School of Sport, Health and Wellbeing

THE GASTROINTESTINAL EXERTIONAL HEAT STROKE PARADIGM: EFFICACY OF ACUTE ORAL GLUTAMINE SUPPLEMENTATION

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Abstract

Exertional heat stroke (EHS) is the most severe form of heat related illness. In military settings, it is considered a largely preventable cause of morbidity, however, prevalence has remained high into the 21st Century. To support disease management, various policy documents provide occupational guidance on effective risk mitigation strategies, however, these can be criticised for focussing solely on the thermoregulatory pathology of the disease. The gastrointestinal (GI) EHS paradigm is a novel pathophysiological model that links EHS to luminal microbial translocation (MT) downstream of structural GI barrier integrity disturbance. Whilst this model is still in its infancy, recent investigations have established practical nutritional interventions that can support GI barrier integrity in populations at risk of EHS. The aims of this thesis were therefore to: (1) characterise the response of GI barrier integrity biomarkers to exertional-heat stress; and (2) examine the efficacy of acute oral $_{L}$-glutamine (GLN) as a nutritional countermeasure to protect GI barrier integrity.

From the experimental evidence reported in this thesis, several major conclusions were derived. First, GI barrier integrity can be reliably examined in blood samples taken at rest and following exertional-heat stress using the dual-sugar absorption test, intestinal-fatty acid binding protein and claudin-3 (chapter 4). Second, GI MT can be reliably examined in blood samples taken at rest and following exertional-heat stress using lipopolysaccharide binding protein and total 16S bacterial DNA, but not *Bacteroides*/total 16S DNA (chapter 4). Third, individuals with high-aerobic fitness experience blunted small intestinal epithelial injury and MT compared with untrained individuals during a fixed load exertional-heat stress test (chapter 5). Fourth, acute GLN supplementation (0.30, 0.60, 0.90 $g{\cdot}kg{\cdot}FFM^{-1}$) causes mild dose-dependent GI symptoms at rest that generally lasted < 4 hours (chapter 6). Fifth, 0.30 $g{\cdot}kg{\cdot}FFM^{-1}$ acute GLN supplementation does not protect GI permeability, small intestinal epithelial injury or MT when consumed 1-hour before either a low-intensity (chapter 7) or high-intensity (chapter 8) exertional-heat stress test. Taken together, GI barrier integrity loss reliably occurred in response to exertional-heat stress, a response that was blunted in individuals with high-aerobic fitness, but not following acute oral GLN supplementation.

Table of Contents

Abstract 2

List of Tables 9

List of Figures 11

List of Equations 13

Publications 14

Acknowledgments 15

Abbreviations 16

Chapter 1 - Introduction 21

1.1 Background 21

1.2 Thesis Structure 23

Chapter 2 – Literature Review 24

2.1 Human Thermoregulation 24

2.2 Heat Balance Theory 25

2.2.1 Heat Storage 25

2.3 Heat Stress and Strain 26

2.4 Thermoregulatory Control 28

2.4.1 Behavioural Thermoregulation 28

2.4.2 Physiological Thermoregulation 30

2.4.2.1 Cardiovascular Function 30

2.4.2.2 Sudomotor Function 31

2.5 Heat Stroke – Classification 32

2.6 Exertional Heat Stroke in the United Kingdom Armed Forces 36

2.6.1 Military Function and Structure 36

2.6.2 Prevention and Management 36

2.6.3 Epidemiology 37

2.6.4 Patient Disposition 40

2.7 The Gastrointestinal Tract 42

2.7.1 Anatomy and Histology 42

2.7.2 Barrier Function and Translocation 44

2.7.3 Microbiome 47

2.8 Gastrointestinal Barrier Integrity Assessment 48

2.8.1 Active Assessment 50

2.8.2 Passive Assessment 54

2.8.3 Microbial Translocation 57
2.9 Exertional Heat Stroke Pathophysiology 60
2.9.1 Heat Cytotoxicity Paradigm 60
2.9.2 Gastrointestinal Paradigm 61
2.9.2.1 Human Evidence 64
2.9.2.2 Animal Evidence 66
2.10 Exertional-Heat Stress and GI Barrier Integrity 68
2.10.1 Gastrointestinal Permeability 68
2.10.2 Small Intestinal Epithelial Injury 74
2.10.3 Microbial Translocation 79
2.11 Aetiology of Gastrointestinal Barrier Integrity Loss 84
2.11.1 Hyperthermia 84
2.11.2 Reactive Oxygen Species 86
2.11.3 Cytokines 87
2.12 Nutritional Countermeasures 88
2.12.1 Carbohydrate 88
2.12.2 Bovine Colostrum 90
2.12.3 Nitric Oxide 91
2.12.4 Probiotics 92
2.12.5 Polyphenols 94
2.12.6 Zinc-Carnosine 95
2.12.7 Future Research Directions 96
2.13 Glutamine 97
2.13.1 Gastrointestinal Metabolism 97
2.13.2 Exercise Metabolism 98
2.14 Glutamine on Gastrointestinal Barrier Integrity 102
2.14.1 Cellular Proliferation and Apoptosis 102
2.14.2 Heat Shock Proteins 104
2.14.3 Glutathione 106
2.14.4 Paracellular Tight Junction Structure 108
2.15 Glutamine, Gastrointestinal Barrier Integrity and Exertional-Heat Stress....109
2.16 Summary 113
2.16.1 Thesis Aims 113

Chapter 3 - General Methodology ... 114
3.1 Research Ethics ... 114
3.2 Health and Safety ... 114
3.2.1 Equipment Sterilisation ... 114
3.2.2 Waste Disposal ... 115
3.3 Participants ... 115
3.3.1 Demographic ... 115
3.3.2 Recruitment ... 115
3.3.3 Medical Criteria ... 115
3.4 Anthropometry ... 116
3.4.1 Height ... 116
3.4.2 Body Mass ... 116
3.4.3 Body Composition ... 116
3.5 Pre-Trial Standardisation ... 118
3.5.1 Lifestyle ... 118
3.5.2 Diet ... 118
3.5.3 Hydration ... 119
3.6 L-Glutamine Supplementation ... 119
3.7 Ambient Environment ... 119
3.8 Exercise ... 120
3.8.1 Clothing ... 120
3.8.2 Fluid Replacement ... 120
3.8.3 Lactate Threshold ... 120
3.8.4 Maximal Oxygen Uptake ... 122
3.8.5 Military Exertional-Heat Stress Test ... 122
3.8.6 High Intensity Exertional-Heat Stress Test ... 124
3.8.7 Forearm Cold-Water Immersion ... 125
3.8.8 Termination Criteria ... 125
3.9 Thermoregulation ... 125
3.9.1 Core Temperature ... 125
3.9.2 Skin Temperature ... 126
3.9.3 Mean Whole-Body Temperature ... 127
3.10 Cardiopulmonary ... 127
3.10.1 Expired Respiratory Gas ... 127
3.10.2 Heart Rate ... 128
3.11 Sudomotor ... 128

3.11.1 Whole-Body Sweat Rate 128
3.12 Perception 128
3.12.1 Rating of Perceived Exertion 128
3.12.2 Thermal Sensation 129
3.12.3 Gastrointestinal Symptoms 129
3.13 Phlebotomy and Haematology 129
3.13.1 Phlebotomy 129
3.13.2 Centrifugation and Biofluid Storage 130
3.13.3 Haemoglobin 130
3.13.4 Haematocrit 131
3.13.5 Plasma Volume 132
3.13.6 Plasma Osmolality 132
3.13.7 Urine Osmolality 133
3.13.8 Urine Specific Gravity 133
3.13.9 Dual-Sugar Absorption Test 134
3.13.10 Intestinal Fatty Acid Binding Protein 135
3.13.11 Claudin-3 136
3.13.12 Lipopolysaccharide Binding Protein 136
3.13.13 Bacterial DNA 136
3.14 Statistical Analysis 137
3.14.1 Sample Size Estimation 137
3.14.2 Randomisation 137
3.14.3 Central Tendency 137
3.14.4 Variation 137
3.14.5 Normality and Sphericity 137
3.14.6 Outliers 138
3.14.7 Tests of Statistical Difference 138
3.14.7.1 Independent and Dependant T-Test 138
3.14.7.2 Mann-Whitney U and Wilcoxon Signed-Rank Tests 138
3.14.7.3 Analysis of Variance 139
3.14.7.4 Post-Hoc Analysis 139
3.14.8 Tests of Reliability 139
3.14.8.1 Correlation 139
3.14.8.2 Coefficient of Variation 140
3.14.8.3 Typical Error of Measurement 140
3.14.8.4 Bland-Altman Mean Bias and Limits of Agreement 140

3.14.8.5 Heteroscedasticity 140
3.14.9 Randomisation Blinding 141

Chapter 4 - Reliability of Gastrointestinal Barrier Integrity and Microbial Translocation Biomarkers in Response to Exertional-Heat Stress.. 142

4.1 Abstract 142
4.2 Introduction..143
4.3 Methods 145
4.4 Results 149
4.5 Discussion 157
4.6 Limitations 162
4.7 Conclusion 162

Chapter 5 - Influence of Aerobic Fitness on Gastrointestinal Barrier Integrity and Microbial Translocation in Response to a Military Exertional Heat Stress Test 164

5.1 Abstract 164
5.2 Introduction..165
5.3 Methods 167
5.4 Results 171
5.5 Discussion 175
5.6 Limitations 178
5.7 Conclusions..179

Chapter 6 - Gastrointestinal Tolerance of Low, Medium and High Dose Acute Oral $_{L}$-Glutamine Supplementation in Healthy Adults... 181

6.1 Abstract 181
6.2 Introduction..182
6.2 Methods 184
6.4 Results 187
6.6 Limitations 195
6.7 Conclusion 196

Chapter 7 - Influence of Low Dose Acute $_{L}$-Glutamine Supplementation on Gastrointestinal Barrier Integrity and Microbial Translocation in Response to a Military Exertional-Heat Stress Test 197

7.1 Abstract 197
7.1 Introduction..198

7.2 Methods 199
7.4 Results 203
7.5 Discussion 207
7.6 Limitations 210
7.7 Conclusion 211

Chapter 8 - Low Dose Acute L-Glutamine Supplementation on Gastrointestinal Barrier Integrity and Microbial Translocation in response to High Intensity Exertional-Heat Stress 213

8.1 Abstract 213
8.2 Introduction 214
8.3 Methods 215
8.4 Results 219
8.5 Discussion 225
8.6 Limitations 228
8.7 Conclusion 228

Chapter 9 - General Discussion 230

9.1 Summary 230
9.2 Gastrointestinal Barrier Integrity Assessment 2322
9.3 Intrinsic and Extrinsic Risk Factors 2366
9.4 Nutritional Intervention 2399

Chapter 10 - Conclusions 2453

References 2474

List of Tables

Table 1. Comparison of CHS and EHS pathophysiology (Leon and Bouchama, 2015) 33

Table 2. Proposed Reference Thresholds for Routine Clinical Biochemistry Analytes and Novel Biomarkers for EHS Diagnosis in Humans 35

Table 3. Mortality from EHS in the UK Armed Forces 41

Table 4. Function of major GI TJ proteins determined by gene knock-down and expression studies (Groschwitz and Hogan, 2009)..46

Table 5. Overview of Common Assessment Techniques (In Vivo) to Determine GI Barrier Integrity (Grootjans et al., 2010). 49

Table 6. Covariates influencing active GI permeability assessment using the DSAT/MSAT (Bjarnason et al., 1995)...51

Table 7. Influence of acute exercise-(heat) stress on small-intestine DSAT responses 71

Table 8. Influence of acute exercise-(heat) stress on systemic I-FABP concentrations 75

Table 9. Influence of acute exercise-(heat) stress on systemic gastrointestinal microbial translocation responses..81

Table 10. Influence of exercise-(heat) stress on systemic glutamine concentration........100

Table 11. Influence of Glutamine Supplementation on GI barrier Integrity Responses to Exertional-Heat Stress 112

Table 12. Demographic Measurements of Male British Armed Forces Defence Personnel 116

Table 13. Reliability of the skin fold thickness body composition assessment (*n* = 12)....117

Table 14. Reliability of the Biosen C-Line automated L-lactate analyser in resting and post lactate-threshold blood samples (*n* = 12)..121

Table 15. UK work/rest guidelines (Military Headquarters of the Surgeon General, 2019) 123

Table 16. UK upper WBGT index for unacclimated service personnel (Military Headquarters of the Surgeon General, 2019) 123

Table 17. US work/rest guidelines (Department of the Army Headquarters, 2016) 124

Table 18. Reliability of the Hemocue® Hb 201+ portable photometric analyser (*n* = 12) 131

Table 19. Reliability of the haematocrit microcapillary technique (*n* = 12) 131

Table 20. Reliability of the Gonotec 3000 freeze-point osmometer in plasma (*n* = 12) ..132

Table 21. Reliability of the Gonotec 3000 freeze-point osmometer in urine (*n* = 12) 133

Table 22. Reliability of the Atago 3741 Pen-Urine S.G handheld refractometer (*n* = 12).134

Table 23. Agreement between the ELH-FABP-1 and DY3078 I-FABP ELISA kits (*n* = 12) ..135

Table 24. Participant demographic characteristics 145

Table 25. Relative and absolute reliability of whole-body physiological responses 151

Table 26. Relative and absolute reliability of all GI barrier integrity biomarkers 154

Table 27. Relative and absolute reliability of all GI barrier integrity biomarkers 157

Table 28. Participant demographic characteristics 167
Table 29. Participant demographic characteristics 184
Table 30. Assessment of trial blinding 187
Table 31. Individual GI symptoms over time, incidence (%) and as a total 24-hour accumulated score following LOW, MED or HIGH GLN ingestion 190
Table 32. Participant demographic characteristics 200
Table 33. Participant demographic characteristics 216
Table 34. Incidence and severity of gastrointestinal symptoms during the EHST 224
Table 35. Primary study hypotheses and outcomes......231
Table 36. Identifiable risk factors and modifiable interventions to protect GI barrier integrity in response to exercise or exertional-heat stress......245

List of Figures

Figure 1. Heat Balance Equation (Parsons, 2014)....................24

Figure 2. The prescriptive zone for compensable heat stress (Lind, 1963)....................26

Figure 3. Example of oesophageal, rectal and GI T_{core} response to 10 minutes submaximal (heart rate = ~150 b·min^{-1}) and 8 minute maximal intensity cycling exercise in 30°C/50% RH (Teunissen et al., 2012)....................27

Figure 4. Annual mean incidence of hospitalisations from HI and HS across the UK Armed Forces. 1 = South Africa (CW) 1979-98 (Simpson, 1909); 2 = Turkey (WW1) 1915-18 (Michell and Smith, 1931); 3 = France (WW1) 1915 (Michell and Smith, 1931); 4= Macedonia (WW1) 1915-16; 5 = South-West Africa (WW1) 1915-18 (Michell and Smith, 1931); 6 = Iraq (WW1) 1915-18 (Michell and Smith, 1931); 7 = India (WW2) 1939-45 (Mellor, 1972); 8 = Iraq (WW2) 1942-43 (Mellor, 1972); 9 = Sudan (WW2) 1942-44 (Mellor, 1972); 10 = Syria (WW2) 1942-45 (Mellor, 1972); 11 = Cyprus (training) 1990-94 (Bricknell, 1996b); 12 = Kuwait (Operation SARA Saraff) 2001 (Bricknell and Wright, 2004); 13 = Iraq (Operation Telic) July 2003 (Bolton et al., 2006); 14 = Iraq (Operation Telic) 2003 (Grainge and Heber, 2005); 15 = Iraq (Operation Telic) 2005 (World and Booth, 2008); 16 = Afghanistan (Operation Herrick) 2011-13 (Cox et al., 2016)....................39

Figure 5. Illustration of the major anatomy of the of the gastrointestinal tract and accessory organs (Biga et al., 2020)....................43

Figure 6. Illustration of the major histology of the of the gastrointestinal tract (Biga et al., 2020)....................44

Figure 7. Illustration of the structure of the gastrointestinal barrier (Turner, 2009)....................45

Figure 8. Schematic illustration of the experimental measurement timings....................146

Figure 9. Whole-body physiological responses to repeated EHSTs: (A) = core temperature; (B) = mean skin temperature (*n* = 13); (C) = mean body temperature (*n* = 13); (D) = heart rate; (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = trial 1, broken line = trial 2. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). 150

Figure 10. GI barrier integrity responses to EHST trial 1 and trial 2: (A) = L/R ratio at 90 minutes; (C) = L/R ratio at 150 minutes; (E) I-FABP (*n* = 13); and (G) = CLDN-3. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). Bland-Altman mean bias and 95% LoA between post EHST trial 1 and trial 2: (B) = L/R ratio at 90- and (D) 150- minutes; (F) I-FABP (*n* = 13); and (H) = CLDN-3....................153

Figure 11. GI MT responses to EHST trial 1 and trial 2: (A) = LBP; (C) total 16S bacterial DNA (*n* = 13); (E) = *Bacteroides* DNA; and (G) = *Bacteroides*/total 16s bacterial DNA (*n* = 13). Significant overall effect of trial (# p ≤ 0.05). Bland-Altman mean bias and 95% LoA between post EHST trial 1 and trial 2: (B) = LBP; (D) = total 16S bacterial DNA (*n* = 13); (F) *Bacteroides* DNA (*n* = 13); and (H) = *Bacteroides*/total 16s bacterial DNA (*n* = 13). 156

Figure 12. Schematic illustration of the experimental measurement timings....................168

Figure 13. Whole-body physiological responses to EHSTs: (A) = core temperature; (B) = mean skin temperature; (C) = mean body temperature; (D) = heart rate; (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = UT, broken line = HT.

Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). Significant group * time interaction (+ p ≤ 0.05; ++ p ≤ 0.01) 172

Figure 14. Gastrointestinal barrier integrity responses pre- and immediately post- the exertional-heat stress tests: (A) = L/R ratio (DSAT) at 90 minutes; (B) I-FABP. UT = untrained group, HT = highly trained group. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). Significant group * time interaction (+ p ≤ 0.05; ++ p ≤ 0.01). 174

Figure 15. Gastrointestinal microbial translocation responses pre- and immediately post- the exertional-heat stress tests: (A) = total 16S DNA; (B) *Bacteroides*/total 16S DNA. UT = untrained group, HT = highly trained group. Significant group * time interaction (+ p ≤ 0.05; ++ p ≤ 0.01). 174

Figure 16. Schematic illustration of the experimental timings. 185

Figure 17. GI symptom scores: (A) = upper-GI symptoms over time; (B) = 24-hour accumulated upper-GI symptoms; (C) = lower-GI symptoms over time; (D) = 24-hour accumulated lower-GI symptoms; (E) = total-GI symptoms over time; (F) = 24-hour accumulated total-GI symptoms. Circles = LOW, squares = MID, Triangles = LOW. On (B), (D), (F) circles = individual participant data. Significant difference between LOW vs. HIGH ([a] p ≤ 0.05), MED vs. HIGH ([b] p ≤ 0.05) and LOW vs. MED ([c] p ≤ 0.05) trials. 189

Figure 18. Microscopic illustration (40x) of the LOW (A), MED (B) and HIGH (C) GLN beverages..192

Figure 19. Schematic illustration of the experimental measurement timings..................200

Figure 20. Whole-body physiological responses to EHSTs: (A) = core temperature; (B) = mean skin temperature; (C) = mean body temperature; (D) = heart rate; (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = GLN, broken line = PLA. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). 205

Figure 21. GI barrier integrity responses to EHSTs: (A) = L/R ratio (DSAT) at 90 minutes; (B) I-FABP. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). Significant trial * time interaction (+ p ≤ 0.05; ++ p ≤ 0.01) 206

Figure 22. GI MT responses to EHSTs: (A) = total 16S DNA; (B) *Bacteroides*/total 16S DNA. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). 207

Figure 23. Schematic illustration of the experimental measurement timings..................217

Figure 24. Whole-body physiological responses to EHSTs: (A) = core temperature; (B) = mean skin temperature; (C) = mean body temperature; (D) = heart rate; (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = GLN, broken line = PLA. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). 221

Figure 25. GI barrier integrity responses to EHSTs: (A) = L/R ratio (DSAT) at 90 minutes; (B) I-FABP. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). Significant effect of trial (# p ≤ 0.05)...222

Figure 26. GI MT responses to EHSTs: (A) = total 16S DNA; (B) *Bacteroides*/total 16S DNA (*n* = 9). Significant overall effect of trial (# p ≤ 0.05) 223

Figure 27. Continuation of the Gastrointestinal Paradigm 244

List of Equations

Equation 1. Calculation of Body Density for adult males 117

Equation 2. Calculation of body fat (%) in humans 117

Equation 3. Calculation of mean skin temperature (Ramanathan, 1964)....................126

Equation 4. Calculation of whole-body temperature (Jay et al., 2007a) 127

Equation 5. Calculation of whole-body sweat rate (SWR) 128

Equation 6. Calculation of plasma volume change (Dill and Costill, 1974) 132

Equation 7. Calculation of the lactulose to L-rhamnose recovery ratio....................135

Publications

Journal Articles

Chapter 2: Ogden, H.B., Child, R.B., Fallowfield, J.L., Delves, S.K., Westwood, C.S. and Layden, J.D. (2020). The gastrointestinal exertional heat stroke paradigm: pathophysiology, assessment, severity, aetiology, and nutritional countermeasures. *Nutrients*, *12*(2), pp.e537.

Chapter 4: Ogden, H.B., Fallowfield, J.L., Child, R.B., Davison, G., Fleming, S.C., Edinburgh, R.M., Delves, S.K., Millyard, A., Westwood, C.S. and Layden, J.D. (2020). Reliability of gastrointestinal barrier integrity and microbial translocation biomarkers at rest and following exertional heat stress. *Physiological Reports*, *8*(5), pp.e14374.

Chapter 5: Ogden, H.B., Fallowfield, J.L., Child, R.B., Davison, G., Fleming, S.C., Delves, S.K., Millyard, A., Westwood, C.S. and Layden, J.D. (2020). Influence of aerobic fitness on gastrointestinal barrier integrity and microbial translocation following a fixed-intensity military exertional heat stress test. *European Journal of Applied Physiology*, *120*(10), pp.2325-2337.

Chapter 6: Ogden, H.B., Child, R.B., Fallowfield, J.L., Delves, S.K., Westwood, C.S., Millyard, A., and Layden, J.D. (2020). Gastrointestinal Tolerance of Low, Medium and High Dose Acute Oral L-Glutamine Supplementation in Healthy Adults: A Pilot Study. *Nutrients*, *12*(10), pp.e2953.

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Chapter 4: Ogden, H.B., Westwood, C.S., Fallowfield, J.L., Delves, S.K., Child, R.B., and Layden, J.D. Reliability of techniques to assess gastrointestinal barrier integrity following a military exercise-heat stress intervention. *International Conference of Environmental Ergonomics (ICEE)*, Amsterdam, Netherlands, July 2019.

Chapter 7: Ogden, H.B., Fallowfield, J.L., Child, R.B., Davison, G., Fleming, S.C., Delves, S.K., Millyard, A., Westwood, C.S. and Layden, J.D. Influence of low dose acute glutamine supplementation on gastrointestinal barrier integrity and microbial translocation in response to exertional-heat stress. *Future Physiology*, Online, July 2020.

