliva sample (which they shouldn't).

Thank you.

Appendix B

Weekly questionnaire used in Chapter 4 (adaption from the Australian Institute of Sport monthly illness log form, gained from Professor Maree Gleeson)

Lauren Baker: LMS/PGR/UH/02940

Name: _____
 Date: _____
 Week: _____

Step 1: Circle your answers for sleep, training, injury and illness sections.
Step 2: Please state what injury/illness, whether you have seen a doctor and what medication you received.
Step 3: To the best of your ability, please number each illness appropriately for the past week (1- Minimal, training was normal, 2- Moderate, training was modified, 3- Severe, did not train). If you had no illness, please leave them blank.

Sleep:
 This week, how often have you met the 7-9 hours of sleep (per day) recommendation?

Everyday	5-6 times a week	3-4 times a week	1-2 times a week	Not once
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How would you rate your quality of sleep, for this week? (1 poor- 10 good)

1	2	3	4	5	6	7	8	9	10
---	---	---	---	---	---	---	---	---	----

How often this week have you felt tired or fatigued, after waking up in the morning?

Everyday	5-6 times a week	3-4 times a week	1-2 times a week	Never
----------	------------------	------------------	------------------	-------

Training

In your opinion, what intensity was swim training this week? (1 low- 10 high)

1	2	3	4	5	6	7	8	9	10
---	---	---	---	---	---	---	---	---	----

In your opinion, what intensity was weight training this week? (1 low- 10 high)

1	2	3	4	5	6	7	8	9	10
---	---	---	---	---	---	---	---	---	----

Injury?

Yes	No
-----	----

If yes, please state:

Illness?

Yes	No
-----	----

If yes, please state what:

Did you see a doctor? Yes/No	Have you taken any medication? Yes/No If yes, please state what:
------------------------------	---

	Thursday	Friday	Saturday	Sunday	Monday	Tuesday	TODAY
Upper Respiratory - blocked or runny nose, sore throat, sneezing							
Chest Infection - coughing, sputum, chest congestion, wheezing, high temperature							
Muscles and joints - aching or swollen (not related to injury)							
General fatigue - lethargy, tiredness							
Head - headache, migraine, dizziness, vision impairment, vertigo, glare/light intolerance							
Ears - ear ache, ringing in the ears, hearing loss							
Rashes - specify - localised (L) or widespread (W)							
Skin infections - sores, boils, deep abscesses, infected blisters, athlete's foot							
Gastrointestinal problems - nausea, vomiting, diarrhoea, abdominal pain, bloating, painful swallowing, loss of appetite							
Cardiovascular - feeling of fast heartbeat, shortness of breath, blackouts							
Eye irritation - itchiness, redness, sticky discharge, watery eyes							
Psychological - feeling depressed or anxious (not related to major event), poor sleeping pattern							
Urinary tract - increased frequency of passing urine, pain/burning, bleeding, menstrual irregularity							
Other (please specify):							

Appendix C

Actigraph instructions given to participants in Chapter 4

ACTIGRAPH INSTRUCTIONS

1. Sign the agreement for loss/breakage of the device
2. Take the device home Wednesday and keep next to your bed (then you cannot lose it)
3. Put the device on your LEFT wrist BEFORE you get into bed
4. **IMPORTANT:** Please record the exact time you get into bed to go to sleep (e.g. 10:49pm), either on a notepad or in notes on your phone. If you read or watch TV in bed, please record the time you turn the light off to go to sleep.
5. **IMPORTANT:** Please record the exact time you get out of bed, either on a notepad or in notes on your phone (e.g. 6:17am).
6. Take the device off your wrist before going out, IT ONLY NEEDS TO BE WORN WHILST SLEEPING
7. These instructions also apply if you nap, before or after training. So please keep the device next to your bed.
8. Wear the device every night and every time you nap (from Wednesday night for a week), recording the exact times for “time in bed” and “time out of bed”.
9. Next Wednesday, please bring the device back to training to give to the next participant.
10. Either text or give Lauren your recorded in and out of bed times, for the whole week, next Wednesday.



Appendix D

Example sleep period breakdown report, given to participants in Chapter 4.