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Abbreviations

ADP	Adenosine Diphosphate
AJ	Adherens Junction
α-GST	Alpha-Glutathione S-Transferase
ALT	Alanine Aminotransferase
AMPK	Adenosine-Activated Protein Kinase
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
BactDNA	Bacterial DNA
B-A LoA	Bland-Altman Limits of Agreement
BASES	British Association of Sport and Exercise Science
BI	Bang Blinding Index
BIL	Bilirubin
C	Convection
Caco-2	Colonic Adenocarcinoma
CBRN	Chemical, Biological, Radiological, Nuclear
CFU	Colony Forming Units
CHO	Carbohydrate
CHS	Classic Heat Stroke
CK	Creatine Kinase
CLDN	Claudin
CLDN-3	Claudin-3
CNS	Central Nervous System
CO_2	Carbon Dioxide
COSHH	Control of Substances Hazardous to Health
Cr	Creatinine
^{51}Cr-EDTA	Chromium-labelled ethylenediaminetetraacetic acid
CV	Coefficient of Variation
DAO	Diamine Oxidase
DIC	Disseminated Intravascular Coagulation
DMS	Defence Medical Surveillance
DSAT	Dual Sugar Absorption Test
E	Evaporation
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor

EHS	Exertional Heat Stroke
EHST	Exertional Heat Stress Test
ELISA	Enzyme Linked Immunosorbent Assay
ESPEN	European Society for Parenteral and Enteral Nutrition
FFM	Fat Free Mass
FODMAP	Fermentable Oligo-, Di-, Mono-Saccharides and Polyols
GALT	Gut-Associated Lymphoid Tissue
GFR	Glomerular Filtration Rate
GI	Gastrointestinal
GIS	Gastrointestinal Symptoms
GLN	L-Glutamine
GLS	Phosphate-Dependant Glutaminase
GS	Glutamine Synthetase
GSH	Glutathione
GSSG	Glutathione Disulfide
HI	Heat Illness
HIGH	High Glutamine Trial
HPLC	High Performance Liquid Chromatography
HR	Heart Rate
HS	Heat Stroke
HSF	Heat Shock Factor
HSP	Heat Shock Protein
HSP70	Heat Shock Protein 70
HT	Highly Trained
I-BABP	Ileal Bile-Acid Binding Protein
ICD	International Classification of Disease
I-FABP	Intestinal Fatty-Acid Binding Protein
IFNγ	Interferon Gamma
IGF-1	Insulin-Like Growth-Factor-1
I-HSP	Intracellular Heat Shock Protein
IL	Interleukin
ISAK	International Society for the Advancement of Anthropometric Kinanthropometry
ISO	International Organisation for Standardisation
JAAM	Japanese Association for Acute Medicine
JSP	Joint Services Publication
K	Conduction
LAL	Limulus Amoebocyte Lysate
LBP	Lipopolysaccharide Binding Protein

L-FABP	Liver Fatty-Acid Binding Protein
L/M	Lactulose/$_D$-Mannitol
LOW	Low Glutamine Trial
LPS	Lipopolysaccharide
L/R	Lactulose/$_L$-Rhamnose
LT	Lactate Threshhold
M	Metabolic Rate
MED	Medium Glutamine Trial
MHP	Metabolic Heat Production
MLC	Myosin Light-Chain
MLCK	Myosin Light-Chain Kinase
MoD	Ministry of Defence
MSAT	Multiple Sugar Absorption Test
MT	Microbial Translocation
mTOR	Mechanistic Target of Rapamycin
mVAS	Modified Visual Analogue Scale
NATO	North Atlantic Treaty Organisation
NF- κB	Nuclear Factor Kappa-Light-Chain-Enhanced
NH_4	Ammonium
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NO_2	Nitrite
NO_3	Nitrate
NSAID	Non-Steroid Anti-Inflammatory
O_2	Oxygen
PAMP	Pathogen Associated-Molecular Pattern
PBS	Phosphate-Buffered Saline
PCT	Procalcatonin
PEG	Polyethylene Glycol
Pi	Inorganic Phosphate
PKC	Protein Kinase C
PLA	Placebo
P_{osmo}	Plasma Osmolality
PPP	Pattern Recognition Receptor
qPCR	Quantitative Polymerase Chain Reaction
R	Radiation
RES	Reticuloendothelial System
RH	Relative Humidity

RMCORR	Repeated Measures Correlation
ROS	Reactive Oxygen Species
RPE	Rate of Perceived Exertion
rRNA	Ribosomal RNA
S	Net Heat Storage
SAPS	Simplified Acute Physiology Score
sCD14-ST	Soluble CD14 subtype
SD	Standard Deviation
SEM	Sensor Electronics Module
SGLT	Sodium-Dependent Glucose Cotransporter
SIRS	Systemic Inflammatory Response Syndrome
SkBF	Skin Blood Flow
SR	Sweat Rate
S/R	Sucrose/$_{L}$-Rhamnose
SST	Serum Separator Tube
S100b	Calcium-Binding Protein B
T_a	Air Temperature
T_{amb}	Ambient Temperature
T_{body}	Mean Body Temperature
TCA	Tricarboxylic Acid Cycle
T_{core}	Core Body Temperature
T_d	Dry Bulb Temperature
TEM	Typical Error of Measurement
TER	Transepithelial Electrical Resistance
T_g	Black Bulb Temperature
T_{GI}	Gastrointestinal Temperature
TJ	Tight Junction
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor Alpha
T_w	Wet-Bulb Temperature
T_{os}	Oesophageal Temperature
TPN	Total Parenteral Nutrition
T_{skin}	Mean Skin Temperature
TS	Thermal Sensation
UK	United Kingdom
UN	United Nations
U_{osmo}	Urine Osmolality
US	United States

USG	Urine Specific Gravity
UT	Untrained
UTCI	Universal Climate Index
$\dot{V}O_2$	Oxygen Uptake
$\dot{V}O_{2max}$	Maximal Oxygen Uptake
W	Work
WBSR	Whole-Body Sweat Rate
WGBT	Wet-Bulb Global Temperature
WW1	World War 1
WW2	World War 2
ZnC	Zinc Carnosine
ZO	Zonula-Occludin

Chapter 1 - Introduction

1.1 Background

Humans aim to maintain core body temperature (T_{core}) around a tight set-point of 37.0 ± 1.0 °C (Cheung, 2010a). To achieve this thermal equilibrium, a combination of behavioural and autonomic effectors continuously regulate bi-directional heat transfer to the environment (Parsons, 2014). Exertional heat stroke (EHS) is the most hazardous condition along a continuum of heat-related illness, characterised by thermoregulatory failure following arduous physical exertion (Bouchama and Knochel, 2002).

Military personnel are an exemplar population who are susceptible to EHS given frequent occupational risk exposure (Epstein et al., 2012). The annual mean prevalence of EHS in the British Armed Forces is *circa* 0.75 cases per 1000 person years (Stacey et al., 2016), though this rate rises considerably during specialist fast-paced operations conducted in hot ambient environments (Bolton et al., 2006). It is anticipated that the prevalence of EHS in the military will continue to follow a recent upward trajectory without additional intervention in future, based on predictions of elevated ambient global surface temperature (Armed Forces Health Surveillance Centre, 2020). Whilst whole-body cooling within 1-hour of symptom onset is very effective in preventing direct mortality from EHS (Belval et al., 2018), disease prevention is still preferable to the military given the significant health, financial, and operational constraints of the disease (Parsons et al., 2019).

To support risk mitigation, various policy documents provide occupational guidance on effective EHS management in the military (e.g. Spitz et al., 2012). Whilst such guidance undoubtedly helped reduce EHS prevalence in military populations over the last century (Casa et al., 2010b), non-proponents critically claim these thermoregulatory centric approaches might be over-simplistic (Lim and Mackinnon, 2006). The gastrointestinal (GI) EHS paradigm is a secondary pathophysiological pathway proposed in the 1990s, which links structural GI barrier integrity loss with worsened clinical outcome in hospitalised EHS patients (Gisolfi, 1993). The GI tract is the human body's largest mucosal interface, which extends the length of the stomach to the colon. Residing inside the GI lumen is *circa* 10^{14} microorganisms – termed the GI microbiota – which perform numerous functions synergistic to human health (Thursby and Juge, 2017). The GI barrier is a semi-permeable multi-layered interface that functions to simultaneously permit dietary nutrient absorption but contain the GI microbiota to the luminal space (Wells et al., 2016).

In states of good health, the GI barrier is largely effective in preventing GI microbial translocation (MT) into the systemic circulation (Wells et al., 2016). In comparison, exertional-heat stress enhances GI MT, through disturbance of physical GI barrier structures (Pires et al., 2017). In animal models of classic (non-exertional) heat stroke, prior treatment with antibiotic, steroid and anti-lipopolysaccharide medications improve clinical outcome, seemingly by blocking the deleterious effects of GI MT (Walter and Gibson, 2020a, 2020b). On this basis, recent scientific investigations have looked to establish safe and practical nutritional interventions that protect GI barrier integrity in response to exertional-heat stress in humans.

L-glutamine (GLN) is the most abundant free amino acid in the human body, which comprise 40-60% of the total intracellular amino acid pool (Soeters and Grecu, 2012). It is classified as a conditionally essential nutrient given that whole-body concentrations can become depleted during severe catabolism (Lacey and Wilmore, 1990). Exertional-heat stress is one stimulus that suppresses whole-body GLN concentrations by up to 20-50% (Gleeson, 2008). Low plasma GLN concentration ($\leq$ 420 µmol·l^{-1}) is a well-characterised risk factor for morbidity in clinical care patients (Rodas et al., 2012), and normalisation of GLN status through supplementation typically improves health outcome (McRae, 2017). Protection of GI barrier integrity is one mechanism how GLN potentially exerts these health benefits, possibly through upregulating GI: cell proliferation; heat-shock protein and glutathione expression; and paracellular tight junction stability (Wang et al., 2014).

High-dose (0.9 g·kg·FFM^{-1}) acute oral GLN supplementation has previously been shown to strengthen GI barrier integrity when taken 2-hours prior to sub-clinical exertional-heat stress (Zuhl et al., 2015; Pugh et al., 2017b). This intervention might have practical relevance for the management of EHS in the military, especially in situations where alternative mitigation strategies are unfeasible, for example: short-notice operational deployment; in poorly conditioned personnel; and during field operations where opportunity to cool casualties is lower. Despite promise, questions remain whether GLN retains its effectiveness during severe exertional-heat stress. Moreover, it is unclear whether improved GI barrier integrity translates to blunted MT. Finally, high-dose acute oral GLN supplementation ($\geq$ 0.5 g·kg·FFM^{-1}) is poorly tolerated by some individuals, thus limiting practical application (Ward et al., 2003). As such, GLN supplementation requires further examination as a potential countermeasure to protect GI barrier integrity during exertional-heat stress before consideration for widespread implementation.

1.2 Thesis Structure

The aims of this thesis were to:

(1) Characterise the nature of GI barrier integrity responses to subclinical exertional-heat stress in healthy physical-active adults with demographic characteristics comparable to British Armed Forces personnel serving in ground combat roles; and

(2) Investigate whether acute GLN supplementation is an effective nutritional intervention to protect GI barrier integrity and prevent GI MT.

Chapter 2 reviews the relevant literature, including: (1) human thermoregulation and heat illness classification; (2) EHS in the British Armed Forces as an exemplar population with high occupational risk exposure; (3) the anatomy of the GI barrier and functional assessment; (4) the GI EHS paradigm; and (5) the potential for acute GLN supplementation as an effective countermeasure to protect GI barrier integrity. Chapter 3 details the common methodologies applied across later experimental studies. Chapter 4 aimed to determine the reliability of biomarkers of GI barrier integrity and MT at rest and following exertional-heat stress. This chapter informs prospective study design, including individual biomarker performance and anticipated statistical power. Chapter 5 aimed to determine the influence of aerobic fitness on GI barrier integrity and MT in response to a fixed-intensity exertional-heat stress test. This chapter informs whether demographic characteristics influence susceptibility to GI barrier disturbance, and in whom limited resources for targeting GI barrier integrity are best targeted. Chapter 6 aimed to determine the time-course of GI symptoms in response to low (0.30 $g \cdot kg \cdot FFM^{-1}$), medium (0.60 $g \cdot kg \cdot FFM^{-1}$) and high (0.90 $g \cdot kg \cdot FFM^{-1}$) dose GLN supplementation. This chapter informs understanding of the optimal GLN dose that could be administered as a practical countermeasure within the military. Based on chapter 6, chapters 7 and 8 aimed to determine the influence of a 0.30 $g \cdot kg \cdot FFM^{-1}$ dose of acute oral GLN bolus on GI barrier integrity and MT in response to low-intensity and high-intensity exertional-heat stress. Chapter 9 discusses the cumulative findings of experimental chapters and provides direction for future research on this topic.

Chapter 2 – Literature Review

2.1 Human Thermoregulation

The thermal environment is a stressor that most earth dwelling species must accommodate (Lieberman et al., 2015). As homeotherms, humans attempt to maintain core body temperature (T_{core}) around a tight set-point of 37.0 ± 1.0 °C (Cheung, 2010a). To achieve this thermal equilibrium, the human body continually counter regulates metabolic heat production and bidirectional environmental heat transfer (Parsons, 2014). Fluctuation of T_{core} either above (>40°C; hyperthermia) or below (<35°C; hypothermia) the normal homeostatic range threatens survival (Cheshire, 2016). Throughout evolution, humans developed a complex thermoregulatory system capable of maintaining homeostasis under situations of extreme hyperthermia. This system comprises both behavioural (e.g. clothing) and physiological (e.g. skin blood flow) effectors (Sawka et al., 2011). The relative contribution of distinct responses is situational specific, which can be depicted through the conceptual heat balance equation (Figure 1).

$$S\ [W \cdot m^2] = M - (\pm W) \pm R \pm C \pm K - E$$

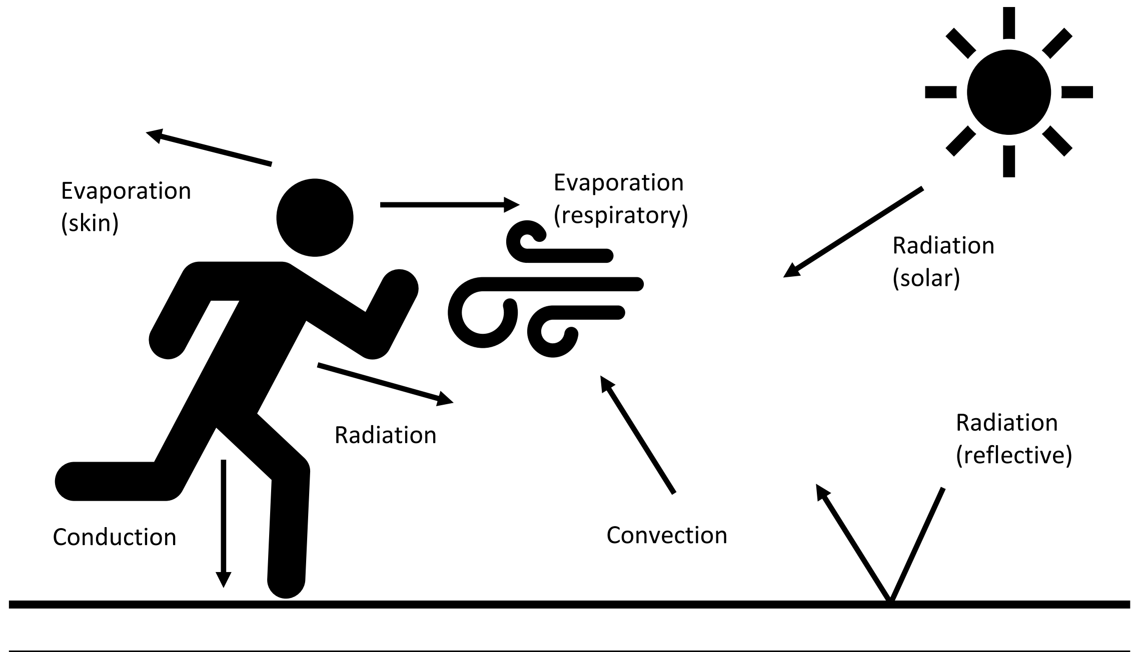

Figure 1. Heat Balance Equation (Parsons, 2014).

Where: S = body heat storage; M = metabolic rate; W = mechanical work; R = radiation; C = convection; K = conduction; E = evaporation.

2.2 Heat Balance Theory

Heat exchange between objects is determined by the biophysical laws of thermodynamics (Parsons, 2014). The first law states energy (heat) cannot be created or destroyed, only transferred between entities. The second law states heat transfer always occurs in the direction of a cooler entity. Conceptually, the heat balance equation applies these laws when modelling the governance of: (1) metabolic heat production inside the body; (2) heat transfer to the external environment; and (3) net heat storage (Figure 1).

Based on the first law of thermodynamics, metabolic heat production (MHP), defined as the production of waste heat during conversion of metabolic rate (M) to mechanical energy (W), is the primary driver of internal heat gain (Kenny and Jay, 2011). In its broadest sense MHP defines the energy released during the hydrolysis of adenosine triphosphate (ATP), which is determined by the rate of oxygen uptake (O_2) and respiratory fuel source (Kenny and Jay, 2013). The exchange of heat between the body (skin and respiratory tract) and external environment takes place along relative temperature gradients, based on the second law of thermodynamics. The bi-lateral transfer of heat between the body and external environment is mediated through the combined processes of conduction (K), convection (C) and radiation (R). In comparison, evaporation (E) defines the uni-lateral transfer of heat away from the body, which is the primary mechanism for heat dissipation when relative humidity is below 100% (Gagnon et al., 2013). Biophysical characteristics of the environment, including: air temperature, radiant temperature, relative humidity, air movement and clothing, all influence the direction and rate of heat transfer (Sawka et al., 2011).

2.2.1 Heat Storage

Heat Storage (S) defines the overall sum of MHP and net heat dissipation. Whilst humans maintain neutral S in most encountered circumstances whilst resting, physical activity increases S via elevating MHP (Cheung, 2010). Over time, S increases T_{core} in a time- and intensity- dependant manner, whist is influenced to a lesser extent by body mass and composition (Ravanelli et al., 2017). The term *compensable* heat stress defines circumstances where S can be realigned to achieve heat balance, whereas the term *uncompensable* heat stress defines circumstances of positive S (Parsons, 2014). The concept of a prescriptive zone has been promoted for over 50 years, which demarcates

various combinations of physical activity intensity (e.g. MHP) and ambient conditions where compensable heat stress is possible (Figure 2; Lind, 1963).

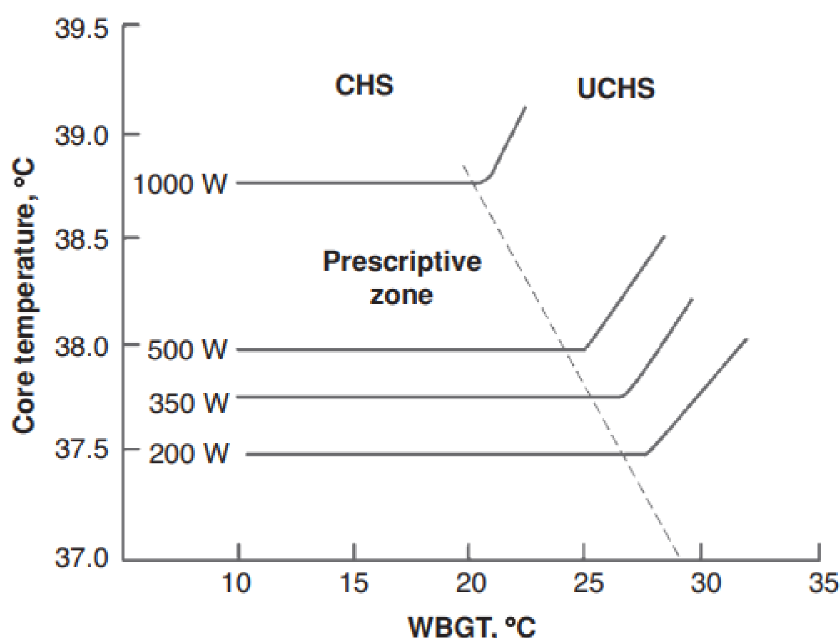

Figure 2. The prescriptive zone for compensable heat stress (Lind, 1963).

2.3 Heat Stress and Strain

Heat stress defines the environmental and metabolic conditions that influence S (Sawka et al., 2011). It is determined by four major meteorological factors: ambient temperature (T_{amb}), relative humidity (RH), air velocity and solar radiation (Havenith and Fiala, 2011). Despite this clear definition, application of a universal index to classify heat stress remains an ongoing source of debate, with over 160 indices having been developed to the present day (Freitas and Grigorieva, 2015). Wet bulb globe temperature (WGBT) is the heat stress index promoted by the International Organisation for Standardisation (Gou et al., 2018) and has received widespread application since development by the United States Armed Forces in the 1950s (Yaglou and Minard, 1957). WGBT is calculated from three sensors: natural wet bulb- (T_w), black globe- (T_g) and dry bulb- (T_d) temperature. WGBT has been validated to predict heat strain in widespread circumstances (Wyndham, 1969). One recent alternative to WGBT that is proposed to better predict heat stress, is the universal thermal climate index (UTCI; Fiala et al., 2012). The UTCI incorporates both passive (e.g. meteorological data, clothing) and active (e.g. metabolic heat, sweat rate) thermoregulatory modelling. Whilst the UTCI offers promise, future research is required to validate this model in response to intense and/or prolonged (> 2 hours) physical activity (Brode et al., 2017).

Heat strain defines the physiological response to heat stress (Sawka et al., 2011). T_{core} assessment is fundamental for heat strain categorisation, which can be taken at a variety of deep-tissue locations (e.g. oesophagus, rectum, gastrointestinal), with no specific indices being universally accepted (Byrne and Lim, 2007). To this point, considerable temperature gradients exist across gross anatomical locations, given regional variability in MHP and cardiovascular perfusion. Irrespective of the selected anatomical site, T_{core} rapidly increases at the start of physical activity before heat dissipation mechanisms are fully active, albeit with somewhat different kinetics and absolute values (Figure 3). Mean skin temperature (T_{skin}) assessment, calculated relative to body surface area is a useful measure for informing thermoregulatory behavioural effector input and to estimate convective/radiative heat exchange (Sawka et al., 2011). The assessment of T_{skin} also enables the calculation of body heat content (Jay and Kenny, 2007). Measurement of T_{skin} is typically made using surface thermistors across 12-26 sites (Liu et al., 2011), though as little as 4 sites have been validated for circumstances where logistical constraints (e.g. locomotion) compromise application (Ramanathan, 1964; Bach et al., 2015).

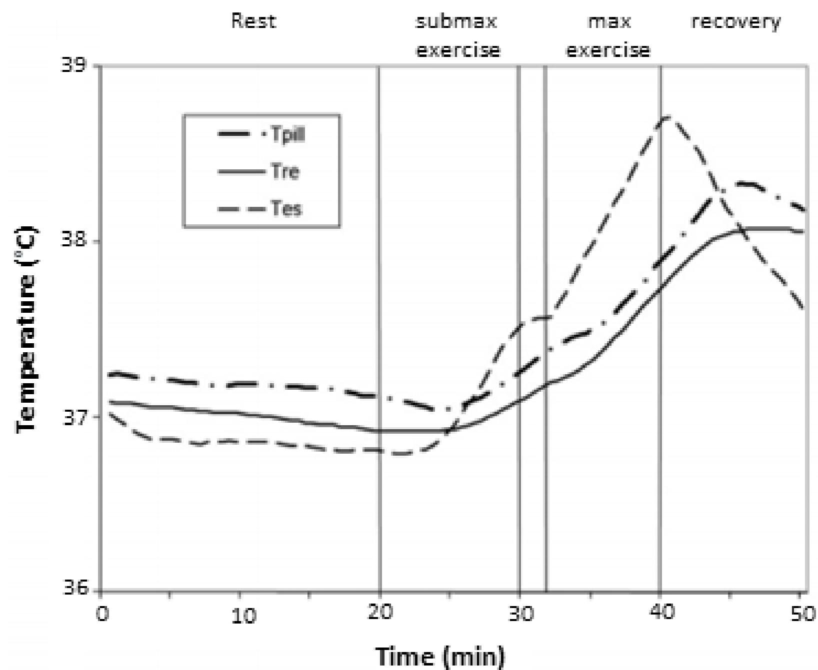

Figure 3. Example of oesophageal, rectal and GI T_{core} response to 10 minutes submaximal, followed-by 8 minute maximal intensity cycling exercise in 30 °C/50% RH (Teunissen et al., 2012).

Body heat content (DHb) defines the product of mean body temperature (T_{body}), body mass and the average heat capacity of tissue (Kenny and Jay, 2013). The determination of DHb is important for assessment of thermal imbalance during heat stress.

Indeed, DHb is commonly utilised as a criterion for maximal heat exposure (Blockley et al., 1954), and for assessment of thermal heterogeneity between indviduals (Saunders et al., 2005). In theory, measurement of body heat exchange can only be made using simultaneous direct and indirect calorimetry (Kenny and Jay, 2013). However, on the basis that direct calorimetry is seldomly available, several indirect two-compartmental (T_{core} and T_{skin}) and three-compartmental (T_{core}, T_{skin} and muscle temperature) weighted equations provide a practical alternative, albeit with poor accuracy (e.g. Jay et al., 2007a, 2007b; Kenny and Jay, 2013).

2.4 Thermoregulatory Control

Thermal homeostasis is achieved through the combined processes of behavioural and physiological thermoregulation. Behavioural thermoregulation describes conscious alterations in behaviour that influence heat storage. Physiological thermoregulation operates autonomically and includes the control of: (1) MHP; (2) cardiovascular heat redistribution; and (3) sweating.

2.4.1 Behavioural Thermoregulation

Behavioural thermoregulation is limitless in its capacity to promote the optimal conditions for heat transfer (Flouris, 2011). Numerous examples of thermal behaviours are readily identifiable, which can be broadly classified as simple and complex behaviours. Examples of simple behaviours include: microenvironment selection, redirection of body orientation/position and locomotion (Schlader et al., 2011). Comparatively, complex behaviours include: home building, wearing insulative clothing and air-conditioning (Flouris, 2011). In addition to behavioural modification of the physical environment, humans may also modify MPH via voluntarily terminating fixed-intensity physical activity at a T_{core} *circa* 40 °C (Gonzalez-Alonso et al., 1999) and reducing self-select exercise intensity in the heat (Tatterson et al., 2000). The importance of thermo-behavioural responses in relation to human health is illustrated by epidemiological evidence that shows heat illness to be most prevalent in circumstances where normal behavioural responses are overridden (Sawka et al., 2011). Specific examples include: occupational (e.g. military) personnel during group-paced exercise (Abriat et al., 2014; Stacey et al., 2015) and young/elderly populations with comorbidities/disabilities that restrict behavioural responses when exposed to high ambient temperature (Vandentorren et al., 2003).

Thermal perception is pivotal in understanding behavioural thermoregulation. Humans sense temperature both peripherally through transient receptor potential ion channels located in cellular plasma membranes (Guler et al., 2002; Romanovsky, 2007) and centrally within several brain regions, including the medulla oblongata, pons, midbrain and cortex (Holmes, 1960; Craig et al., 1994; 2011). Whilst warming of the central brain regions is a direct trigger of thermoregulatory behaviour, recent evidence suggests peripheral thermosensors to be the primary driver of thermal behaviour through mediating thermal perception (Craig, 2011; Flouris, 2011). The perception of temperature includes both an affective and discriminative component (Flouris and Schlader, 2015). The affective component relates to thermal comfort, defined as a subjective indifference with the thermal environment, whilst the discriminative component relates to thermal sensation, defined as the relative intensity of temperature being sensed (Attia, 1984). Quantification of these indices allows for identification of the combined sensations that humans experience as *pleasantly warm* in the winter and *pleasantly cool* in the summer (Flouris and Schlader, 2015).

During passive heat stress, thermal behaviour is primarily driven by perceptions of thermal comfort (Flouris and Schlader, 2015). In normothermia, thermal comfort is most closely related to T_{skin} (Gagge et al., 1967; Schlader et al., 2009), whilst in hyperthermia T_{core} is the primary driver of thermal comfort (Cabanac et al., 1972; Bleichert et al., 1973). Though scientific understanding of thermal behaviours during passive heat stress is well understood, less is known about the factors mediating these responses during exertional-heat stress (Flouris and Schlader, 2015). To this point, inconsistent associations between thermal comfort and thermal behaviour (e.g. pacing) that are regularly reported during exertional-heat stress, might be partially explained by several external co-factors. These include: learned expectations of discomfort during physical activity (Cabanac, 2006), perceived achievement of physical discomfort (Havenith et al., 2002; Ekkekakis, 2003) and social distraction (e.g. competition; Corbett et al., 2017). Instead, perceived physical exertion has been suggested to be the primary determinant of pacing during exertional-heat stress (Tucker et al., 2006; Schlader et al., 2011). To explain this response, a two-factor model has been proposed (Nybo, 2008; Schlader et al., 2011), which combines the influence of skin sensation (e.g. temperature, wettedness) on thermal perception (Best et al., 2018) and T_{amb} on physiological strain (Nybo et al., 2014).

2.4.2 Physiological Thermoregulation

2.4.2.1 Cardiovascular Function

The movement of blood around the cardiovascular system facilitates convective heat transfer from the deep tissues to the periphery (Rowell et al., 1967, 1969). Prior to the onset of sweating, this pathway is the sole mechanism for external heat dissipation through dry-heat transfer. Likewise, once sweating has begun, skin blood flow (SkBF) assists wet-heat transfer through the delivery of heat for evaporative heat loss (Johnson et al., 2011). In humans, SkBF has a large functional range that fluctuates from close to zero during hypothermia to maximal values *circa* 8 $l \cdot min^{-1}$ during passive heat stress when T_{core} exceeds ~38 °C (Rowell, 1969; Minson et al., 1998). SkBF is controlled by the direct influence of local T_{skin} on cutaneous vascular muscle tone (Wenger et al., 1986) and the indirect influence of whole-body T_{core}/T_{skin} on sympathetic reflex responses (Low et al., 2011). In humans, SkBF is largely under dual vasomotor control (Rowell, 1990). Within glabrous skin (e.g. hands, feet, ears, lips) adrenergic vasoconstrictor fibres are predominant, whereby vasodilation in these regions is almost solely attributable to the withdrawal of vasoconstrictor activity (Blair et al., 1960). In comparison, non-glabrous skin (e.g. torso, back) has near minimal vasoconstrictor activity under conditions of thermal comfort (Fox et al., 1962), therefore, sympathetic stimulation instead primarily mediates vasodilatation (Taylor et al., 1984).

In response to passive heat stress, the cardiovascular system increases SkBF to dissipate body heat, whilst simultaneously maintains central blood volume to protect blood pressure (Crandall and Wilson, 2015). To achieve this outcome, cardiac output increases linearly with heat stress from *circa* 5 $l \cdot min^{-1}$ in temperate conditions to maximal values *circa* 14 $l \cdot min^{-1}$ (Rowell, 1969, 1986). This increase in cardiac output is driven by an elevation in heart rate (HR), which is regulated by the combined influence of sympathetic stimulation and parasympathetic withdrawal on sinoatrial impulse (Crandall et al., 2000). In comparison, stroke volume is largely unchanged during passive heat stress (Brothers et al., 2009) and is offset by a reduction in central venous pressure (Rowell, 1969), left ventricular pressure (Wilson et al., 2007) and diastolic filling time (Trinity et al., 2010). Another major mechanism that helps support SkBF demand during passive heat stress is the redistribution of cardiac output from the splanchnic vascular beds (e.g. gastrointestinal tract, kidney, and liver) towards the central circulation (Rowell et al., 1965, Minson et al., 1998, 1999). During rest, splanchnic blood flow is *circa* 25-50% of total cardiac output, however, during passive heat stress this figure can drop < 10% (Minson et al., 1998; Crandall et al. 2008).

Though healthy individuals regulate the cardiovascular demands of passive heat relatively straightforwardly, exertional-heat stress is a more complex stressor on the cardiovascular system. During exertional-heat stress, cardiac output must simultaneously perfuse both the skin to support heat transfer and the skeletal muscle to support metabolism (Crandall and Gonzalez-Alonso, 2010). These divergent demands for blood flow create competition for cardiac output, which results in a plateau in SkBF at a T_{core} ~38 °C (Brengelmann et al. 1977; Smolander et al. 1987). During low-to-moderate intensity exertional-heat stress (40-60% $\dot{V}O_{2max}$), cardiac output is sustained at a lower arterial blood pressure than during matched intensity exercise performed in a temperate environment (Brothers et al., 2009). However, during high-intensity exercise (≥ 70% $\dot{V}O_{2max}$), cardiac output cannot be adequately maintained in the heat, when compared to temperate conditions (Nadel et al., 1979). Independent of exercise intensity, HR is elevated in hot environments for any given cardiac output, which is determined by the combination of increased SkBF, plasma volume loss and reduced diastolic filling time (Rowell et al., 1969). These effects are particularly pronounced when the T_{core}-to-T_{skin} gradient narrows (Cheuvront et al., 2003). Consistent with the response to passive heat stress, cardiac output is redirected away from the splanchnic organs to support thermoregulation during exertional-heat stress. In this situation, splanchnic redistribution is mediated in a summative manner by both exercise intensity and T_{amb} (Ho et al., 1997).

2.4.2.2 Sudomotor Function

Evaporative heat loss is the primary mechanism of heat dispersion in warm-dry environments. The effectiveness of this system is determined by the body's ability to generate sweat and the skin-to-air pressure gradient (Parsons, 2014). Humans have between 1.4-to-4 million eccrine sweat glands located across the entire surface of the body (Nadel et al., 1971), which are primarily responsible for sustaining wet-heat transfer in response to increased T_{core} (Nadel, 1979), T_{skin} (Nadel et al., 1971) and SkBF (Wingo et al., 2010). Non-thermal stimuli may also play a role in initiating sweat secretion via feed-forward mechanisms, including: muscle mechanoreceptors, osmoreceptors and baroreceptors (Shibasaki et al., 2003).

In response to exertional heat stress, humans produce large quantities of sweat. At sweat onset (T_{core} = ~37.3 °C), sweat rate is initially mediated by an increase in sweat gland recruitment (Kondo et al., 1998). Sweat rate increases linearly with T_{core}, which is primarily driven by an elevation in sweat secretion per gland (Kondo et al., 1998). Regionally, the

chest and back have the greatest sweat rates for a given T_{core}, compared to lower sweat rates on the upper- and lower- limbs (Nadel et al. 1971). In typical occupational exertional-heat stress settings, sweat rate often ranges between 0.5 to 2.5 $l \cdot h^{-1}$ (Montain et al., 1994), with evidence of an upper threshold close to 4.0 $l \cdot hr^{-1}$ in highly-trained, heat acclimated individuals (Armstrong et al. 1986). To preserve total body water content, sweat rate is reduced in response to severe hypohydration (> 3% body mass) (Fortney et al., 1984).

2.5 Heat Stroke – Classification

Heat stroke (HS) is a life-threatening medical condition involving thermoregulatory failure and is the most severe form of heat-related illness (Bouchama and Knochel, 2002). Though one of the oldest recognised medical conditions, with anecdotal records dating back to biblical times (Casa et al., 2010a), the disease still has no universal medical definition (Hifumi et al., 2018). Traditionally, HS classification was based on characteristic symptoms at clinical admission, principally an elevation in T_{core} > 40 °C and central nervous system (CNS) dysfunction (Grogan and Hopkins, 2002). However, over the last two decades, this classification was extended to include a systemic inflammatory response syndrome (SIRS) considered to be inducive of disseminated intravascular coagulation (DIC), necrosis of organ tissue and multiple organ failure (Bouchama and Knochel, 2002; Lim, 2018).

HS can be subclassified as *classic* and *exertional* types, based on the sequalae of events that occur prior to collapse (Bouchama and Knochel, 2002). Classic HS (CHS) is a disease that primarily effects debilitated individuals (e.g. elderly, isolated), who have impaired behavioral and/or physiological thermoregulation (Table 1). The most prominent risk factor for CHS is a high T_{amb}, with most incidences occurring during seasonal heat waves (Semenza et al., 1996; Dematte et al., 1998) or on sojourns to warmer climatic regions (Ghaznawi and Ibrahim., 1987; Al-Ghamdi et al., 2003). In comparisom, exertional HS (EHS) primarily effects young healthy individuals (e.g. military personnel, athletes) when undertaking arduous physical activity (Casa et al. 2012; DeMartini et al. 2014). Since MHP is the primary driver of this form of HS, it can arise most T_{ambs} (Epstein et al., 1999; Bergeron et al., 2005). In general, CHS patients have worse prognosis than EHS patients, though this observation is probably attributable to differences in the patient's health prior to incapacitation and access to medical services (Bouchama and Knochel, 2002). A broad comparison of the pathophysiology of CHS and EHS is presented in Table 1.

Table 1. Comparison of CHS and EHS pathophysiology (Leon and Bouchama, 2015).

Characteristic	Classic Heat Stroke	Exertional Heat Stroke
Age	Very young, elderly	Adolescent, middle aged
Health	Chronic illness	Healthy
Weather	Heat wave	Temperate or hot
Activity	Sedentary	Heavy or sustained exertion
Medication	Common	Rare
Acid-base	Mixed	Metabolic acidosis
Calcium	Normal	Hypocalcemia
Potassium	Normal	Hypokalemia
Phosphate	Hypophosphatemia	Hyperphosphatemia
Glucose	Hyperglycemia	Hypoglycemia
Rhabdomyolysis	Rare	Often severe
Kidney Damage	Rare	Often severe
Liver Damage	Mild	Severe
DIC	Mild	Severe

To disassociate EHS from other heat illnesses (HI), a continuum of progressively severe heat pathologies is broadly defined by the medical community. Less severe HI's include: (1) heat exhaustion, characterised by an inability to sustain cardiac output, hot/dry skin and a markedly raised T_{core} (< 40.0 °C); and (2) heat injury, an intermediate condition between heat exhaustion and EHS, where organ damage is detectable, but there is no evidence of CNS abnormality (Leon and Bouchama, 2015). Furthermore, several mild heat-related syndromes, including heat cramps, heat oedema and heat syncope all have unique heat-related pathologies, however, all fall below the HI continuum (Leon and Bouchama, 2015). Whilst the symptoms outlined in the HI continuum are broadly defined, in clinical practice the absence of a more specific symptomatic grading system has resulted in wide inconsistencies in patient diagnosis.

The present inability to properly anticipate, diagnose, and predict the long-term sequelae of EHS is a serious limitation of modern medicine. Indeed, several controversies oppose the generalisations promoted by mainstream EHS classifications. First, the definition of EHS requires a T_{core} recording of > 40.0 °C (Laitano et al., 2019). Despite this,

asymptomatic athletes regularly present T_{cores} between 40.0-42.5 °C (Ely et al., 2009; Racinais et al., 2019), whereas there are numerous case studies in EHS patients where peak T_{core} was < 40.0 °C when measured at incapacitation (Shapiro et al., 1990; Abriat et al., 2014). In consideration of this point, retrospective analysis of EHS casualties in the US Armed Forces, recently reported the ICD-10 classification to have poor sensitivity (51%, De Groot et al., 2017; 65%, King et al., 2018) and specificity (78%, De Groot et al., 2017; 30%, King et al., 2018) in predicting EHS severity. Variations in the T_{core} assessment location (e.g. rectal, tympanic), assessment timing and cooling intervention also minimise the practical application of upholding specific T_{core} thresholds (Belval et al., 2018). Second, no objective diagnostic criteria exist for determining the presence and/or severity of multiple organ injury following EHS. Historically, routine clinical-biochemistry biomarkers (Table 2) have been used to define tissue injury (Abdelmoety et al., 2018; Ward et al., 2020), though these measures are often confounded by simultaneous skeletal muscle injury (Leon and Bouchama, 2015). In recent years, concentration thresholds for several novel organ specific biomarkers in predicting EHS severity have been proposed (Table 2), however, require further validation. Third, no objective diagnostic criteria exist for determining the presence and/or severity of CNS dysfunction following EHS. A Glasgow Coma Scale rating < 15 has been recently been proposed by the United States Armed Forces (Donham et al., 2020), but again requires further validation.

The Japanese Association for Acute Medicine (JAAM) recently proposed a novel classification system for heat illness (HI) diagnoses in 2017 (Hifumi et al., 2017, 2018). This classification system provides no T_{core} threshold, with the intention to prevent an underestimation in disease prognosis attributable to differences in patient management (e.g. cooling) prior to clinical assessment. Instead, this classification relies on objective criteria for classification of multi-organ injury (ALT, Cr, BUN, platelet count) and CNS dysfunction (JAAM coma scale). Stage I (e.g. heat cramps, oedema) represents any minor heat related illness, classified by symptoms including: dizziness, faintness, muscle pain/cramps and heavy sweating. No impaired consciousness is observed at this stage. Stage II (e.g. heat exhaustion) represents any heat-related illness not covered by Stage I or Stage III, classified by symptoms including: headache, vomiting, fatigue, a feeling of sinking, reduced concentration, and impaired judgment. Stage III (e.g. EHS) represents severe conditions that arise following severe heat exposure. By definition, stage III patients exhibit symptoms of both CNS dysfunction and multi-organ injury. Specific criteria for Stage III

classification include: (1) Glasgow coma score ≤ 14; (2) blood creatinine or total bilirubin concentrations ≥ 1.2 mg·dl^{-1}; and (3) JAAM disseminated intravascular coagulation score (Kondo et al., 2019) ≥ 4. Validation of the JAAM HI classification system was assessed in a prospective, nationwide, multicentre surveillance study, which concluded the JAAM definition to outperform the Bouchama and Knochel (2002) definition in predicting multi-organ injury (ALT, Cr, BUN, platelet count), however, both classification systems were comparable in predicting diseases severity (APACHE II score) and mortality (Yamamoto et al., 2018). The JAAM definition has not yet been validated in EHS patients.

Table 2. Proposed Reference Thresholds for Routine Clinical Biochemistry Analytes and Novel Biomarkers for EHS Diagnosis in Humans.

Traditional Biomarkers	Organ	Reference Threshold (Peak)
Aspartate Aminotransferase (AST)	Liver, Skeletal Muscle	2-fold basal increase (Thai Military; Sithinamsuwan et al., 2009); 3-fold basal increase (US Military; De Groot et al., 2017)
Alanine Aminotransferase (ALT)	Liver, Skeletal Muscle	2-fold basal increase (Thai Military; Sithinamsuwan et al., 2009); 3-fold basal increase (US Military; De Groot et al., 2017)
Bilirubin (BIL)	Liver, Kidney	1.2 mg·dl^{-1} (JAAM; Hifumi et al., 2017)
Creatine Kinase (CK)	Skeletal Muscle	5-fold basal increase (US Military; De Groot et al., 2017)
Creatinine (Cr)	Kidney	1.2 mg·dl^{-1} (JAAM; Hifumi et al., 2017); 1.5 mg·dl^{-1} (US Military; De Groot et al., 2017),
Novel Biomarkers	**Organ**	**Reference Threshold (Peak)**
Calcium-binding protein B (S100B)	CNS	0.61 µg·l^{-1} (Chun et al., 2019)
Procalcitonin (PCT)	Immune	0.2 pg·ml^{-1} (Hausfater et al., 2008)

2.6 Exertional Heat Stroke in the United Kingdom Armed Forces

2.6.1 Military Function and Structure

The United Kingdom (UK) Armed Forces are the military services of the United Kingdom of Great Britain and Northern Island, the UK Overseas Territories, and the Crown Dependencies. The UK Armed Forces also promotes the countries wider international interests, including the support of United Nations (UN) and North Atlantic Treaty Organisation (NATO) operations. As of January 1st 2020, the UK Armed Forces has a professional force with a strength of 132,360 UK Regulars and Gurkhas, 32,850 volunteer Reserves and 7830 other personnel (Ministry of Defence, 2020). The tri-service breakdown of the UK Armed Forces at this date was: 37,150 in the Royal Airforce, 38,980 in the Royal Navy and 116,030 in the British Army (Ministry of Defence, 2020).

2.6.2 Prevention and Management

In military personnel, EHS cases have been observed for millennia, despite widespread classification as a preventable cause of morbidity (Casa et al., 2010a, 2010b). The health issues surrounding EHS in military personnel are especially distressing given that most causalities are young, healthy individuals, many of whom were unaware of the possible dangers of excessive physical exertion (Epstein et al., 2012). In addition to a direct risk to human health, the clinical sequelae following EHS can result in many secondary adverse effects. These include: reduced occupational effectiveness (Bricknell et al., 1996a), an increased susceptibility to military hazards (Parsons et al., 2019) and a signficant financial burden associated with medical treatment and lost work hours (e.g. $100 million·year^{-1} in US Armed Forces; Ward et al., 2020). Based on these issues, the UK (Military Headquarters of the Surgeon General, 2019) and other international militaries (e.g. NATO, Spitz et al., 2012) advocate EHS to be a condition that is more preventable than treatable, through which all means should be taken to reduce the risk of illness to as low as reasonably practicable.

The Joint Services Publication (JSP) 539 for Heat and Cold Injury, is the UK Armed Forces code of practise for the prevention and management of thermal illnesses. It is intended that the JSP 539 should inform the initial management and treatment of climatic casualties at all levels of pre-hospital emergency care. The JSP 539 has two distinct subsections: (1) A directive to be followed in accordance with UK Defence statute; and (2)

guidance surrounding best practice that should assist the user to comply with the directive(s) detailed in part 1.

In the UK Armed Forces, most historical HI incidents (>90%) occur during strenuous physical activity, with CHS almost universally limited to overseas operations conduced in warm climates (Bricknell et al., 1995, 1996a). However, the JSP 539 informs that any operational, training or recreational activity involving either undertaking physical activity, wearing protective clothing (e.g. body armour) or being exposed to a hot T_{amb} should be considered a high-risk scenario for EHS. Consequently, it is promoted that the key to EHS prevention is the effective assessment and management of concurrent risks by military commanders. The Commanders EHS Risk Assessment Checklist provides a list of the major risk factors to be considered with activity planning.

2.6.3 Epidemiology

The UK public health approach to disease prevention involves 5 steps: surveillance, field investigation, laboratory research, program implementation, and policy evaluation (Public Health England, 2020). As the first step, surveillance is central to this approach, because understanding the magnitude of a problem is necessary before later steps can be implemented. Since introduction of the JSP 539 in 2003, UK Armed Forces doctors must report all EHS incidents to the Army Health Unit (Military Headquarters of the Surgeon General, 2019), whilst under UK stature, Armed Forces commanders must additionally notify HI casualties as workplace incidents to the Army Incident Notification Cell (Stacey et al., 2016). Despite this surveillance, the quality of data being collected is subject to severe limitations. First, there is poor uniformity in disease classification and diagnostic assessment (e.g. location of T_{core} assessment). Second, reporting relative incidence data is reliant on both the number of EHS cases (e.g. the numerator) and number of exposed personnel (e.g. the denominator). To this point, relative prevalence is significantly underestimated through the inclusion of personnel whose job requirements (e.g. administration, reserve personnel) would not regularly expose them to situations where EHS commonly arises (Stacey et al. 2015). Third, retrospective cohort analysis is often subject to methodological limitations, including: selection bias, misclassification bias and single-group reporting (Sedgwick, 2014). Finally, intentional underreporting of EHS cases is anecdotally widespread throughout the UK Armed Forces (Hunt and Smith, 2005). This underreporting is likely attributable to a combination of case misdiagnosis, intentional non-reporting to avoid repercussion and delays in incident publication (Stacey et al., 2016).