Sleep Period Breakdown

Sleep Algorithm Used: Sadeh

In Bed	Out Bed	Latency (min)	Efficiency	Total Time in Bed (min)	Total Sleep Time (TST) (min)	Wake After Sleep Onset (WASO)	# of Awakenings	Avg Awakening (min)
11/10/2017 23:07	12/10/2017 08:00	41	80.49%	533	429	63	20	3.15
12/10/2017 22:39	13/10/2017 04:50	39	69.27%	371	257	75	15	5
13/10/2017 10:37	13/10/2017 11:49	6	90.28%	72	65	1	1	1
13/10/2017 22:46	14/10/2017 04:17	10	77.95%	331	258	63	12	5.25
14/10/2017 07:59	14/10/2017 08:40	10	58.54%	41	24	7	3	2.33
14/10/2017 11:17	14/10/2017 11:53	7	61.11%	36	22	7	1	7
14/10/2017 23:41	15/10/2017 10:34	8	85.15%	653	556	89	27	3.3
15/10/2017 15:42	15/10/2017 16:31	8	57.14%	49	28	13	4	3.25
15/10/2017 22:48	16/10/2017 04:24	32	79.17%	336	266	38	12	3.17
16/10/2017 08:35	16/10/2017 09:44	6	72.46%	69	50	13	2	6.5
16/10/2017 16:03	16/10/2017 16:48	6	84.44%	45	38	1	1	1
16/10/2017 22:34	17/10/2017 04:50	15	81.65%	376	307	54	15	3.6
17/10/2017 12:06	17/10/2017 14:03	2	89.74%	117	105	10	4	2.5
17/10/2017 23:55	18/10/2017 09:17	16	79.18%	562	445	101	24	4.21
01:59	09:41	14.71	76.18%	256.5	203.57	38.21	10.07	3.79

Appendix E

Assay protocol flowchart for in-house ELISA development

THE DAY BEFORE

Prepare Coating Buffer	Prepare Wash Buffer	Prepare PBS	Prepare Blocking Buffer	For the Plate- Coating Antibody		TOTAL
1 capsule, 100mL deionized water	1 sachet in 1L of deionized water	1 tablet into 200mL deionized water	18ml PBS with 2mL BSA	Combine 10µl of anti-human IgA with 20mL of coating buffer (full plate): 1:2000	Add 100µl of the anti-human IgA and coating solution to the wells. Incubate at room temperature for 1 hour, cover plate and refrigerate overnight.	
5 mins	5 mins	5 mins	5 mins	5 mins	5 mins	30 mins

ON THE DAY

Prepare IgA Standards	For the Plate						
Prepare standards (2.3mg/mL = 174µL IgA + 326µL Diluent for 800µg/mL standard)	After the overnight incubation, blot and wash 3x with 300µL of wash buffer. Discard fluid in sink and tap plate on tissue.	Add 150µL of blocking protein to the wells.	Incubate plates at RT 22°C.	Wash and blot plate 3x, with 300µL of wash buffer.	Add 100µL of standards, samples, control (plus zero) to the wells in duplicate.	Incubate plates at RT 22°C.	Wash and blot plate 3x, with 300µL of wash buffer.
30 mins	1 min	3 mins	10 mins	3 mins	30 mins	90 mins	3 mins

Prepare IgA Detection Antibody	For the Plate		Create ABTS	For the Plate			TOTAL
Add 12.5µL of anti-human IgA HRP conjugate (25µL 1:2 with glycerol for storage) with 25mL of PBS: 1:2000	Add 100µL of the IgA HRP conjugate to each well	Incubate plates at RT 22°C.	Add 3 ABTS tablets to 15mL of ABTS solution.	Wash and blot plate 6x, with 300µL of wash buffer.	Add 100µL of ABTS to every well.	Incubate plates at RT for 15-20 minutes, then measure absorbance at 405nm.	
3 mins	3 mins	90 mins	←During Incubation	3 mins	3 mins	15 mins	4 hours 50 minutes

Appendix F

Total number of 'yes', 'no' and 'don't know' responses for each AXIS question in Chapter

6

	Question	Yes	No	Don't know/ comment
Introduction				
1	Were the aims/objectives of the study clear?	5	1	0
Methods				
2	Was the study design appropriate for the stated aim(s)?	5	0	1
3	Was the sample size justified?	2	3	1
4	Was the target/reference population clearly defined? (Is it clear who the research was about?)	6	0	0
5	Was the sample frame taken from an appropriate population base so that it closely represented the target/reference population under investigation?	5	1	0
6	Was the selection process likely to select subjects/participants that were representative of the target/reference population under investigation?	6	0	0
7	Were measures undertaken to address and categorise non-responders?	0	2	4
8	Were the risk factor and outcome variables measured appropriate to the aims of the study?	6	0	0
9	Were the risk factor and outcome variables measured correctly using instruments/measurements that had been trialled, piloted or published previously?	6	0	0
10	Is it clear what was used to determined statistical significance and/or precision estimates? (e.g., p-values, confidence intervals)	6	0	0
11	Were the methods (including statistical methods) sufficiently described to enable them to be repeated?	6	0	0
Results				
12	Were the basic data adequately described?	6	0	0
13	Does the response rate raise concerns about non-response bias?	2	3	1
14	If appropriate, was information about non-responders described?	0	3	3
15	Were the results internally consistent?	5	1	0
16	Were the results presented for all the analyses described in the methods?	6	0	0
Discussion				
17	Were the authors' discussions and conclusions justified by the results?	6	0	0
18	Were the limitations of the study discussed?	5	1	0
Other				
19	Were there any funding sources or conflicts of interest that may affect the authors' interpretation of the results?	0	5	1
20	Was ethical approval or consent of participants attained?	6	0	0