The first report of EHS in the UK Armed Forces was published in the 19th century (Bricknell, 1996a). At this point in history, evidence was primarily limited to first-person solider accounts and anecdotal medical assessments. In non-wear years between 1902 and 1911, an absolute incidence of 3219 heat related hospital admissions (0.79/1000 personnel) were reported across the British Army (Pembrey, 1914). During World War 1 (WW1; 1914-1918), the UK Armed Forces were deployed across many regions (e.g. West Africa, Mediterranean Europe) where high T_{amb} posed a significant risk to health. The Mesopotamian campaign (1915-1918) fought in Iraq is said to be the single most devastating operation in terms of EHS related morbidity in the history of the UK Armed Forces, with the prevalence of heat-related hospital admissions reported to be 68/1000 personnel·year^{-1} (Hill, 1920; Willcox, 1920). Whilst less prevalent than in Mesopotamia, the prevalence of heat related hospitalisations was high across other WW1 military theatres in Mediterranean Europe and South-Western Africa (Figure 4). In comparison to WW1, improvements in EHS management markedly reduced the impact of EHS on the health of UK Armed Forces personnel during WW2 (Bricknell, 1995). In total, there was *circa* 1.0/1000 personnel·year^{-1} heat related hospitalisations across the entire UK Armed Forces throughout WW2, with the highest prevalence of hospital admissions occurring during campaigns fought in the Middle-East (Figure 4). Following WW2, the UK Armed Forces continued to be deployed on operations worldwide, however, the introduction of strategic overseas military bases (e.g. Kenya, Brunei) and more extensive preventative practise (Bricknell, 1996a), reduced the relative incidence of EHS during high-risk activities such as the Anti-British Liberation War in Malaya (Renbourn, 1959).

The burden of heat illness in the UK military was surveyed between 1981-1991 (Dickinson et al., 1994). Overall, the annual prevalence was 124 (0.75/1000 personnel·year^{-1}), 17 (0.16/1000 personnel·year^{-1}), and 1 (0.01/1000 personnel·year^{-1}) for HI, EHS hospitalisation and EHS mortalities, respectively. A tri-service breakdown determined HI prevalence to be far greater in British Army (0.73/1000 personnel·year^{-1}), compared to both the Royal Navy (0.14/1000 personnel·year^{-1}) and Royal Air Force (0.05/1000 personnel·year^{-1}). Following a near two-decade absence of EHS reporting in the UK Armed Forces, Stacey et al. (2015) conducted a 7-year analysis of EHS incidence in the British Army between 2007-2014. In total, 361 incidents were reported, which included 277 hospital outpatients (0.38/1000 personnel·year^{-1}) and 137 inpatients (0.19/1000 personnel·year^{-1}).

Of these cases, 66% occurred in the UK and 34% overseas. Worryingly, a follow-up study highlighted only 13% concordance between two independent military medical incident reporting databases, suggestive of significant case underreporting (Stacey et al., 2016). When incidences from both databases were compiled between 2009-2003, 565 unique heat illness incidents were identified (0.76/1000 personnel·year^{-1}). Like previous reports, most cases (61%) occurred in the UK, with greater prevalence in recruits (2.23/1000 personnel·year^{-1}) than fully-trained personnel (0.59/1000 personnel·year^{-1}). EHI severity was not reported, though the authors suggested a reference of ~70% of cases resulted in hospitalisation (0.53/1000 personnel·year^{-1}). Concordant with previous points in history, EHS prevalence reported from field-hospitals during fast-paced operations overseas has remained high (Figure 4). For example, during the liberation of Iraq in 2003 - 2004 (Operation Telic), the incidence of HI was 131.4/1000 personnel·year^{-1} (Figure 4).

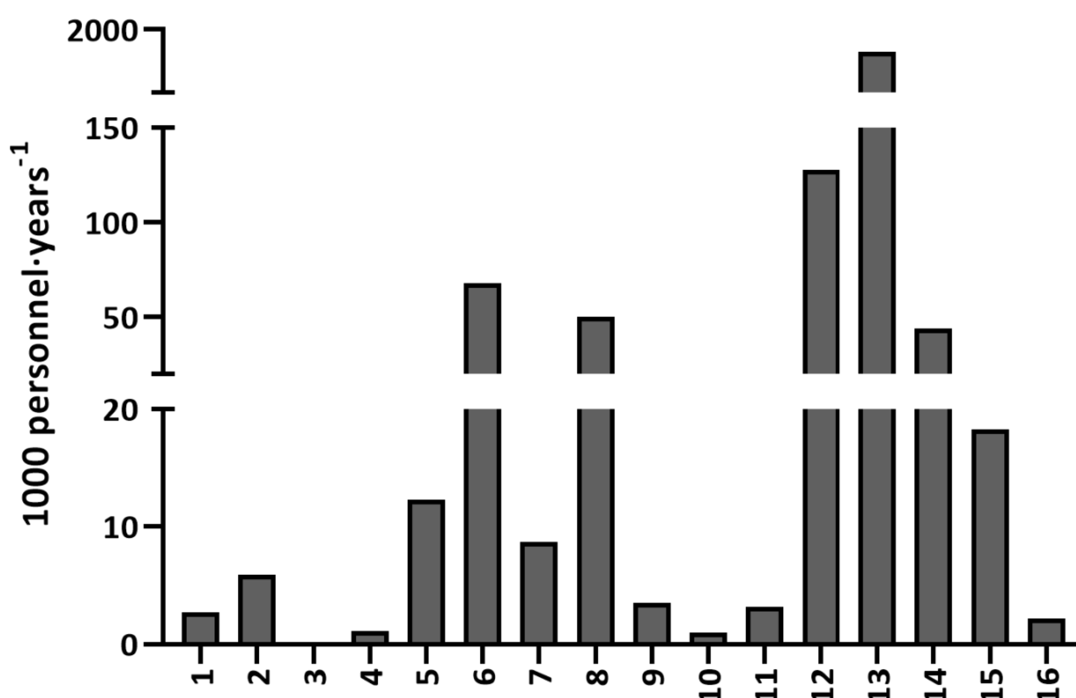

Figure 4. Annual mean incidence of hospitalisations from HI and HS across the UK Armed Forces. 1 = South Africa (CW) 1879-98 (Simpson, 1909); 2 = Turkey (WW1) 1915-18 (Michell and Smith, 1931); 3 = France (WW1) 1915 (Michell and Smith, 1931); 4 = Macedonia (WW1) 1915-16; 5 = South-West Africa (WW1) 1915-18 (Michell and Smith, 1931); 6 = Iraq (WW1) 1915-18 (Michell and Smith, 1931); 7 = India (WW2) 1939-45 (Mellor, 1972); 8 = Iraq (WW2) 1942-43 (Mellor, 1972); 9 = Sudan (WW2) 1942-44 (Mellor, 1972); 10 = Syria (WW2) 1942-45 (Mellor, 1972); 11 = Cyprus (training) 1990-94 (Bricknell, 1996b); 12 = Kuwait (Operation SARA Saraff) 2001 (Bricknell and Wright, 2004); 13 = Iraq (Operation Telic) July 2003 (Bolton et al., 2006); 14 = Iraq (Operation Telic) 2003 (Grainge and Heber, 2005); 15 = Iraq (Operation Telic) 2005 (World and Booth, 2008); 16 = Afghanistan (Operation Herrick) 2011-13 (Cox et al., 2016).

2.6.4 Patient Disposition

Direct mortality from EHS is relatively infrequent in military settings, with only 2-3% of total hospitalisations broadly reported to be fatal (Leon and Bouchama, 2015). Accumulated evidence from the UK Armed Forces over the last 150 years largely aligns to this rule of thumb (Table 3). Overall, EHS fatalities dropped from 11.2% of hospitalised cases between 1902-1911 (Permbrey, 1914), through 5.9% of cases between 1981-1991 (Dickinson et al., 1994), to 0.5% of cases between 2009-2013 (Stacey et al., 2016). Although these data suggest a reduction in EHS mortality in recent years, there has been large variation in reported disease prognosis across time (Table 3). For example, during the Confederations War (1879-1898), the mortality rate from EHS was 0.9% (Simpson, 1909), whilst there were 3 fatalities during special forces recruit training on a single day in the UK in 2013 (Ministry of Defence, 2015). For sake of comparison, the incidence of EHS related mortality across international militaries is relatively consistent with the UK. This includes: the French Armed Forces (2004-2006; 2/282 cases; Abriat et al., 2014); Indian Paratroopers (2012-2014; 0/78 cases; Deshwal et al., 2017); a Thai military hospital (1995-2007: 2/28 cases; Sithinamsuwan et al., 2009); the Chinese Armed Forces (2002-2012; 18/69; Zhao et al., 2013); and the US Armed Forces (1980-02; 37/5246; Carter et al., 2005; 2007-14; 1/48 cases; Donham et al., 2020).

There are several potential explanations that might explain the heterogeneity in EHS mortality. First, the primary factor upheld by medical consensus guidance for poor EHS outcome is delayed or inadequate post-exertional cooling (Belval et al., 2018). This risk factor is common in military settings where patients are either isolated and/or there was medical triage (Rav-Acha et al., 2004). In support of this notion, data collected from the Falmouth 7-mile road race (1984-2011), found immediate (< 2 mins) whole-body cold-water (10°C) immersion prevented mortality in 100% of cases in recreational athletes (De Martini et al., 2015). Second, the influence of intrinsic risk factors, such as aerobic fitness, heat acclimation status and health co-morbidities all likely influence EHS prognosis, despite being poorly understood (Leon and Bouchama, 2015). These intrinsic risk factors almost certainly contribute to the increased rate of mortality (20-80%) in elderly individuals from CHS, when compared to EHS patients who would be considered healthy prior to incapacitation (Dematte et al., 1990; Misset et al., 2006).

Table 3. Mortality from EHS in the UK Armed Forces.

Reference	Population	Classification	Medical Care	Mortality (%)
Simpson (1909)	UK Armed Forces – Confederations War (1879-1898)	Hospitalisation following physical activity in heat	Not Reported	0.9% (15/1625 cases)
Pembrey (1914)	British Army (1902-1911)	Loss of consciousness and a rise in T_{core} (unspecified)	Cold bath or water sponging	11.2% (360/3219 cases)
Michell and Smith (1931)	UK Armed Forces – WW1 (1914-1918)	Hospitalisation following physical activity in heat	Not Reported	Turkey = 0% (0/680 cases); France = 0% (0/262 cases); Macedonia= 0.8% (4/506 cases); Iraq = 3.0% (659/21,976 cases); Africa = 0.2% (1/406 cases)
Henderson et al. (1986)	British Army – Hong Kong (1985)	T_{core} > 41.0 °C and CNS dysfunction	Stripped and dosed with water	0% (0/12 cases)
Dickinson et al. (1994)	UK Armed Forces (1981-1991)	International Classification of Disease code 9-992.0 code	Not Reported	5.9% (11/188 cases)
Bricknell (1996b)	British Army – Cyprus (1990-1994)	Hospitalisation following physical activity in heat	Not Reported	0% (0/10 cases)
Bolton et al. (2006)	UK Armed Forces – Operation Telic II (2003)	International Classification of Disease 9-992.0 code	Water spray and fanning	0% (0/5 cases)
Stacey et al. (2016)	British Army (2009-2013)	Incapacitation following a rise in T_{core} (unspecified)	Not Reported	0.5% 3/565 cases)
Cox et al. (2016)	UK Armed Forces – Operation Herrick (2011-2013)	International Classification of Disease 10-T67 code	Not Reported	0% (0/44 cases)

The UK Armed Forces standard of care for return to action following EHS medical discharge is graduated, and relative to the severity of initial injury sustained following incapacitation (Military Headquarters of the Surgeon General, 2019). In severe cases, patients are referred for an initial medical examination. Following discharge, patients are restricted from exercise for 1-month, before enrolment onto a primary care rehabilitation programme. The intention of this programme is to safely rebuild physical fitness prior to reintegration into full active service (Military Headquarters of the Surgeon General, 2019). Data published by the UK Regular Armed Forces Official Statistics, reported 17 EHS casualties who later went on to be medically discharged between 2014-2019 (Ministry of Defence, 2019). Despite this seemingly low prevalence of health/career threatening cases, the economic burden associated with EHS remains high. For example, in the US Armed Forces, the combined costs of direct medical treatment, rehabilitation, time off duty, compensation and legal costs were estimated at $1 billion over the decade 2008-2018 (Ward et al., 2020). Furthermore, there is growing concern that EHS casualties experience long-term health complications caused by residual organ damage. For example, EHS patients frequently display cardiovascular (e.g. irregular electrocardiogram, myocardial lesions; Kew et al., 1969; Costrini et al., 1979), central nervous system (e.g. cerebellar, sensory and behavioural abnormalities; Rav-Acha et al., 2007; Sithinamsuwan et al., 2009; Yang et al., 2017), hepatic (e.g. necrosis, steatosis; Bianchi et al., 1972; Giercksky et al., 2009; Bi et al., 2019), renal (e.g. tubule atrophy; Kew et al., 1967; Satirapoj et al., 2016) and thermoregulatory (e.g. heat intolerance; Mitchell et al., 2019) irregularities that may persist for several months. Longitudinally, a 30-year retrospective cohort study in the US Armed Forces reported EHS patients to have a 40% increased risk of all-cause mortality, in comparison to a control group of hospitalised for appendicitis (Wallace et al., 2007).

2.7 The Gastrointestinal Tract

2.7.1 Anatomy and Histology

The gastrointestinal (GI) tract is an open-ended organ, organised into both longitudinal and transverse segments. It is the largest mucosal barrier with an area of 250-400 m^2, which separates the human host from the external environmental. The gross anatomy of the GI tract can be broadly divided into two major segments: the upper- and lower- GI tracts (Figure 5). The upper- GI tract spans the mouth, pharynx, oesophagus and stomach. In comparison, the lower GI tract spans the small- (duodenum, jejunum, and ilium) and large- (cecum, colon, rectum, and anal canal) intestines. The primary function of

the GI tract is to transport, digest, absorb and extract nutrients. A variety of accessory organs support the GI tract in these primary processes, including the tongue, salivary glands, liver, pancreas, and gall bladder. In the mouth, food is mechanically masticated, moistened, and digestion begins via salivary enzymes (e.g. amylase). After swallowing, the bolus is transported through the pharynx and oesophagus relatively unchanged. The gastric phase of digestion takes place in the stomach, where the bolus is converted to chyme through mixing with gastric acid that contains powerful digestive enzymes (e.g. pepsinogen). The small intestine is the primary site for digestion and subsequent nutrient absorption into the circulating blood. To increase the surface area for absorption the small intestine is highly folded containing plicae circulares, villi, and microvilli. Finally, the bolus reaches the large intestine where it is fermented by high specialised microbiota, water is reabsorbed, and eventually faeces is excreted (van De Graaff, 1986).

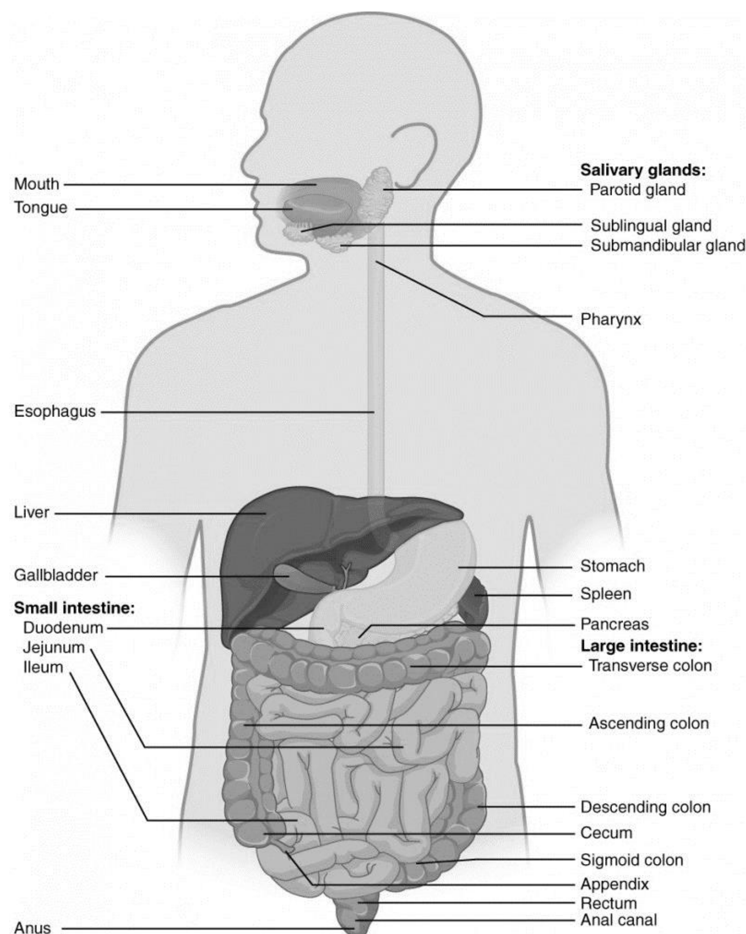

Figure 5. Major anatomy of the of the gastrointestinal tract and accessory organs (Biga et al., 2020).

The histology of the GI tract is broadly divided into four concentric layers (Figure 6; Bischoff et al., 2014). The innermost layer of the GI wall is the mucosa, which comprises the epithelium, lamina propria and muscularis mucosae. This layer comprises the GI barrier, which performs two primary functions. First, it regulates the selective movement of water, electrolytes, and nutrients between the GI lumen and circulating blood. Second, it prohibits the translocation of harmful products (e.g. antigens, digestive enzymes) outside of the lumen. The lamina propria contains capillaries that supply the epithelium with blood, whilst the muscularis mucosae supports the movement of mucus. Next, the submucosa is a thin layer of connective tissue that contains larger blood vessels, lymphatics, and nerves to supply the mucosa. The muscularis externa covers the submucosa. It is formed of an inner layer of circular muscle and outer layer of longitudinal muscle, which controls the movement of chyme via peristalsis. Finally, the outermost layer is made up of supportive loose connective tissue, termed the serosa if adjacent to the peritoneal cavity, or the adventitia if attached to surrounding tissue. This layer contains larger blood vessels, lymphatics, and nerves.

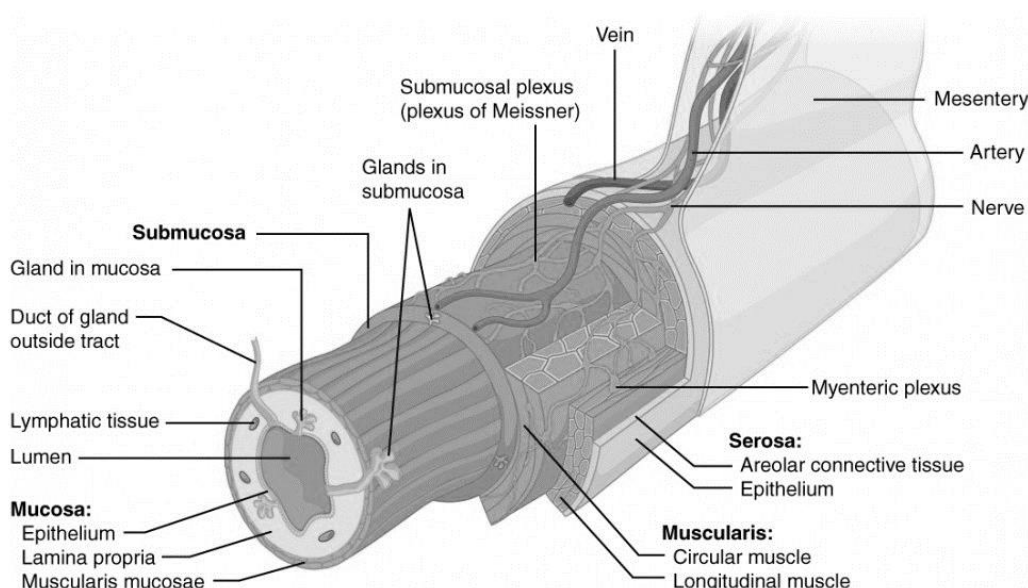

Figure 6. Major histology of the of the gastrointestinal tract (Biga et al., 2020).

2.7.2 Barrier Function and Translocation

The gastrointestinal (GI) barrier (i.e. mucosa), is a multi-layered physical and immunology structure (Figure 7). The first line of defence comprises constitutive secretory proteins that prevent direct contact between the internal milieu and epithelium. These include mucosal glycoproteins (mucins) secreted by goblet cells, which prevent adherence of enteric microbes to the epithelium (McCauley and Guasch, 2015). This is supported by Paneth cells, which produce several antimicrobial proteins, such as α-defensins and

lysozyme (Zopp et al., 2015). Below the mucosal layer, a monolayer of epithelial cells forms an impermeable physical barrier separating the lumen from the host tissue. This barrier is continually reformed via the differentiation of multipotential stem cells in the epithelial crypts (van der Flier and Clevers, 2009). The combination of rapid epithelial cell turnover, peristalsis and microfold cells remove entrapped microbes from the GI wall (Ohno, 2016). The GI epithelium maintains a selective semipermeable barrier through the formation of complex intra-membrane networks that mechanically link adjacent cells, including: tight junctions (TJs), adherens junctions (AJs) and desmosomes. At the apical-lateral membrane, TJs comprised of transmembrane proteins, scaffolding proteins, and signalling complexes, modulate paracellular permeability. Meanwhile, at the lateral membrane, AJs comprised of transmembrane proteins, adaptor proteins and the cytoskeleton, predominately support cell-to-cell adhesion, and epithelial restitution with support from desmosomes. The final line of defence is gut-associated lymphoid tissue (GALT). The GALT, consisting of Peyer's patches, isolated lymphoid follicles, and the appendix, contains several immune cell types, including macrophages, dendritic cells, plasma cells and neutrophils (Turner, 2009). The function of individual immune cells is extensive, though include: antigen presentation, pathogen neutralisation, apoptosis and natural antibody synthesis (Mantis et al., 2011).

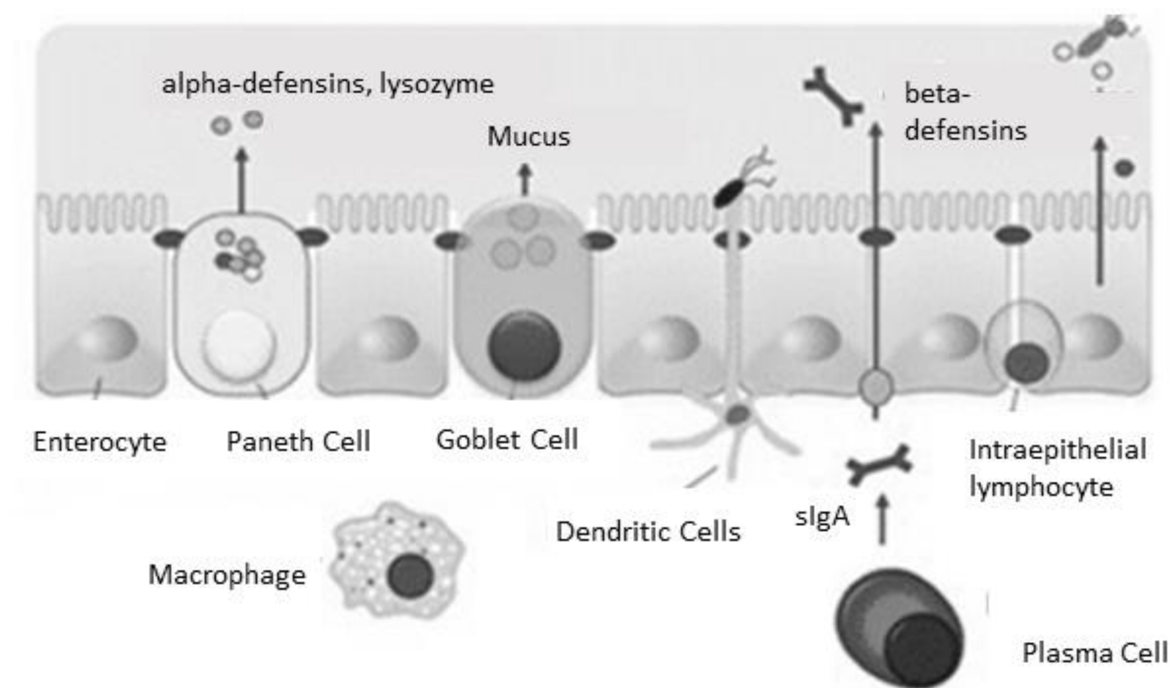

Figure 7. Illustration of the structure of the gastrointestinal barrier (Turner, 2009).

The movement of solutes across the intestinal epithelium occurs through two distinct pathways (Ulluwishewa et al., 2011). The transcellular pathway refers to energy-

dependent transport of charged solutes (e.g. glucose) through epithelial cells. This pathway is predominantly regulated by nutrient specific transporter proteins. Second, the paracellular pathway refers to the passive transport of solutes against electrochemical or concentration gradients. This pathway is regulated by intercellular TJs. TJ's comprise a network of > 50 proteins that connect the actin cytoskeleton to junctional cell-membrane spanning proteins. Transmembrane TJ proteins include: occludin, claudins, junction adhesion molecule, tricellin, whilst intracellular TJ scaffolding proteins comprise the zonula occludins. The function of specific TJ proteins on regulating GI permeability has been demonstrated in gene knock-down and expression research (Table 4). Expression of TJ's vary across regional GI anatomy, whilst phosphorylation mediates complex stabilisation. Broadly, the large intestine has greater transepithelial resistance (i.e. lower permeability) than the small intestine, which is due to a greater expression of claudins- 1, 3, 4, 5 and 8 (Groschwitz and Hogan, 2009).

Table 4. Function of major GI TJ proteins determined by gene knock-down and expression studies (Groschwitz and Hogan, 2009).

Name	Type	Primary Function
Occludin	Transmembrane	Strengthens GI paracellular permeability via the leak pathway. Modulates expression of other TJ proteins.
Claudin-1, -3, -4, -5, -8, -11, -14, -18	Transmembrane (sealing)	Strengthens GI paracellular permeability via the pore pathway.
Claudin-2, -10, -15, -16, -17	Transmembrane (pore forming)	Weakens GI paracellular permeability via the pore pathway
Junctional Adhesion Molecule-1, -2, -3	Transmembrane	Unknown influence on GI paracellular permeability. Single transmembrane spanning domain.
Tricellin	Transmembrane	Unknown influence on GI paracellular permeability. Localizes TJs between 3 epithelial cells.
Zonula Occludin-1, -2, -3	Intracellular Scaffolding	Necessary for the correct assembly of functional TJs. Connect transmembrane proteins to the actin cytoskeleton.

2.7.3 Microbiome

The lumen of the GI tract contains the densest microbial ecosystem in the human body. It is estimated from stool analysis that the GI microbiome comprises more than 10^{14} cells (Sender et al., 2016). Though various micro-organisms including archaea, viruses, phages, yeast and fungi can all be harboured from stool, bacteria account for by far the largest proportion (>98%) of microbial biomass (Nash et al., 2017). Information on microbiota density is based on two relatively recent advances in DNA-sequencing technologies. These are: 16S ribosomal RNA (rRNA) gene sequencing that provides information on microbiome membership, and metagenomic data that portrays functional potential (Poretsky et al., 2014). Groups of bacteria are organised on a phylogenetic tree with taxonomical categories ranging from low resolution (phylum) to high resolution (genus, species) taxa. Applying high-resolution shotgun metagenomic analysis across large populations has allowed characterisation of the anatomy, function and temporal variability of the GI microbiota (Qin et al., 2010; Li et al., 2014; Hugon et al., 2015; Lloyd-Price et al., 2017).

To date, population metagenomic analysis has characterised 12 phyla and 2172 species of common bacteria within the GI microbiome (Thursby and Juge, 2017). Of these phyla, *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* account for > 95% of total microbial composition. The density of micro-organisms measured in mucosal samples across GI segments display a proximal-to-distal concentration gradient (Cani, 2018). This ranges from < 10^3 cell·g·mucosa^{-1} in the duodenum, 10^7-10^8 cell·g·mucosa^{-1} in the ilium and 10^{10}-10^{11} cell·g·mucosa^{-1} in the colon (Hillman et al., 2017). This biogeography of GI colonisation relates to chemical, nutritional, and immunological concentration gradients across the GI tract (Donaldson et al., 2015). For example, in the small intestine only fast growing, facultative anaerobes can survive the highly acidic environment, whereas, in the large intestine the slow flow, neutral pH and low oxygen saturation support a greater abundance of microbial communities (Seekatz et al., 2019). In adulthood, microbial abundancy remains largely stable at the phylum level, however, at the genus level there is considerable inter- and intra-individual variability (Thursby and Juge, 2017). Several host and environmental covariates have been identified to influence GI microbial composition. These include: age, ethnicity, diet, antibiotics and body composition (Hugon et al., 2015; Zhernakova et al., 2016; Mach and Fuster-Botella, 2017; Falony et al., 2019).

The GI microbiome is involved in many regulatory processes that are symbiotic to human health (Thursby and Juge, 2017). First, microbial regulated carbohydrate fermentation produces various metabolites that support host metabolism and immunity (Husted et al., 2017; Rastelli et al., 2018). These include: vitamins (e.g. riboflavin [B_2], folate [B_9], cobalamin [B_{12}]), indoles, secondary bile acids, trimethylamine-N-oxide, short-chain fatty acids and neurotransmitters (e.g. serotonin). For example, short-chain fatty acids (e.g. propionate, butyrate, acetate) have functional roles including being an important energy source for GI barrier integrity (Corrêa-Oliveira et al., 2016) and modulating secretion of intestinal peptides (e.g. peptide YY) involved in glucose metabolism (McKenzie et al., 2017). Second, the microbiota can directly modulate GI barrier function. This includes influencing epithelial cell regeneration, mucosal thickness, TJ phosphorylation and local immunity (Natividad and Verdu, 2013). This knowledge has been derived from research in germ-free rodents (Smith et al., 2007). Third, commensal microbiota compete with pathogens for nutrients, thus reducing susceptibility to infection (Buffie and Palmer, 2013). Despite these synergistic microbiota-host interactions, this relationship appears fragile. To this point, alternations in GI microbiota composition and/or barrier integrity have both been associated with poor health (Bischoff et al., 2014).

2.8 Gastrointestinal Barrier Integrity Assessment

Various techniques are available to safely assess GI barrier integrity *in vivo* at a systemic level (Bischoff et al., 2014). Available methods are generally categorised as either: (1) *active* tests (i.e. GI permeability), based upon the oral ingestion and extracellular recovery of inert, water-soluble, molecular probes over a set time-period (Bjarnason et al., 1995); or (2) *passive* tests (i.e. GI injury) based upon monitoring extracellular biomarkers assumed to be indicative of GI barrier integrity loss (Table 5). The assessment of systemic GI luminal microbial products (i.e. microbial translocation [MT]) is one widely applied example of a *passive* GI barrier integrity test.

Table 5. Overview of Common Assessment Techniques (In *Vivo*) to Determine GI Barrier Integrity (Adapted from: Grootjans et al., 2010).

Technique	Fluid		Site	Limitations
Active Techniques				
Dual-Sugar Absorption Test (DSAT)	Urine or blood	HPLC (+) MS	Small intestine permeab-ility	Gold-standard. Good reliability. Time-consuming (5 hr urine, >1.5 hour blood). No standard protocol. Well-studied.
Multi-Sugar Absorption Test (MSAT)	Urine or blood	HLPC (+) MS	Segmental intestine permeab-ility	Gold-Standard. Segmental GI integrity. Time-consuming (5 hr urine, >1.5 hour blood). No standard protocol. Few studies.
Polyethylene Glycol (PEG) Absorption Test	Urine or Blood	HLPC (+) MS	Whole GI tract permeab-ility	Validated. Can include multiple weight PEGs (e.g. 100, 400, 4000 kDa). Time-consuming (5 hr urine). Few studies.
Passive Techniques				
Intestinal Fatty Acid Binding Protein (I-FABP)	Urine or Blood	ELISA	Epithelial injury	Small intestine specific (duodenum and jejunum). 11 minute half-life. Well-studied.
Ileal Bile-Acid Binding Protein (I-BABP)	Urine or Blood	ELISA	Epithelial injury	Ilium specific. Moderate correlations with I-FABP. Few studies.
Diamine Oxidase (DAO), Alpha-Glutathione s-Transferase (α-GST)	Blood	ELISA	Epithelial injury	Non-GI specific. Few studies.
Claudin-3 (CLDN3)	Urine or Blood	ELISA	TJ Integrity	Non-GI specific. Few studies.
Zonulin	Blood	ELISA	TJ Integrity	Non-GI specific. Assay cross-reactivity. Few studies.
Microbial Translocation (MT)				
Endotoxin (LPS)	Blood	LAL assay	MT	GI specific. Sample contamination risk. Well-studied.
Bacterial 16S DNA	Blood	PCR assay	MT	GI specific. Sample contamination risk. Few studies.
LPS Binding Protein (LBP)	Blood	ELISA	MT	Predominately GI specific. Indirect endotoxin marker. Long half-life (12-14 hours). Few studies.
Soluable-CD14-subtype (sCD14-ST)	Blood	ELISA	MT	Predominately GI specific. Indirect endotoxin marker. Few studies.
D-lactate	Blood	ELISA	MT	Predominately GI specific. Few studies.

Abbreviations: HPLC, high performance liquid chromatography; MS, mass spectrometry; ELISA, enzyme-linked immunosorbent assay; LAL, limulus amoebocyte lysate assay; qPCR, quantitative polymerase chain reaction.

2.8.1 Active Assessment

The Dual Sugar Absorption Test (DSAT) is the gold-standard *active* GI permeability test (Bischoff et al., 2014). It has received almost universal application as a method to examine GI permeability within the field of exercise physiology (Costa et al., 2017). The test standardly involves oral ingestion of a large non-dietary disaccharide (5 grams), typically lactulose (342 kDa) or cellobiose (342 kDa); and a small non-dietary monosaccharide (2 grams), typically $_L$-rhamnose (164 kDa) or $_D$-mannitol (182 kDa). The disaccharide probe only transverses the GI tract paracellularly upon barrier integrity loss, however, the monosaccharide probe freely transverses the GI tract transcellularly independent of barrier integrity (Menzies et al., 1999). Both sugars are measured in urine for 5-hours following probe ingestion and are assumed to be equally affected by confounding factors (Table 6) during excretion (Bjarnason et al., 1995). The endpoint of the DSAT is the total urinary excretion ratio of the monosaccharide to disaccharide probe, relative to the ingested dose. Since conceptual introduction in the 1970s (Menzies et al., 1974), several meta-analyses have concluded the DSAT to have strong sensitivity and specificity in diagnosing GI tissue integrity in patients with various clinical GI diseases (Tibble et al., 2000; Deitch et al., 2012). Despite widespread application, several theoretical assumptions of the DSAT are still contentious. For example, lactulose/cellobiose and $_L$-rhamnose/$_D$-mannitol kinetics are not always equal (Rao et al., 2011), especially if the time-period for biofluid collection is lengthy. To this point, it is suggested that 2-hours urine collection can offer improved test sensitivity relative to traditional 5-hour collections (Akram et al., 1998; Camilleri et al., 2010; van Wijck et al. 2011a, 2012a, 2012b, 2014). Whilst healthy basal DSAT ratios are typically reported *circa* 0.01-0.04, there is little consistency in absolute responses within the literature. Consequently, it is recommended that every laboratory have its own pre-defined reference range (Wells et al., 2017).

Table 6. Covariates influencing active GI permeability assessment using the DSAT/MSAT (Bjarnason et al., 1995).

	Disaccharide	Monosaccharide
Pre-mucosal		
Dose Ingestion	Equal	Equal
Gastric Dilution	Equal	Equal
Gastric Emptying	Equal	Equal
Intestinal Dilution	Equal	Equal
Intestinal Transit	Equal	Equal
Microbial Degradation	Equal	Equal
Unstirred Water Layer	Equal	Equal
Hydrolysis	None	None
Mucosal		
Route of Permeation	Paracellular	Transcellular
Intestinal Blood Flow	Equal	Equal
Post Mucosal		
Metabolism	Inert	Inert
Endogenous Production	None	None
Tissue Distribution	Equal	Equal
Renal Function	Equal	Equal
Timing of Blood Collection	Equal	Equal
Timing of Urine Collection	Equal	Equal
GI Microbial Degradation	Equal	Equal
Analytical Performance	Equal	Equal

Validation of the DSAT in blood samples has been proposed as an alternative to urine. This method reduces the time-course of biofluid collection and consequently patient burden. Pioneering work demonstrated the serum DSAT to be sufficiently sensitive to quantify histologically proven GI barrier integrity loss in patients undergoing jejunal biopsy at each 1-, 1.5- and 2- hours following probe ingestion (Fleming et al., 1996). This study concluded the 1.5-hour time-point best predicted the 5-hour urine DSAT ratio (r = 0.88), with the method considered accurate enough for the two methods to be utilised interchangeably. Follow-up evidence subsequently confirmed plasma/serum DSAT ratios to be relatively stable when assessed between 1-3 hours following probe ingestion (Cox et al., 1997, 1999; Fujii et al., 2001; van Wijck et al., 2012b; Wong et al., 2019), though in the case of moderate-to-severe intensity exercise, there might be a delay in probe transit by *circa* 1-hour (van Wijck et al., 2011a). In paediatric patients hospitalised with severe

diarrhoea, the serum DSAT was shown to accurately predict the 5-hour urine DSAT (r = 0.76; limits of agreement = 0.011) at 1.5-2 hours following probe ingestion, whilst offered high levels of sensitivity (81%) and specificity (89%) if assuming the urine DSAT to be the criterion method (Haase et al., 2000). Most recently, pharmaceutical (van Wijck et al., 2011b) and exercise (van Wijck et al., 2011a; Janssen-Duijghuijsen et al., 2017a; Pugh et al., 2017a) gastroenterology research has promoted the serum/plasma DSAT to be more responsive than the urine DSAT for detecting small transient losses in GI barrier integrity. A limitation of the serum DSAT has been the application of inconsistent time-points to assess probe recovery.

The multiple-sugar absorption test (MSAT) is an extension of the DSAT, which allows a more detailed examination of segmental GI permeability (van Wijck et al., 2011b). The MSAT involves simultaneous ingestion of 5 molecular sugar probes and exploits knowledge surrounding site specific degradation (Meddings and Gibbons, 1998). To this point, lactulose, L-rhamnose and D-mannitol are all fermented by the microbiota upon entering the large intestine (caecum), meaning that the traditional DSAT primarily assesses small intestinal integrity. In comparison, sucrose (342 kDa) is immediately hydrolysed in the small intestine following gastric excretion, thus making it a suitable probe for assessment of gastroduodenal integrity (Meddings et al., 1993). Typically, sucrose is assessed 1-2 hours following probe ingestion in urine and 1-hour in plasma (van Wijck et al., 2011b, 2013). Ideally, sucrose should be co-ingested with either L-rhamnose or D-mannitol as reference probes. Sucralose (397 kDA) is resistant to degradation across the entire GI tract and can therefore be applied to examine large intestinal integrity. Typically, sucralose assessment is made over a 5-24 hour period following probe ingestion in urine to allow time for probe transit to the colon (Camilleri et al., 2010). Alternatively, large intestinal integrity can be calculated by subtracting 24-hour lactulose excretion from 24-hour sucralose excretion (Anderson et al., 2004). The assessment of large intestinal integrity in blood has not presently been validated. To control for small intestinal sucralose absorption, erythritol (122 kDa) provides a suitable reference probe (van Wijck et al., 2013). Until recently, simultaneous assessment of segmental GI barrier integrity was considered infeasible without affecting GI motility through administering hyperosmolar probe mixtures (Bjarnason et al., 1995). However, this issue has been recently addressed with the development of high-sensitivity analytical techniques (van Wijck et al., 2011b) and validation of a reduced probe dosage (van Wick et al., 2013).

Application of the DSAT/MSAT has never gained a place in routine clinical practise for GI barrier diagnostic assessment. This is largely due to the impracticality of test administration. First, the test is undertaken following an overnight fast, which is sustained until the completion of sample collection. If baseline measurements are required, this procedure is required to be replicated on multiple days or at least separated for > 7 hours for serum assessment (Pugh et al., 2019). Second, quantitative analysis using high performance liquid chromatography (HPLC) with or without mass spectrometry (HPLC + MS) is expensive, not routinely available and requires technical expertise. Third, there is no universal standardisation of probe solution osmolality, with hyperosmolar doses (typically defined >1500 mOsm.kg^{-1}) known to artificially increase GI permeability (Uil et al., 2000). Fourth, basal sugar concentrations from dietary sources are often uncontrolled (Bjarnason et al., 1995) and with specific relevance to sucralose/$_{D}$-mannitol may severely impact test results (Grover et al., 2016). Fifth, the test (in urine) might be unsuitable for overweight individuals given the impact of elevated glomerular filtration upon test results (Teixeira et al., 2014). Sixth, the size of high-molecular weight disaccharides (i.e. lactulose [342 kDa], sucrose [342 kDa], sucralose [398 kDa]) are relatively small in comparison to immunomodulatory GI luminal products (e.g. endotoxin, food antigens), therefore application of larger molecular weight probes (400-15,000 kDa) might have greater relevance in predicting MT (Vojdani, 2013). Finally, in exercise settings little is known regarding the influence of probe ingestion timing (e.g. pre, during or post exercise) around GI barrier disruption on test outcome (van Wijck et al., 2012c).

The assessment of GI permeability using active probe-based techniques is not unique to the DSAT/MSAT and can be performed using several alternative molecular probes (Bjarnason et al., 1995). The most common of these include: PEGs, radioisotopes and iohexol. One limitation of these probes is their ability to transverse the entire GI tract unchanged, consequently preventing segmental GI permeability assessment. Polyethylene glycols (PEGs) are low-cost synthetic materials available in various molecular weights (e.g. 100, 400, 4000 kDa). Generally, PEGs return comparable results to the MSAT (van Wijck et al., 2012a), albeit the absolute magnitude of PEG permeability largely exceed that of lactulose (Maxton et al., 1986; Bjarnason et al., 1994). This finding is likely attributable to: (1) PEGs reduced cross-section area relative to molecular mass; and (2) PEGs lipid solubility, which consequently might permit transcellular permeation (Bjarnasson et al., 1995). Further limitations of PEG assessment include, cross-contamination from artificial food

products (e.g. soft drinks, toothpaste) and poor patient palatability (Castle et al. 1988). Chromium-labelled ethylenediaminetetraacetic acid (^{51}Cr-EDTA) is a radioisotope (~340 kDa) that is easily detectable within humans and can be monitored either in isolation or in combination with $_{L}$-rhamnose/$_{D}$-mannitol (Maxton et al., 1986; Elia et al., 1987). The major limitation of this method is the radioactive nature of the probe (~0.12 mSv) (Bjarnason et al., 1995). Iohexol is a water-soluble contrast agent (821 kDa) commonly used in X-ray based imaging techniques. Though uncommonly applied for GI barrier integrity assessment, Iohexol display comparable results to the DSAT over a 5-hour urine collection period (Halme et al., 1993, 1997, 2000). One strength of iohexol assessment, is the potential for inexpensive analysis using commercial enzyme-linked immunosorbent assays (ELISA).

2.8.2 Passive Assessment

Passive blood-based biomarkers of GI barrier integrity have been evaluated with the aim to provide specific information relating to either: (1) injury to the GI epithelial monolayer (e.g. fatty acid-binding proteins; alpha glutathione s-transferase, citrulline), or (2) paracellular GI TJ breakdown (e.g. claudin-3, zonulin). Evaluation of these biomarkers has received varying levels of empirical support, though given the significant practical drawbacks associated with *active* GI barrier integrity assessment, their use has recently popularised within the field of exercise physiology (Costa et al., 2017). Despite this increased application, the validity, reliability, and reference ranges of these biomarkers has never been thoroughly examined. The main advantage of *passive* GI barrier integrity assessment is the ability to measure basal concentrations immediately prior to GI barrier integrity challenge, which can be inexpensively undertaken using a commercial ELISA kit (Grootjans et al., 2010).

Fatty acid-binding proteins (FABP's) are cytosolic proteins involved in fatty acid metabolism. They are located on the outer villi of mature enterocytes and are rapidly shed into the circulation following injury (Furuhashi and Hotamisligil, 2008). To date, nine distinct FABP isoforms have been identified, which are classified based on the tissue location (e.g. liver, heart, brain) of initial detection (Pelsers et al. 2005). It must be acknowledged, however, that these classifications are somewhat misleading, given that some FABP's are expressed in multiple tissues, whilst some tissues express multiple FABP's (Pelsers et al. 2005). In the GI tract, liver (L-FABP), intestinal (I-FABP) and ileal (I-BABP) FABP are all expressed (Pelsers et al. 2005). Evidence from human autopsy studies has shown L-

FABP/I-FABP to be most prominent in the duodenum and jejunum (Pelsers et al. 2003; Derikx et al. 2009), whilst I-BABP is only expressed in the ileum (Watanabe et al. 1995). Importantly, whereas I-FABP and I-BABP are specific to the GI tract, L-FABP is more concentrated in the liver (Watanabe et al., 1995). In healthy individuals, basal I-FABP and I-BABP concentrations reflect the physiological turnover rate of GI enterocytes and have a calculated half-life of 11 minutes (van de Poll et al. 2007). For I-FABP, basal concentrations vary widely with commercial ELISAs supplied from separate manufactures (e.g. Hycult Biotechnology = 0.2-1.0 ng·ml^{-1}; D.S. Pharma Biomedical = 1.0-50.0 ng·ml^{-1}) (Treskes et al., 2017). In various clinical studies, plasma I-FABP weakly predicts both histological GI barrier integrity (Derikx et al., 2009; Adriaanse et al., 2013) and *active* GI permeability assessment (van Wijck et al., 2012c, 2013). Less is understood about basal I-BABP concentrations, and its ability to accurately predict histological GI barrier integrity status (Thuijls et al., 2011).

There are several alternative biomarkers that can predict GI epithelial injury besides FABPs. Alpha-Glutathione s-Transferase (α-GST) is a protein involved in cell antioxidation and is highly expressed on the outer membrane of GI enterocytes (Sundberg et al., 1993; Coles et al., 2002). Healthy basal α-GST concentrations have been clearly defined (0.5 ng·ml^{-1}; van Wijck et al., 2011b), whilst increase rapidly following GI ischemia (Delaney et al., 1999; Khurana et al., 2002). Unfortunately, α-GST is not tissue specific unlike I-FABP (Campbell et al., 1991) and consequently its clinical utility appears limited to situations of local GI disruption (Grootjans et al., 2010). Diamine oxidase (DAO) is the principle histamine catalysing enzyme, which despite being expressed across multiple organ tissues (e.g. GI tract, kidney, thymus), has been broadly classified to predict GI epithelial injury (Wollin et al., 1981; Luk et al., 1983). Healthy basal DAO concentrations have been defined (0.5-2 ng·ml^{-1}; Boehm et al., 2017), and increase following GI barrier integrity loss (Song et al., 2009; Leber et al., 2012). Citrulline is a non-protein amino acid that is synthesised almost exclusively by small intestinal enterocytes through glutamine metabolism (Curis et al., 2007). Healthy basal citrulline concentrations have been defined (20-25 µmol·l^{-1}) and appear to be suppressed in various clinical states where functional GI enterocyte mass is reduced (Fragos et al., 2018). Despite increasing empirical evidence, no universal analytical method is presently available to assess plasma citrulline (Kartaram et al., 2018).

Claudins are a highly abundant family of low molecular weight (21-34 kDa) TJ proteins that have been extensively examined as candidate biomarkers of paracellular TJ breakdown (Gunzel and Yu, 2013). Specifically, claudin-3 (CLDN-3) is a pore-sealing family

member, which is highly concentrated across GI (particularly colonic) enterocytes (Rahner et al., 2001; Marcov et al., 2010). The importance of CLDN-3 on GI barrier integrity is demonstrated given that tissue expression is downregulated in clinical settings where functional GI barrier integrity loss takes place (Zeissing et al., 2007). Though evidence is limited, preliminary research has shown a strong relationship between histologically proven GI tight junction breakdown and urinary CLDN-3 concentration in humans with relapsed irritable bowel disease (Thuijls et al., 2009). Due to ease of collection, plasma/serum has become the principle biofluid to assess CLDN-3. Indeed, research has shown plasma CLDN-3 concentrations to increase in patients following acute (Typoo et al., 2015) and chronic (Sikora et al., 2018) GI disturbance. Unfortunately, these studies report large variations in healthy basal CLDN-3 concentrations (~0-50 ng·ml^{-1}), whilst the sensitivity/specificity of CLDN-3 in predicting GI barrier integrity is unknown. This said, plasma/urinary CLDN-3 is positively assoicated with both the DSAT (r = 0.45; Typoo et al., 2015) and urinary I-FABP (r = 0.38; Habes et al., 2017) in patients following major abdominal surgery. In addition to CLDN-3, the other candidate TJ proteins (e.g. occludin) should be examined in future research.

Zonulin is a precursor protein of haptoglobin (pre-HP2), which is claimed can reversibly disassemble GI TJs (Sturgeon and Fasano, 2016). Since discovery, zonulin has been implicated within the pathogenesis of various GI and metabolic diseases (Sturgeon and Fasano, 2016). How zonulin disassembles GI TJs is poorly understood, although one study did show zonulin reduced GI transepithelial electrical resistance via activation of epidermal growth factor receptors and G protein-coupled receptors (Tripathi et al., 2009). Though demarcations of haptoglobin occur within the endoplasmic reticulum (Wicher and Fries, 2004), it is supposedly possible to detect zonulin in low concentrations within the systemic circulation (Tripathi et al., 2009). Indeed, research in both clinical (Sturgeon and Fasano, 2016) and exercise (Janssen-Duijghuijsen et al., 2016; Karhu et al., 2017) settings has shown positive associations between systemic zonulin concentration with alternative GI barrier integrity biomarkers. Despite zonulin's recent popularity as a biomarker of GI barrier integrity, the two-sole commercial zonulin assays available (e.g. Cusabio, Wuhan, China; Immunodiagnostic, Bensheim, Germany) have been shown to exhibit unacceptable analytical cross-creativity (Ajamian et al., 2019).

2.8.3 Microbial Translocation

Passive biomarkers of GI microbial translocation (MT) provide indirect information relating to: (1) GI barrier integrity and (2) subsequent immune activation. The term MT was traditionally applied to define the migration of live bacteria from the GI lumen into the mesenteric lymph, however, given the invasiveness of this technique, this definition was later broadened to include the detection of microbial products/fragments within the circulating blood (Fukui, 2016). Whilst there is presently no gold-standard *in vivo* technique to assess MT, various direct (e.g. endotoxin, bacterial 16S DNA) and indirect (e.g. lipopolysaccharide binding protein, soluble CD14, D-lactate) biomarkers have been validated. Despite this, there are several issues that limit the assessment of such GI MT products are biomarkers of GI barrier integrity. These include: (1) hepatic detoxification before most MT products reach the systemic circulation; (2) incorporating of MT products in chylomicrons that permits systemic translocation via the lymphatic duct; and (3) the influence of the host immune response on the rate clearance/production of certain MT biomarkers (Wells et al., 2017). These covariates likely explain the weak correlations (r = 0.1-0.6) typically reported between GI barrier integrity and MT biomarkers in response to exertional-heat stress (Yeh et al., 2013; March et al., 2019).

Endotoxin is the hydrophobic domain of lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria (Gnauck et al., 2016). Traditionally, endotoxin was the primary biomarker of GI MT, due to its well-characterised antigenic properties (Raetz and Whitfield, 2002). Endotoxin is detectable within the systemic circulation following disassociation from gram-negative bacteria, which occurs during both cell lysis and division (Petsch et al., 2000). Analytical assessment is typically performed using the chromogenic limulus amoebocyte lysate (LAL) assay, a US food and drug association approved method (Gnuack et al., 2016). Whilst practical, this method is prone to limitations that frequently lead to both false-positive and false-negative results (Dullah and Ongkudon 2017). Specifically, false-positive results may arise from cross-reactivity with non-target bacterial components (e.g. [1,3]-β-D-glucan) within the assay or contamination from external sources (e.g. collection tubes) during sample handling. In comparison, false-negative results may arise through assessment of systemic blood following hepatic detoxification and/or the presence of endotoxin neutralizing factors (e.g. lipopolysaccharide binding protein) within the sample (Gnauck et al., 2016). Together, these issues have prevented the classification of a universal healthy reference-range (0.15-

61 EU·ml^{-1} [1 EU = 120 pg·ml^{-1}]; Gnuack et al., 2016) and likely contribute to the poor test-retest reproducibility shown in basal healthy individuals (Guy et al., 2016). Despite these shortcomings, numerous studies demonstrate endotoxin to predict GI barrier integrity when compared to both histological and *in vivo* permeability techniques (Gnuack et al., 2016). Likewise, in cases of acute GI barrier integrity loss (e.g. exertional-heat stress), it is possible to minimise the influence of assay shortcomings through basal sample correction.

Bacterial 16S DNA is a stable bacterial component, which can be quantified through real-time quantitative polymerase chain reaction (qPCR) of target genes following extraction of plasma cell free DNA (Fukui, 2016). In comparison to alternative GI MT biomarkers, one major advantage of bacterial 16S DNA assessment is a lack of hepatic clearance (Mortensen et al., 2013), thus permitting valid assessment in systemic blood. Early evidence assessing 16S bacterial DNA utilised insensitive PCR techniques, which were only able to detect bacterial DNA concentrations in a proportion of patients with serve acute illnesses (de Madaria et al., 2005; Bossola et al., 2009; Gutierez et al., 2011, 2014). Despite growing empirical evidence, a lack of universal analytical procedure (e.g. target primers, controls) and rapid improvements in analytical sensitivity has made understanding the clinical relevance of bacterial DNA assessment complex (Fukui, 2016). To this point, where early research focused on sequencing the entire 16S gene, a procedure that offers poor methodological specificity (Castillo et al., 2019), recent techniques may offer better specificity through targeting shorter GI specific subregions (Bala et al., 2014; Buford et al., 2018). In particular, given that the GI microbiota is dominated by gram-positive *Firmicutes* and gram-negative *Bacteroidetes* (Eckburg et al., 2005) phyla, primers specific to these subregions appear to offer the greatest promise. One study recently demonstrated an acute increase in *Bacteroides* DNA (the dominant *Bacteroidetes* genus; Wexler and Goodman, 2017) following 1-hour of moderate-intensity (70% $\dot{V}O_{2max}$) exertional-heat stress (March et al., 2018). Relevantly, this technique corrected *Bacteroides* to total 16S bacterial DNA concentrations (Paisse et al., 2016; Qiu et al., 2019) to help minimise the influence covariates including: DNase concentration (Velders et al., 2014), non-GI mediated MT (e.g. from lung, leukocytes; Paisse et al., 2016) and the efficiency of DNA extraction/purification (March et al., 2018).

Lipopolysaccharide Binding Protein (LBP) is a hepatic type-1 acute phase protein (Mussap et al., 2011). The major function of LBP is to transport systemically derived endotoxin to immune cells bearing CD14 receptors (e.g. macrophages). Consequently,

plasma LBP concentration typically increases following systemic endotoxin exposure (Schumann, 2011). Whilst LBP has been widely proposed as a surrogate biomarker of endotoxemia, it perhaps better represents overall GI MT, given that it is activated by several other microbial-associated molecular patterns (Mussap et al., 2011). These include: gram-positive bacteria (e.g. peptidoglycan, lipoteichoic acid), mycobacteria (e.g. lipoproteins and lipomannans) and spirochetes (e.g. glycolipids and glycoproteins) (Schumann, 2011). In healthy individuals, habitual LBP concentrations (2-15 $\mu g \cdot ml^{-1}$) sensitise the immune system to microbial exposure (Schumann et al., 1990), however paradoxically, LBP concentrations increase (50-100 $\mu g \cdot L^{-1}$) linearly in response to acute septic shock concordant with the severity of GI MT (Opal et al., 1994, 1999; Schafer et al., 2002; Rojo et al., 2006; Schiffrin et al., 2008). Assessment of LBP can be reliably quantified by ELISA, consequently overcoming analytical limitations associated with endotoxin assessment (Mussap et al., 2011). Importantly, LBP has been consistently associated with histological GI atrophy (Farras et al., 2018), GI permeability (Reibeger et al., 2013; Farras et al., 2018) and systemic endotoxemia during acute-stress (Schafer et al., 2002; Rojo et al., 2006; Moreno-Navarrete et al., 2012; Nier et al., 2017). Despite this evidence, sparse research has assessed the sensitivity, specificity, and kinetics of LBP in predicting acute changes in GI barrier integrity loss.

Soluble CD14 subtype (sCD14-ST) is a multifunctional cell surface acute phase protein (Mussap et al., 2011). Following LBP facilitated microbial ligand- CD14 coreceptor binding, sCD14-ST is subsequently cleaved into the systemic circulation upon CD14 proteolysis (Mussap et al., 2011). The biological role of sCD14-ST is incompletely understood, though membrane bound CD14 modulates host immune responses through the activation of toll like receptors (Mussap et al., 2011). Given these characteristics, sCD14-ST can be considered a surrogate marker of MT. In healthy individuals, sCD14-ST is detectable in the systemic circulation (100-400 $ng \cdot ml^{-1}$), though like LBP increases in response GI MT (500-2000 $ng \cdot ml^{-1}$) during acute septic shock (Schafer et al., 2002; Rojo et al., 2006). In clinical populations, sCD14-ST has been associated with systemic endotoxin responses in most (Schafer et al., 2002; Rojo et al., 2006; Nier et al., 2017), but not all (Schafer et al., 2002; Farras et al., 2018) cases. In accordance with LBP, there is little evidence to characterise the sensitivity, specificity, and kinetics of SCD14-ST in predicting GI barrier integrity.

Lactate is a hydroxycarboxylic acid that exists inside the human body as two stereoisomers: L(+)-lactate and D(-)-lactate. Unlike L-lactate, human tissue does not express the enzyme D-lactate dehydrogenase, therefore systemic D-lactate concentrations are hypothesised to be derived from the GI microbiota (Ewaschuk et al., 2005). To this point, healthy basal L-lactate concentrations are *circa* 90 $\mu g \cdot ml^{-1}$ (Vincent et al., 2016), whereas D-lactate concentrations are *circa* 10-60 $\mu g \cdot ml^{-1}$ (Herrera et al., 2008; Ficek et al., 2017; Nier et al., 2017). Given that D-lactate has an estimated half-life of 21 minutes in the systemic circulation (de Vrese et al., 1990), analysis has potential to provide real-time GI MT assessment. In rodent trauma models, D-lactate has been found to strongly predict histological GI barrier integrity loss (Murray et al., 1993; Sun et al., 2001; Szalay et al., 2003; Sobhian et al., 2012). In humans, moderate associations have been reported between D-lactate with: GI ischemia (Poeze et al., 2003), the DSAT (Fang et al., 2007), I-FABP- (Shi et al., 2015) and endotoxin (Ruan et al., 2004) concentrations in various clinical settings. Despite promise, the low molecular weight (0.09 kDa) of D-lactate might permit transcellular GI barrier permeation independent of GI barrier integrity.

2.9 Exertional Heat Stroke Pathophysiology

Historically, the pathophysiology of EHS has been widely attributed to the direct influence of hyperthermia on multiple-organ injury (Malamud et al., 1946; Knochel, 1989). These models were constructed based on evidence of histological injury to all major organs from autopsies of deceased EHS patients, without consideration of the potential involvement of a simultaneous immune response. However, in more recent decades, these models have been criticised for an inability to adequately explain many of the clinical manifestations of EHS (Bouchama and Knochel, 2002).

2.9.1 Heat Cytotoxicity Paradigm

A major issue with traditional models of EHS pathophysiology, is the wide heterogeneity in T_{core} reported in casualties immediately following collapse (De Groot et al., 2017; King et al., 2018). To this point, if EHS was primarily caused by hyperthermia induced cytotoxic injury, then the relationship between these two variables would be near linear (Laitano et al., 2019). Whilst measurement error in T_{core} assessment (e.g. site, timing) might partially explain some variability (Laitano et al., 2019), there is significant overlap in T_{core} responses between EHS casualties and healthy individuals when assessed during physical exertion (Lim, 2018). For example, T_{core} (rectal) has been reported <40 °C in severe

EHS case reports when measured without delay (< 15 minutes; Shapiro and Seidman, 1990), whereas, many athletes appear to tolerate a T_{core} between 41-43 °C during prolonged exertion without clinical manifestation (Maron et al., 1977; Bynre et al., 2006; Racinais et al., 2019). *In vitro* cell lines from various mammalian organs, including the cerebrum, heart, liver and kidney, have all been shown to tolerate hyperthermia 40-43 °C for extended periods (30-60 minutes) with minimal cellular denaturation (Burger and Fuhrman, 1964; Harmon et al., 1990). Likewise, i*n vivo* cellular apoptosis is unchanged in the heart, lung, kidney, liver, pancreas, and major endocrine glands of rodents exposed to 2-hours passive hyperthermia at 41.5 °C (Sakaguchi et al., 1995). Together, these findings suggest whilst T_{core} is an important driver of EHS, it is unlikely that thermal injury is sole cause of morbidity/mortality given that in most cases T_{core} remains below the temperature threshold for cellular denaturation (>45°C, Buckley, 1972). Inconsistent with this hypothesis, variability in cellular thermotolerance (e.g. heat shock protein expression; Horowitz and Robinson, 2007), secondary stress exposure (e.g. acidosis, energy depletion; Leopock, 2003) and/or the duration of hyperthermia (Lepock, 2003) may all partially explain intra-individual variability in cellular denaturation.

2.9.2 Gastrointestinal Paradigm

The GI paradigm was first conceptualised within the pathophysiology of EHS by Moseley and Gisolfi (1993), who upon reviewing multiple lines of evidence described associations between immune function, multi-organ injury, and DIC during extreme hyperthermia. Since then, this model was included within conventional medical classifications of EHS since 2002 (Bouchama and Knochel, 2002) and has more recently been a focus of several dedicated reviews (Lim and Mackinnon, 2006; Lim and Suzuki, 2017; Armstrong et al., 2018; Lim, 2018). The broad scientific basis of the GI EHS paradigm centres on the notion that sustained physical exertion with or without heat stress can distrupt the GI barrier, which subsequently enhances the translocation of pathogenic luminal microbial products into the systemic circulation. Compared with other major organs that are resistant to heat cytotoxicity at temperatures < 43°C, the GI tract appears more sensitive to injury at temperatures below this threshold (Chao et al., 1981; Sakaguchi et al., 1995). These luminal products include a variety of microbes and/or their fragments, though most research has focused on endotoxin assessment (Leon and Bouchama, 2015). However, in addition to endotoxin, there are other less-well characterised components of bacteria (e.g. flagellin,

DNA, peptidoglycan, lipoteichoic acid), fungi (e.g. [1→3]-β-D-glucan) and food antigens that also stimulate a potent immune response (Fukui, 2016; Armstrong et al., 2018).

The human body comprises several lines of defence that neutralise and/or sequestrate GI microbes that have translocated into the hosts circulation. The hepatic reticuloendothelial system (RES) is the first line of defence, which detoxifies a large proportion of GI microbes drained from portal blood (Nolan, 1981). In the RES, hepatic macrophages (Kupffer cells) phagocytose GI microbes (Freudenberg et al., 1985), before enzymatic neutralisation (Treon et al., 1993). Once neutralised, hepatocytes uptake the GI microbes (Freudenberg et al., 1985), which subsequently stimulates the secretion of various proteins involving in detoxification. These include: opsonin's (e.g. c-reactive protein, serum amyloid-A), endotoxin signalling regulators (e.g. LBP, sCD14-ST), iron-regulatory proteins (e.g. lipocalin-2, hepcidin), clotting factors and proteinase inhibitors (Zhou et al., 2016). The importance of the RES in counter regulating GI MT, can be demonstrated given that endotoxin concentrations are ~80% greater in portal *versus* systemic blood (Lumsden et al., 1988; Tachiyama et al., 1988). Despite this, the RES has only a limited capacity for microbial neuralisation, before microbes are able to spill-over into the systemic blood (Mathison and Ulevitch, 1979). Alternatively, a second hypothesis suggests that GI microbes may bypass the RES altogether, instead translocating directly into the systemic circulation via the mesenteric lymph nodes (Chakraborty et al., 2010). Once in the systemic circulation, GI microbes are neutralised through multiple host-binding and degradation pathways. These include: natural antibodies (e.g. immunoglobin G and M), leukocyte granular proteins (e.g. bactericidal permeability increasing protein, lactoferrin) and low-density lipoproteins (Munford, 2004). Although poorly characterised, aspects of microbial detoxification have been shown to be suppressed in response to exertional-heat stress. These include: RES dysfunction (Gathiram et al., 1987c), lymphatic cytotoxicity (Sakaguchi et al., 1995) and systemic antimicrobial capacity (Lim and Mackinnon, 2006).

Once present in the systemic circulation, pathogen associated-molecular patterns (PAMP) located on GI microbes will bind to toll-like receptors (TLR) located on leukocyte cell surface membranes. This binding stimulates a potent immune response, which is unique to the PAMP and TLR involved (Munford, 2016). For example, TLR2 detects peptidoglycan and lipoteichoic acid, TLR3 detects double-strand DNA, TLR4 detects endotoxin, and TLR5 detects flagellin (Fukui, 2016). Downstream of TLR-PAMP binding, the inducible transcription factor, nuclear factor kappa-light-chain-enhancer (NF-κB) becomes

activated (Liu and Malik, 2006). Here, NF-κB mediates pro-inflammatory (interleukin [IL] 1-β, IL-2, IL-6, IL-8 and tumour-necrosis factor [TNF]-α, Interferon [IFN]γ) and anti-inflammatory (e.g., IL-1 receptor agonist, IL-4, sIL-6 receptor, IL-10, IL-13, soluble TNF receptor) gene transcription. The threshold of GI MT required to induce a systemic inflammatory response syndrome (SIRS) is poorly characterised, though will be somewhat dependant on the type and/or structure of antigens present (Gnauck et al., 2016). Downstream of the subsequent SIRS response, a complex interplay of interactions culminate in haemorrhagic shock via reduced vascular tone and plasma volume; DIC via endothelial-cell activation; CNS abnormalities via increased intercranial pressure; and finally, multiple organ failure via intracellular leukocyte activation (Leon and Helwig, 2010; Heled et al., 2013). These responses operate through a vicious positive feedback cycle, which promotes further GI MT, cytokine production, and potentially culminates in fatal septic shock (Leon and Helwig, 2010; Epstein and Roberts, 2011).

To date, scientific understanding of the GI EHS paradigm is limited to a small heterogenous body of research (Lim, 2018). This lack of evidence is surprising given that GI MT was first characterised to influence EHS mortality in the 1970s (Graber et al., 1971; Bynum et al., 1979). However, when considering the ethical and methodological challenges faced by scientists in this field, the progress made to date appears more understandable. In humans, the ethical principles outlined in the World Medical Association's *Declaration of Helsinki*, prohibits research where participants health cannot be adequately safeguarded. Based on this, human research has been heavily reliant on opportunistic monitoring of hospitalised EHS patients following self-selected field activities. In animals, the ethical principles outlined in the *Declaration of Helsinki* are more lenient, where scientists are fully compliant with animal welfare regulations. Historically, most animal research has involved rodent models of CHS, which has limited reproducibility to humans. For example, rodents have vastly distinct GI anatomy to humans (e.g. a forestomach; Hugenholtz and de Vos, 2018), whilst it is difficult to separate the influence of experimental stress caused by invasive instrumentation (e.g. rectal thermistors), anaesthesia and/or physical restraint (King et al., 2015). In recent years, a more robust rodent EHS model has been developed, where animals are forced to perform wheel running until a humane T_{core} of 42.5 °C is achieved (King et al., 2015). To minimise intra-species heterogeneity, primates have been used on occasion within CHS models, given that they display similar clinical

manifestations as humans in response to heat stress (Hales et al., 1979; Bouchama et al., 2005).

2.9.2.1 Human Evidence

Pioneering evidence researching the influence of GI barrier integrity on EHS pathophysiology came from pathological reports undertaken in hospitalised EHS patients (Lim, 2018). These early data demonstrated histological GI barrier injury to occur in response to EHS, however given simultaneous injury to other major organs, direct mechanistic causality could not be attributed (Bouchama and Knochel, 2002). For example, in a cohort of 125 fatal EHS cases from the US military during WW2, autopsies revealed petechiae, peptic ulceration and gross haemorrhages across the length of the GI tract (Malamud et al., 1946). Likewise, in 7 fatal EHS cases reported in the Israeli military between 1956-1966, 4 patients displayed extensive small intestinal petechiae, 3 patients displayed small intestinal peptic ulceration and 1 patient displayed duodenal congestion (Shibolet et al., 1967). More recently, small intestinal haemorrhages were reported in autopsies from EHS patients in the Singapore (Chao et al., 1981) and Israeli (Hiss et al., 1994; Rav-Acha et al., 2004) Armed Forces. In 1971, seminal evidence reported severe systemic MT in a case-report of a single fatal EHS patient (Graber et al., 1971). This patient presented with a T_{core} of 43.1 °C on hospital admission, GI bleeding on day 2, and a positive blood culture for *Providencia alcalifaciens* (enteric gram-negative bacteria) on day 4 shortly before a fatal cardiac arrest. This patient had a systemic endotoxin concentration of ~500 $ng{\cdot}ml^{-1}$ and symptomology of septic shock (e.g. phenomena) during autopsy (Graber et al., 1971). Unfortunately, the limited time-course of blood sample assessment and limitations in plasma endotoxin analysis prevented a more detailed interpretation of the sequalae of events before fatality.

More extensive evidence demonstrating systemic GI MT in response to semi-exertional HS, was reported in patients visiting local hospitals during Islamic pilgrimage to Mecca during the 1980s. In one study, analysis of blood samples collected in 17 HS patients (peak T_{core} = 42.1 ± 0.2°C) during the 1989 pilgrimage season, found plasma endotoxin concentration to be ~1000-fold greater (8.6 ± 1.19 $ng{\cdot}ml^{-1}$ *versus* < 0.009 $ng{\cdot}ml^{-1}$) than a healthy control group ($p < 0.01$; Bouchama et al., 1991). Furthermore, plasma endotoxin weakly correlated with the severity of pro-inflammatory cytokinemia in these patients (TNF-α, $r = 0.46$; IL-1α, $r = 0.47$). For sake of illustration, the severity in endotoxemia reported in these HS patients was ~ 25-fold greater than what was reported using an

identical assay in athletes without clinical *sequalae* following an 89.4 km ultra-marathon (pre: 0.08 ng·ml^{-1}; post = 0.294 ng·ml^{-1}; Brock-Utne, 1988) or ultra-triathlon (pre: 0.08 ng·ml^{-1}; post = 0.329 ng·ml^{-1}; Bosenberg et al., 1988). In a follow-up study, analysis of 18 HS patients (peak T_{core} = 41.0 ± 0.2°C) during the 1990 pilgrimage season, revealed plasma IL-6 to correlate (r = 0.52) with EHS severity determined using the Simplified Acute Physiology Score (SAPS) (Bouchama et al., 1993). Likewise, Hasihim et al. (1997) provided more extensive cytokine analysis during the first 24 hours (0, 6, 12 and 24 hours) following hospital admission in 26 HS patients (peak T_{core} = 42.3 ± 0.5°C) during the 1994 Mecca pilgrimage. In this study, plasma IL-6 and soluble TNF receptor (sTNFR) concentrations were more elevated in non-survivors (n = 6) than survivors (n = 20) throughout their stay in intensive care ($p < 0.01$). Finally, in 17 military EHS patients (peak T_{core} = 41.2 ± 1.2°C), IL-2 (r = 0.56), IL-6 (r = 0.57), IFN-γ (r = 0.63) and monocyte chemoattract protein 1 (r = 0.78) all correlated with SAPS severity, though no such association was evident with all other cytokines assayed (IL-1β, IL-2ra IL-4, IL-8, IL-10; TNF-α) (Lu et al., 2004).

A major limitation of human investigation into the GI EHS paradigm, has been the lack of direct evidence examining GI barrier integrity in clinical care patients, instead largely relying on indirect plasma cytokine analysis. To date, only Bouchama et al. (1991) quantified GI MT in their patient cohort, however, they did not go as far as to assess the sensitivity/specificity of endotoxin in predicting disease prognosis. In future, validated biomarkers of GI barrier integrity (e.g. I-FABP, LBP, bacterial DNA) might provide more ample opportunity for extensive examination of GI EHS paradigm. One potentially relevant biomarker that has been applied for this purpose is procalcitonin (PCT), a pro-inflammatory acute phase reactant, which has strong sensitivity/specificity in predicting sepsis prognosis (Wacker et al., 2013). In a retrospective analysis of 68 EHS casualties in the Chinese Military, plasma PCT measured within 2-hours of hospital admission, was able to independently predict mortality (p = 0.02) when examined using a univariate regression model (Tong et al., 2012). In addition, plasma PCT was positively associated with EHS severity measured via the APACHE II inventory (r = 0.59). In support of this finding, Yang et al. (2019) found PCT predicted (odds ratio = 1.02; $p < 0.01$) EHS mortality using univariate regression analysis in 102 EHS patients. In opposition to these findings, comparable plasma PCT concentrations were reported between fatal (0.16-4.71 pg·ml^{-1}) and non-fatal (0.12-1.61 pg·ml^{-1}) CHS cases during a major European heat wave (Hausfater et al., 2008).

2.9.2.2 Animal Evidence

If EHS and sepsis share a common pathway, then treatment strategies aimed at preventing GI MT should improve EHS prognosis, like what had been demonstrated in sepsis patients (Gibson and Walter, 2020a, 2020b). The first study that examined this notion was conducted in sedated dogs, who were passively heated until their T_{core} reached 43.5 ± 0.4 °C (Bynum et al., 1979). In this study, animals receiving prior antibiotic treatment, exhibited lower GI microbial stool concentrations and an improved rate of survival (71% *versus* 20%; $p < 0.01$) compared to controls. This research was followed by a series of CHS (peak T_{core} = ~43.5 °C) studies in primates. The first study in this series, subjected sedated monkeys to a T_{amb} of 41.0 °C (100% RH) until death (Gathiram et al., 1987a). In this model, plasma endotoxin increased linearly with T_{core} until 42.0-43.0 °C, with concentrations sharply increasing until a peak of 0.35 ± 0.2 ng·ml^{-1} at death. To examine the contribution of GI MT on HS prognosis, follow-up studies tested the efficacy of several anti-endotoxin treatments. In the second study, 5-days of antibiotics suppressed basal endotoxin concentration (0.007 ng·ml^{-1}) compared to controls (0.044 ng·ml^{-1}), which also did not increase throughout heat stress (death = 0.005 ng·ml^{-1}), unlike controls who responded similarly to study 1 (Gathiram et al., 1987b). The next study examined clinical prognosis following administration of equine anti-endotoxin hyperimmune plasma prior to CHS. In part 1, the treatment group demonstrated improved survival at a T_{core} of 43.5 °C (6/6 *versus* 1/6 animals; $p < 0.01$), whilst in part 2 animals receiving treatment survived longer ($p < 0.01$; 428 ± 61 minutes) than controls (*versus* 81 ± 34 minutes) during lethal hyperthermia (T_{core} = 44 °C). The final study in this series administered a single corticosteroid dose (30 mg·kg^{-1} Methylprednisolone) prior to CHS (peak T_{core} = 43.5 °C), which again attenuated plasma endotoxin concentration ($p < 0.01$) and improved survival ($p < 0.01$; 6/6 *versus* 2/6 animals) in comparison to controls (Gathiram et al., 1988).

More recently, rodents have largely been used to examine the role of GI MT on HS prognosis (DuBose et al., 1983; Leon et al., 2005, 2006, 2010; Chang et al., 2006; Hsu et al., 2006; Chen et al., 2008). Dexamethasone, a corticosteroid known to blunt GI MT (Auphan et al., 1995), is one treatment that offers opportunity to assess the relationship between GI MT, inflammation, and HS prognosis. In a rat CHS model (T_{core} = 42°C), Lim et al. (2007) found Dexamethasone prevented mortality in 100% of animals, in comparison to a mortality rate of 37.5% in controls ($p < 0.01$). These authors suggested the benefits of Dexamethasone to be primarily attributable to an attenuation of GI MT. Since then, several

studies have shown Dexamethasone treatment before (Liu et al., 2004; Kobayashi et al., 2018) or immediately following (Yang et al., 2010, 2016; Liu et al., 2004, 2014; Xia et al., 2017) CHS (T_{core} = 42.5-43°C) to dramatically improve survival rate, prolong survival duration and/or reduced organ injury in a dose-dependent manner (4-10 mg·kg^{-1}). The most detailed of these studies was conducted by Xia et al. (2017), who found Dexamethasone to simultaneously strengthen histological GI barrier integrity and reduce GI permeability (plasma endotoxin, D-lactate) during CHS. Alternative pharmacological pre-treatments, such as Ulinastatin (a multivalent enzyme inhibitor; Ji et al., 2017) and recombinant IL-6 (Phillips et al., 2015) also appear to protect GI barrier integrity (e.g. endotoxin, DAO, histology) during CHS, but their influence on disease prognosis is yet to be examined.

In comparison to the positive effects of Dexamethasone on GI barrier integrity, non-steroidal anti-inflammatory drugs (NSAIDs; e.g. ibuprofen, indomethacin) disturb GI barrier integrity (Bjarnason and Hayllar, 1993). In a recent study, treatment with high dose (5 mg·kg^{-1}) indomethacin prior to CHS (T_{core} = 42.4°C) in mice, dramatically increased gross morphological GI haemorrhage compared to controls (Audet et al., 2017). Furthermore, the mortality rate in this study was 45% indomethacin treatment, in comparison to 0% of controls. Relevantly, indomethacin did not exacerbate hepatic injury (ALT, AST), with these authors suggesting that the increased mortality with treatment to be most likely attributable to increased GI MT. Earlier sublethal (T_{core} = 41.5°C) CHS research on rats supports this finding, whereby indomethacin (6 and 12 mg·kg^{-1}) unexpectedly caused dose-dependent fatalities (9% and 20% of cases), during 1-hour of routine physiological monitoring (Caputa et al., 2000). In support, direct endotoxin injection during recent sub-lethal CHS models in mice had a comparably determinantal influence on pro-inflammatory cytokines and multi-organ injury (Lin et al., 2009).

Contrasting to the extensive body of literature examining CHS in rodents, research applying EHS models is far less extensive to date. Early studies in this field lead to the generation of T_{core} dose-response curves, where it was concluded that the threshold for fatality was *circa* 40.5 °C (Hubbard et al., 1977; 1978; 1979a). These authors suggested splanchnic perfusion, metabolic stress, and enhanced organ injury, to all contribute to a reduction in thermal tolerance in comparison with CHS models (Hubbard et al., 1979b). After a near four-decade gap in the literature, this research team published an updated EHS mouse model in 2015, which found animals consistently experienced symptom-limited

maximal T_{core}'s of 42.1-42.5 °C irrespective of T_{amb} (King et al., 2015). In their initial study, King et al. (2015) performed histopathological assessment on organ tissue collected from animals sacrificed post-EHS, revealing extensive damage to the small intestine 30-minutes after collapse. For the first time, plasma I-FABP concentrations were simultaneously monitored to assess GI epithelial injury, which showed a > 12-fold (~11 ng·ml^{-1}) and > 22-fold increase in concentration following EHS (peak T_{core} = 42.1°C) and CHS (peak T_{core} = 42.4°C), respectively. Whilst the peak T_{core} response was identical between the two forms of HS, a ~200% greater thermal area (409 ± 71°C·min^{-1} *versus* 144 ± 22°C·min^{-1}) with CHS likely explains the increased rate of epithelial injury in this group ($p < 0.01$). In this EHS model, both the relative (2-4 fold) and absolute (0.5-5 ng·ml^{-1}) increase in I-FABP concentration are significantly greater than those reported during sub-clinical (e.g. peak T_{core} 39-39.5°C) exertional-heat stress in humans (Pires et al., 2017). To date, only one investigation has attempted to determine the influence of manipulating GI barrier integrity on EHS prognosis, however, this study found no additional influence of ibuprofen on histological GI barrier injury and survival comparison to EHS alone (Garcia et al., 2020).

2.10 Exertional-Heat Stress and GI Barrier Integrity

Given the hypothesised relevance of GI barrier integrity within the pathophysiology of EHS, the impact of sub-clinical exertional-heat strain (T_{core} < 39.5°C) on GI barrier integrity in humans has been a specific topic of investigation (Table 7, 8 and 9). This research has primarily involved assessment of small intestinal permeability using the DSAT, though attempts have been made to quantify gastroduodenal and large intestinal permeability using the MSAT (Costa et al., 2017). Over the last decade, the increasing availability of passive GI and/or MT biomarkers has permitted more extensive investigation of GI barrier integrity, whilst overcomes some of the analytical limitations of traditional GI permeability assessment (van Wijck et al., 2012c). In most situations, I-FABP has been monitored to assess small intestinal epithelial integrity, and endotoxin to assess GI MT. The exercise models assessed are disparate, ranging from 45-minutes brisk walking (Nieman et al. 2018) to a multiday desert ultramarathon (Gill et al., 2015b).

2.10.1 Gastrointestinal Permeability

The seminal study to assess the influence of exercise on GI barrier integrity was undertaken by Ryan et al. (1996), who monitored small intestinal permeability using the urine DSAT in response to 1-hour of moderate intensity (69% $\dot{V}O_{2max}$) treadmill running in

temperate conditions (T_{amb} = 21°C). This exercise protocol evocated moderate whole-body physiological strain (e.g. peak T_{core} = 38.7 ± 0.3°C), however, did not significantly ($p > 0.05$) elevate the L/M ratio (0.029 ± 0.012) above rest (0.012 ± 0.004). In a follow-up study conducted by this group, the influence of exercise-intensity (40%, 60%, 80% $\dot{V}O_{2max}$) on urine DSAT responses was compared during 1-hour of treadmill running (Pals et al., 1997). As hypothesised, small intestinal permeability was increased in an intensity-dependant manner (Table 7), whilst this response was positively associated with the peak T_{core} attained (r = 0.48; p = 0.03). Later studies monitoring GI barrier integrity following exercise in temperate conditions corroborated earlier research, with low-to-moderate intensity (40-60% $\dot{V}O_{2max}$) exercise having little influence on DSAT results when compared to rest (e.g., van Nieuwenhoven et al., 2000; JanssenDuijghuijsen et al., 2017a); whereas moderate-to-high intensity (~70-120% $\dot{V}O_{2max}$) exercise of durations ≥ 20 minutes increases permeability by 100-250% (e.g., Zuhl et al., 2015; Davison et al., 2016). Unfortunately, more specific conclusions cannot be drawn when accumulating all available evidence, given large intra-study variability in absolute DSAT ratios, attributable to modifications in the DSAT procedure and/or a frequent lack of basal permeability correction (Table 7).

Despite large intra-study variability in DSAT implementation, various studies have highlighted the importance of specific aspects of the exercise stimulus on GI permeability. For example, increased DSAT ratios were demonstrated following: running *versus* cycling (van Nieuwenhoven et al., 2004); permissive dehydration *versus* rehydration (van Nieuwenhoven et al., 2000a; Lambert et al., 2008); and following ingestion of NSAIDs (Ryan et al., 1996; Lambert et al., 2001, 2007; van Wijck et al., 2012b). In comparison, biological sex (Snipe and Costa, 2018a) does not influence GI permeability during exercise, whilst other co-variates such as aerobic fitness, biological age, heat-acclimation status, and GI microbiota composition have not been examined in this setting. To date, only two published studies have directly compared the influence of T_{amb} on GI barrier permeability (Snipe et al., 2018a, 2018b). In conflict with the *a priori* hypothesis, 2-hours of moderate intensity (60% $\dot{V}O_{2max}$) treadmill running in temperate (22 °C/44 % RH) *versus* mild hyperthermia (30 °C/35 % RH) caused comparable GI permeability (0.025 ± 0.010 vs. 0.026 ± 0.008) (Snipe et al., 2018a). However, these results are potentially explained by a similar T_{core} response between the supposed disperse trials (e.g., peak T_{core} = 38.1 °C *versus* 38.4 °C). A follow-up trial on the same subjects compared the results of the temperate exercise condition (22 °C/44 % RH) to a third trial conduced in a more severe hyperthermia (35

°C/26% RH) (Snipe et al., 2018b). The DSAT data (0.032 ± 0.010) remained statistically indifferent from the temperate condition, despite a greater elevation in T_{core} (e.g. peak T_{core} = 39.6 °C).

In comparison to literature examining small intestinal permeability using the DSAT, few studies have assessed either gastroduodenal or large intestinal permeability responses to exercise (Costa et al., 2017). The only published evidence applying the MSAT around exercise was conducted by van Wijck et al. (2011a, 2012b). In the first of their studies, large intestinal permeability measured using the 5-24 hour urine S/E test was unchanged (basal = 0.012 ± 0.003; post-exercise = 0.010 ± 0.003) following 1 hour cycling at 70% peak power output in temperate conditions (~22°C) (van Wijck et al., 2011a). For sake of comparison, the 5-hour urine L/R ratio was also unchanged following this exercise protocol (van Wijck et al., 2011a). In a follow-up study that applied an identical experimental protocol, neither gastroduodenal (2-hour S/R ratio) or small intestinal (2-hour L/R ratio) permeability were influenced by exercise. Given the mild stress response evoked by these exercise protocols, it was not possible to determine whether exercise induced GI barrier integrity loss is consistent across GI subregions. In comparison, earlier studies have shown gastroduodenal permeability measured using a single sugar-probe (sucrose) test to be unaltered from rest in response to exercise where small intestinal permeability was simultaneously increased (Pals et al., 1997; Lambert et al., 2001, 2007). No additional studies have measured large intestinal integrity in response to acute exercise using a single sugar-probe (sucralose), however, 2 studies reported large intestinal permeability to increase at rest following several days arduous military training in the Norwegian (Karl et al., 2017) and Singaporean (Li et al., 2013) Armed Forces. Investigation of large intestinal permeability warrants future investigation within the context of the GI-EHS paradigm given that microbial biomass is several magnitudes greater in this segment of the GI tract (e.g., duodenum = $<10^3$, ilium 10^3–10^7, colon = 10^{12}–10^{14}) (Thursby and Juge, 2017).

Table 7. Influence of acute exercise-(heat) stress on small-intestine DSAT responses.

Author	Subjects	Exercise Protocol	Peak T_{core} (°C)	Biofluid, L/R or L/M (timepoint)
van Nieuwenh-oven et al. (1999)	10 male (MT)	90 minutes cycling at 70% $Watt_{max}$ (fasted) in T_{amb} 19 °C (RH = N/A)	-	Urine L/R (5hr): 0.007 [s]
van Nieuwenh-oven et al. (2000a)	10 male (MT)	90 minutes cycling at 70% $Watt_{max}$ (fasted) in T_{amb} 19 °C (RH = N/A)	38.8	Urine L/R (5hr): 0.008 [nb, c]
van Nieuwenh-oven et al. (2000b)	10 male (MT)	90 minutes cycling at 70% $Watt_{max}$ (fasted) in T_{amb} 19 °C (RH = N/A)	-	Urine L/R (5hr): 0.009 [nb, c]
Nieman et al. (2018)	20 male (UT)	45 minutes walking uphill (5% grade) at 60% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	-	Urine L/R (5hr): 0.009 [nb, c]
Smetanka et al. (1999)	8 male (HT)	Chicago marathon (42.2 km) in T_{amb} (fed) 22 °C (48% RH)	-	Urine L/R (5hr): 0.020 [ns]
Shing et al. (2014)	10 male (HT)	~33 minutes running to fatigue at 80% VE (fed) in T_{amb} 35 °C (40% RH)	39.4	Urine L/R (5hr): 0.022 [nb, c]
Janssen-Duijghuijsen et al. (2017a)	11 male (HT)	90 minutes cycling at 50% $watt_{max}$ (fed) in T_{amb} not reported following a *sleep-low* glycogen depletion regime	-	Urine L/R (5hr): ~0.022 [ns] Plasma L/R (1hr): ~0.110 [s]
Snipe et al. (Part A) (2018a, b)	6 male, 4 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 22 °C (44% RH)	38.5	Urine L/R (5hr): 0.025 [nb]
Snipe et al. (Part B) (2018b)	6 male, 4 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 30 °C (25% RH)	38.6	Urine L/R (5hr): 0.026 [nb]
van Wijck et al. (2014)	10 male (MT)	60 minutes cycling at 70% $watt_{max}$ (fasted) in T_{amb} not reported	-	Urine L/R (2hr): 0.027 [nb,c]
Snipe and Costa (Part A) (2018a)	13 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (25% RH)	38.8	Urine L/R (5hr): 0.028 [nb]

Ryan et al. (1996)	7 males (MT)	60 minutes running at 69% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	-	Urine L/M (6hr): 0.029 [ns, c]
van Nieuwenh-oven et al. (2004)	9 male, 1 female (MT)	90 minutes cycling at 70% $Watt_{max}$ (fasted) in T_{amb} 19 °C (RH = N/A)	-	Urine L/R (5hr): 0.030 [ns]
van Wijck et al. (2012b)	9 male (MT)	60 minutes cycling at 70% $watt_{max}$ (fasted) in T_{amb} not reported	-	Urine L/R (2hr): 0.030 [s, c]
Pugh et al. (2017a)	11 male (MT-HT)	18x 400 metre sprint at 120% $\dot{V}O_{2max}$ (fed) in T_{amb} not reported	-	Urine L/R (2hr): 0.030 [ns] Serum L/R (2hr): ~0.051 [s]
Snipe and Costa (Part B) (2018a)	11 male (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (25% RH)	39.1	Urine L/R (5hr): 0.030 [nb]
Buchman et al. (1999a)	17 male, 2 female	Competitive Marathon (fed) in T_{amb} 2 °C with freezing rain	-	Urine L/R (6hr): 0.030 [ns, c]
Snipe et al. (Part B) (2018a)	6 male, 4 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35°C (26% RH)	39.6	Urine L/R (5hr): 0.032 [nb]
Snipe et al. (2017)	6 male, 5 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (30% RH)	39.3	Urine L/R (5hr): 0.034 [nb, c]
March et al. (2017)	9 male (MT)	20 minutes running at 80% $\dot{V}O_{2peak}$ (fasted) in T_{amb} 22 °C (37% RH)	38.4	Urine L/R (5hr): 0.035 [s, c]
Pals et al. (Part A) (1997)	5 male, 1 female (MT)	60 minutes running at 40% $\dot{V}O_{2peak}$ (fasted) in T_{amb} 22 °C (50% RH)	38.0	Urine L/R (5hr): 0.036 [ns]
Flood et al. (2020)	7 male, 7 female (MT)	90 minutes cycling at 45% $\dot{V}O_{2max}$ (fasted), then 15 minute time trial in T_{amb} 32 °C (70% RH)	39.1	Urine L/R (5hr): 0.038 [nb, c]
Marchbank et al. (2010)	12 male (MT)	20 minutes running at 80% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	38.3	Urine L/R (5hr): 0.038 [s, c]
van Nieuwenh-	10 male (MT)	90 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 19 °C (RH = N/A)	-	Urine L/R (5hr): 0.040 [s]

oven et al. (2000a)				
van Wijck et al. (2011a)	6 male (HT)	60 minutes cycling at 70% $watt_{max}$ (fasted) in T_{amb} not reported	-	Urine L/R (5hr): 0.040 [ns] Plasma L/R (2.4hr): 0.060 [s]
Lambert et al. (Part A) (2008)	11 male, 9 female (MT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 22 °C (48% RH)	38.5	Urine L/R (5hr): 0.049 [ns, c]
Lambert et al. (2001)	13 male, 4 female (HT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 22 °C (48% RH)	38.3	Urine L/R (5hr): 0.050 [nb, c]
Pugh et al. (2020)	7 male (MT)	120 minutes cycling at 55% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	-	Plasma L/R (1.5hr): 0.052 [nb, c]
Zuhl et al. (2014)	4 male, 3 female (LT/MT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 30 °C (12-20% RH)	39.4	Urine L/R (5hr): 0.060 [s, c]
Zuhl et al. (2015)	2 male, 5 female (LT/MT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 30 °C (12-20% RH)	39.5	Urine L/R (5hr): 0.060 [s, c]
Lambert et al. (Part B) (2008)	11 male, 9 female (MT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 22 °C (48% RH) without fluid ingestion	38.5	Urine L/R (5hr): 0.063 [s, c]
Pals et al. (Part B) (1997)	5 male, 1 female (MT)	60 minutes running at 60% $\dot{V}O_{2peak}$ (fasted) in T_{amb} 22 °C (50% RH)	38.7	Urine L/R (5hr): 0.064 [ns]
Lambert et al. (2007)	8 male (MT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 22 °C (48% RH)	38.3	Urine L/R (5hr): 0.065 [nb, c]
Davison et al. (2016)	8 male (MT/HT)	20 minutes running to fatigue at 80% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	39.3	Urine L/R (5hr): 0.065 [s, c]
Buchman et al. (1999b)	15 male, 8 female (LT-HT)	Road marathon (42.2 km) (fed) in T_{amb} not reported	-	Urine L/M (6hr): 0.070 [ns, c]
Pugh et al. (2019)	10 male, 2 female (MT)	42.4 km track marathon (247 ± 47 minutes; fed) in T_{amb} 16-17 °C (N/A RH)	-	Serum L/R (1hr) 0.081 (37%) [s, c]

Lambert et al. (Part A) (1999)	12 female (LT-HT)	Hawaii Ironman (fed) in T_{amb} not reported	-	Urine L/R (5hr): 0.087 [nb]
Pugh et al. (2017b)	10 male (MT)	60 minutes at 70% $\dot{V}O_{2max}$ running (fasted) in T_{amb} 30 °C (40-45% RH)	38.5	Serum L/R (1.5hr): ~0.090 [s, c]
Janssen-Duijghuijsen et al. (2017b)	4 male,6 female (LT)	60 minutes cycling at 70% $watt_{max}$ (fed) in T_{amb} not reported	-	Plasma L/R (1hr): ~0.100 [s]
Lambert et al. (Part B) (1999)	29 male (LT-HT)	Hawaii Ironman (fed) in T_{amb} not reported	-	Urine L/R (5hr): 0.105 [nb]
Pals et al. (Part C) (1997)	5 male, 1 female (MT)	60 minutes running at 80% $\dot{V}O_{2peak}$ (fasted) in T_{amb} 22 °C (50% RH)	39.6	Urine L/R (5hr): 0.107 [s]

LT = Low-trained (35-49 ml·kg·min^{-1} $\dot{V}O_{2max}$); MT = Moderate-trained (50-59 ml·kg·min^{-1} $\dot{V}O_{2max}$); HT = High-trained (60+ ml·kg·min^{-1} $\dot{V}O_{2max}$). s = significant change post-exercise ($p < 0.05$); ns = non-significant change post-exercise ($p > 0.05$); nb = no basal data; c = control/placebo trial of study.

2.10.2 Small Intestinal Epithelial Injury

Introduction of I-FABP as a biomarker of small intestinal epithelial injury in exercise settings was reported in a series of studies conducted by van Wijck et al. (2011a, 2012b). These studies found plasma I-FABP concentration to increase throughout 1-hour of moderate-intensity (70% $Watt_{max}$) cycling in temperate conditions. The peak concentration was ~2-fold (Δ 200 pg·ml^{-1}) greater than basal concentrations at exercise termination, before gradually recovering over the subsequent 1-2 hours. Relevantly, I-FABP responses weakly correlated with I-BABP (i.e., ileum injury) and the DSAT (van Wijck et al., 2011a). Since these early findings, many studies (Table 8) have shown low intensity exercise (~50% $\dot{V}O_{2max}$) performed in temperate environments to have little effect on I-FABP concentrations (e.g. Trommelen et al., 2017), but moderate-to-high intensity exercise (60–120% $\dot{V}O_{2max}$) elevates concentrations by 50–250% (e.g.; Jonvik et al., 2019). Like the DSAT, various individual studies have been able to highlight the importance of specific aspects of the exercise stimulus on small intestinal epithelial injury. For example: increased I-FABP concentrations have been demonstrated in response to matched intensity/duration exercise interventions comparing: hypoxia (F_iO_2 = 0.14) *versus* normoxia (Lee and Thake, 2017; Hill et al., 2019); permissive dehydration *versus* rehydration (Costa et al., 2019; Kartaram et al., 2019); high *versus* low aerobic fitness (Morrison et al., 2014) and post NSAID ingestion (van Wijck et al., 2012b). In comparison, biological sex (Snipe and Costa, 2018a) does not influence small intestinal epithelial injury during exercise. As a biomarker

of small intestinal epithelial function, plasma citrulline concentrations increased with a standard amino-acid bolus following 60 minutes low-intensity (50% $watt_{max}$) cycling, however, were unchanged following 60 minutes moderate intensity (70% $watt_{max}$) cycling with permissive dehydration (Kartaram et al., 2019).

Several studies have attempted to characterise the influence of T_{amb} on GI epithelial injury (Snipe et al., 2018a, 2018b; Osborne et al., 2019a). In response to 2-hours moderate intensity (60% $\dot{V}O_{2max}$) cycling in temperate conditions (22 °C/44% RH; peak T_{core} 38.1 °C) where I-FABP increased by 127%, matched exercise in both mild (30 °C/35% RH) and severe (35 °C/26% RH) hyperthermia (Snipe et al., 2018a, 2018b) triggered a greater increase in I-FABP, by 184% and 432%, respectively ($p < 0.01$). Furthermore, a moderate correlation (r = 0.63; $p < 0.01$) was shown between peak T_{core} and I-FABP concentration in this study. These findings were substantiated by Osborne et al. (2019a) who found plasma I-FABP concentration to increase by 140% immediately-following 1-hour mixed-intensity (50 and 70% $watt_{max}$ 2-minute splits) cycling in the heat (35 °C/53% RH), but was unchanged when performed in temperate (20 °C/55% RH) conditions.

Table 8. Influence of acute exercise-(heat) stress on systemic I-FABP concentrations.

Author	Subjects	Exercise Protocol	Peak T_{core} (°C)	I-FABP Δ pre-to-post exercise (%)
Pugh et al. (2020)	7 male (MT)	120 minutes cycling at 55% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	-	-350 pg·ml^{-1} (~-47%) [ns, c]
Janssen-Duijghuijsen et al. (2017a)	11 male (HT)	90 minutes cycling at 50% $watt_{max}$ (fed) in T_{amb} not reported following a *sleep-low* glycogen depletion regime	-	~-90 pg·ml^{-1} (~-65%) [c]
Kartaram et al. (Part A) (2019)	15 male (MT)	60 minutes cycling at 50% $watt_{max}$ (fed) in T_{amb} not reported	-	~-50 pg·ml^{-1} (~-10%) [ns]
Lee and Thake (Part A) (2017)	7 male (MT)	60 minutes cycling at 50% $\dot{V}O_{2max}$ (fed) in T_{amb} 18 °C (35% RH)	37.9	28 pg·ml^{-1} (8%) [ns,c]
Trommelen et al. (2017)	10 male (HT)	180 minutes cycling at 50% $watt_{max}$ (fasted) in T_{amb} 18-22 °C (55-65% RH)	-	N/A pg·ml^{-1} (20%) [ns,c]

Edinburgh et al. (Part A) (2018)	12 male (MT)	60 minutes cycling at 50% $\dot{V}O_{2max}$ (fed) in T_{amb} 18 °C (35% RH)	-	70 pg·ml^{-1} (34%) ^{s}
Sessions et al. (2016)	5 male, 2 female (HT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 30 °C (12-20% RH)	39.5	88 pg·ml^{-1} (35%) $^{ns, c}$
Edinburgh et al. (Part B) (2018)	12 male (MT)	60 minutes cycling at 50% $\dot{V}O_{2max}$ (fasted) in T_{amb} 18 °C (35% RH)	-	88 pg·ml^{-1} (20%) ^{s}
Osborne et al. (Part A) (2019a)	8 male (MT-HT)	30 minutes cycling at 50/70% $Watt_{max}$, then 30 minutes at 50% $watt_{max}$ (fasted) in T_{amb} 20 °C (55% RH)	38.5	138 pg·ml^{-1} (29%) ^{ns}
Salvador et al. (2019)	12 male (MT-HT)	120 minutes cycling at 60% $\dot{V}O_{2max}$ (fed) then 30-40 minutes (20 km) time trial in T_{amb} not reported	37.9	N/A pg·ml^{-1} (~50%) $^{s, c}$
van Wijck et al. (2014)	10 male (MT)	60 minutes cycling at 70% $watt_{max}$ (fasted) in T_{amb} not reported	-	153 pg·ml^{-1} (72%) ^{s}
Nava et al. (2019)	7 male, 4 female (LT-MT)	56 minutes mixed intensity (~55% $\dot{V}O_{2max}$) firefighting exercises (fed) in T_{amb} 38 °C (35% RH)	38.7	~160 pg·ml^{-1} (46%) $^{ns, c}$
Mooren et al. (2020)	20 male (LT)	10 minutes 60% $\dot{V}O_{2max}$, 25 minutes 70% $\dot{V}O_{2max}$ and 25 minutes 80% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	-	176 pg·ml^{-1} (23%) $^{s, c}$
van Wijck et al. (2012b)	9 male (MT)	60 minutes cycling at 70% $watt_{max}$ (fasted) in T_{amb} not reported	-	179 pg·ml^{-1} (61%) ^{s}
Lee and Thake (Part C) (2017)	7 male (MT)	60 minutes cycling at 50% $\dot{V}O_{2max}$ (fed) in T_{amb} 18 °C (35% RH) and F_iO_2 = 0.14%	38.2	193 pg·ml^{-1} (43%) ^{s,c}
Lis et al. (2015)	8 male, 5 female (MT)	45 minutes cycling at 70% $watt_{max}$ and 15 min cycling time trial (fed) in 20 °C (40% RH)	-	210 pg·ml^{-1} (223%) ^{s,c}
Pugh et al. (2017b)	10 male (MT)	60 minutes at 70% $\dot{V}O_{2max}$ running (fasted) in T_{amb} 30 °C (4-45% RH)	38.5	250 pg·ml^{-1} (71%) ^{s,c}
Snipe et al. (Part A) (2018a)	6 male, 4 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 22 °C (44% RH)	38.5	274 pg·ml^{-1} (127%) ^{s}
Sheahen et al. (Part A) (2018)	12 male (MT)	45 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 20 °C (40% RH)	38.2	281 pg·ml^{-1} (49%) ^{s}

Lee and Thake (Part B) (2017)	7 male (MT)	60 minutes cycling at 50% $\dot{V}O_{2max}$ (fed) in T_{amb} 40 °C (25% RH)	38.7	282 pg·ml^{-1} (76%) ^{s,c}
Morrison et al. (Part B) (2014)	8 male (UT)	30 minutes cycling at 50% heart rate reserve (HRR), 30 minutes jogging at 80% HRR and 30-minute running time trial (fed) in T_{amb} 30°C (50% RH)	38.6	283 pg·ml^{-1} (276%) ^{s,c}
Barberio et al. (2015)	9 male (MT)	~24 minutes running at 78% $\dot{V}O_{2max}$ (fed) in T_{amb} 40 °C (40% RH)	39.0	297 pg·ml^{-1} (46%) ^{s,c}
Hill et al. (2019)	10 male (MT)	60 minutes running at 65% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	-	300 pg·ml^{-1} (50%) ^{ns,c}
van Wijck et al. (2011a)	15 male (HT)	60 minutes cycling at 70% watt$_{max}$ (fasted) in T_{amb} not reported	-	306 pg·ml^{-1} (61%) ^{s}
Kashima et al. (2017)	5 male, 3 female (MT)	30 intermittent 20 second cycle sprints at 120% watt$_{max}$, with 40 seconds recovery between each (fed) in T_{amb} 23 °C (40% RH)	-	343 pg·ml^{-1} (266%) ^{s}
Pugh et al. (2017a)	11 male (MT-HT)	18x 400 metre sprint at 120% $\dot{V}O_{2max}$ (fed) in T_{amb} not reported	-	348 pg·ml^{-1} (72%) ^{s}
March et al. (2017)	9 male (MT)	20 minutes running at 80% $\dot{V}O_{2peak}$ (fasted) in T_{amb} 22 °C (37% RH)	38.4	350 pg·ml^{-1} (61%) ^{s,c}
Janssen-Duijghuijsen et al. (2017b)	4 male, 6 female (LT)	60 minutes cycling at 70% watt$_{max}$ (fed) in T_{amb} not reported	N/A	~350 pg·ml^{-1} (~77%) ^{s,c}
Sheahen et al. (Part B) (2018)	12 male (MT)	45 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 30 °C (40% RH)	38.3	369 pg·ml^{-1} (63%) ^{s}
Costa et al. (2019)	11 male (MT-HT)	120 minutes running at 70% $\dot{V}O_{2max}$ (fed) in T_{amb} 25 °C (35% RH)	-	371 pg·ml^{-1} (86%) ^{ns,c}
Osborne et al. (2019b)	12 male (MT-HT)	33 minutes (20 km) cycling time trial (fasted) in 35 °C (50% RH)	39	441 pg·ml^{-1} (83%) ^{s,c}
Costa et al. (2020a)	11 male (MT-HT)	120 minutes running at 70% $\dot{V}O_{2max}$ (fed) in T_{amb} 25 °C (43% RH)	-	470 pg·ml^{-1} (120%) ^{s,c}
Kartaram et al. (Part B) (2019)	15 male (MT)	60 minutes cycling at 70% watt$_{max}$ (fed) in T_{amb} not reported	-	~500 pg·ml^{-1} (~66%) ^{s}

Kartaram et al. (Part C) (2019)	15 male (MT)	60 minutes cycling at 85/55% $watt_{max}$ (fed) in T_{amb} not reported	-	~500 pg·ml^{-1} (~66%) [s]
McKenna et al. (2017)	10 male (MT)	46 minutes running at 95% ventilatory exchange threshold (fasted) in T_{amb} 40 °C (50% RH)	39.7	516 pg·ml^{-1} (52%) [s,c]
Karhu et al. (2017)	17 male (MT-HT)	90 minutes running at 80% of best 10 km race time (fed) in T_{amb} not reported	-	531 pg·ml^{-1} (151%) [s]
Snipe and Costa (2018b)	6 male, 6 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 30 °C (35% RH)	38.8	573 pg·ml^{-1} (184%) [s,c]
Snipe et al. (Part B) (2018a)	6 male, 4 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 30 °C (25% RH)	38.6	~ 580 pg·ml^{-1} (184%)
Hill et al. (2019)	10 male (MT)	60 minutes running at 65% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported (F_iO_2 = 13.5%)	N/A	700 pg·ml^{-1} (168%) [ns,c]
Osborne et al. (Part B) (2019a)	8 Male (MT-HT)	30 minutes cycling at 50/70% $Watt_{max}$, then 30 minutes at 50% $watt_{max}$ (fasted) in T_{amb} 35 °C (53% RH)	39.5	608 pg·ml^{-1} (140%) [s]
Szymanski et al. (2017)	6 male, 2 female (LT/MT)	60 minutes running at 68% $\dot{V}O_{2max}$ (fasted) in T_{amb} 37 °C (25% RH)	39.0	800 pg·ml^{-1} (87%) [s,c]
Morrison et al. (Part A) (2014)	7 male (HT)	30 minutes cycling at 50% heart rate reserve (HRR), 30 minutes jogging at 80% HRR and 30 minute running time trial (fed) in T_{amb} 30 °C (50% RH)	38.6	806 pg·ml^{-1} (663%) [s,c]
Snipe et al. (2017)	6 male, 5 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (30% RH)	39.3	897 pg·ml^{-1} (288%) [s,c]
Snipe et al. (Part B) (2018b)	6 male, 4 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (26% RH)	39.6	1230 pg·ml^{-1} (432%) [s]
Pugh et al. (2019)	10 male, 2 female (MT)	42.4 km track marathon (247 ± 47 minutes; fed) in T_{amb} 16-17 °C (RH not reported)	-	1246 pg·ml^{-1} (371%) [s, c]

March et al. (2019)	12 male (MT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 30 °C (60% RH)	39.3	1263 pg·ml^{-1} (407%) $^{s, c}$
Snipe and Costa (Part A) (2018a)	11 male (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (25% RH)	39.1	1389 pg·ml^{-1} (479%) ^{s}
Snipe and Costa (Part B) (2018a)	13 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (25% RH)	38.8	1445 pg·ml^{-1} (479%) ^{s}
Jonvik et al. (2019)	16 male (HT)	60 minutes cycling at 70% watt$_{max}$ (fasted) in T_{amb} not reported	-	1745 pg·ml^{-1} (249%) ^{s}
Gaskell et al. (2019b)	10 male, 8 female (MT-HT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (25% RH)	38.6	1805 pg·ml^{-1} (710%) $^{s, c}$

LT = Low-trained (35-49 ml·kg·min^{-1} $\dot{V}O_{2max}$); MT = Moderate-trained (50-59 ml·kg·min^{-1} $\dot{V}O_{2max}$); HT = High-trained (60+ ml·kg·min^{-1} $\dot{V}O_{2max}$). s = significant change post-exercise ($p < 0.05$); ns = non-significant change post-exercise ($p > 0.05$); c = control/placebo trial of study.

2.10.3 Microbial Translocation

Endotoxin is the most frequently applied biomarker to examine GI MT in exercise settings (Table 9). Seminal research reported plasma endotoxin concentration to increase by a comparable magnitude to hospitalised patients with sepsis (~50-500 pg·ml^{-1}) when measured following competitive ultra-endurance exercise. These events included an: ultra-triathlon (Bosenberg et al., 1988), 90 km ultra-marathon (Brocke-Utne et al., 1988) and 100-mile cycle race (Moore et al., 1995). In contrast to these seminal findings, more modest increases in plasma endotoxin concentration (< 20 pg·ml^{-1}) were reported by follow-up studies involving comparable duration ultra-endurance competitions (Jeukendraup et al., 2000; Gill et al., 2015a, 2015b). These discrepant findings are most likely attributable to analytical inconsistencies within the LAL assay, most notably including biochemical cross-reactivity for β-D-glucan. In laboratory settings, moderate intensity (50-70% $\dot{V}O_{2max}$) exercise of ≤ 2 hours duration performed in a temperate environment generally does not influence endotoxin concentration (e.g. Yeh et al., 2013; Zuhl et al., 2015; Karhu et al., 2017). Similar responses are reported when LBP (Moncada-Jimenez et al., 2009; Jonvik et al., 2017) and sCD14-ST (Costa et al., 2019, 2020a) are applied as indirect biomarkers of endotoxemia during this form of exercise. Indeed, such is the poor sensitivity of GI MT analysis in systemic blood, few studies have attempted to determine the influence of specific aspects of the exercise stimulus on GI MT. Together, accumulation of all available data (Table 9) suggests that a presently undefined threshold of GI barrier integrity loss is

required to induce GI MT in response to exercise, given that GI MT frequently unmeasured despite concurrent rises in DSAT or I-FABP concentrations (Zuhl et al., 2015; Karhu et al., 2017; Snipe et al., 2018a).

Several investigations have attempted to characterise the influence of heat stress on GI MT (Selkirk et al., 2008; Yeh et al., 2013; Snipe et al., 2018a, 2018b). Applying an eloquent research design, Selkirk et al. (2008) assessed plasma endotoxin and LBP concentrations at 0.5 °C increments in T_{core} during *uncompensable* (40 °C/30% RH) treadmill walking (4 km·h^{-1}) whilst wearing CBRN personal protective equipment. In this study, participants displayed progressively elevated GI MT at every T_{core} increment examined until fatigue. A subsequent study by Yeh et al. (2013) supported this finding, demonstrating a 54% increase in plasma endotoxin concentration in response to 1-hour of moderate intensity treadmill running (70% $\dot{V}O_{2max}$) in the heat (33 °C/50% RH), but was reduced by 10% during matched-intensity exercise performed in a temperate environment (22 °C/ 62% RH). In studies conducted by Snipe et al. (2018a, 2018b), who reported I-FABP concentrations to increase by 127%, 184% and 432% following 2-hours moderate intensity (60% $\dot{V}O_{2max}$) cycling in a 22 °C (44% RH), 30 °C (35% RH) and 35 °C (26% RH) T_{amb} respectively, endotoxin concentration only increased marginally (11%) in the 35 °C environment. This finding corroborates similar studies where endotoxin (Gill et al., 2016) and sCD14-ST (Costa et al., 2019, 2020a) concentrations were unchanged during 2-hours moderate intensity (60% $\dot{V}O_{2max}$) cycling in T_{amb}'s ≤ 30°C. Numerous other studies have measured GI MT following exertional-heat stress, however, large inconsistencies in research design make it difficult to make precise recommendations regarding the typical magnitude of GI MT to be anticipated in response to exertional-heat stress (Table 9). In studies where GI MT is measurable, systemic concentrations generally peak immediately following exercise termination (Lim et al., 2009; Yeh et al., 2013; Barberio et al, 2015; March et al., 2019).

Table 9. Influence of acute exercise-(heat) stress on systemic gastrointestinal microbial translocation responses.

Author	Subjects	Exercise Protocol	Peak T_{core} (°C)	Endotoxin Δ pre-to-post exercise (%)
Antunes et al. (Part A) (2019)	19 male (MT)	56 ± 7 minutes cycling at 90% of first ventilatory threshold (fasted) in 22.1 °C (55% RH)	-	-3 $pg \cdot ml^{-1}$ (-3%) ^{ns}
Yeh et al. (Part B) (2013)	15 male, 1 female (LT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fed) in T_{amb} 22 °C (66% RH)	38.4	-1.1 $pg \cdot ml^{-1}$ (-10%) ^{ns}
Zuhl et al. (2015)	2 male, 5 female (LT/MT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 30 °C (12-20% RH)	39.5	-0.2 $pg \cdot ml^{-1}$ (-7%) $^{ns, c}$
Karhu et al. (2017)	17 males (MT-HT)	90 minutes running at 80% of best 10 km race time (fed) in T_{amb} not reported	-	0.3 $pg \cdot ml^{-1}$ (~ 1%) $^{ns, c}$
Kuennen et al. (2011)	8 male (MT)	100 minutes walking (6.3 $km \cdot h^{-1}$) at 50% $\dot{V}O_{2max}$ (fasted) in T_{amb} 46.5 °C (20% RH)	39.3	~0.5 $pg \cdot ml^{-1}$ (10%) $^{ns, c}$
Ng et al. (2008)	30 male (HT)	Half-marathon (fed) in T_{amb} 27 °C (84% RH)	40.7	0.6 $pg \cdot ml^{-1}$ (32%) ^{s}
Sessions et al. (2016)	5 male, 2 female (HT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 30 °C (12-20% RH)	39.5	0.6 $pg \cdot ml^{-1}$ (6%) $^{ns, c}$
Jeukendrup et al. (2000)	29 male, 1 female (HT)	Ironman (3.8 km swim; 185 km cycle; 42.2 km run) (fed) in T_{amb} 9-32 °C	-	1.7 $pg \cdot ml^{-1}$ (666%) ^{s}
Guy et al. (2015)	20 male (LT-MT)	10 minutes cycling at 50%, 60%, and 70% $watt_{max}$, then 5 km (fasted) in T_{amb} 35 °C (70% RH)	38.9	2 $pg \cdot ml^{-1}$ (9%) ^{ns}
Mooren et al. (2020)	20 male (LT)	10 minutes 60% $\dot{V}O_{2max}$, 25 minutes 70% $\dot{V}O_{2max}$ and 25 minutes 80% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	-	2.3 $pg \cdot ml^{-1}$ (7%) $^{ns, c}$
Selkirk et al. (Part B) (2008)	12 male (HT)	To fatigue (~122 minutes) uphill walk at 4.5 $km.h^{-1}$ (fasted) in T_{amb} 40 °C (30% RH)	39.7	~3 $pg \cdot ml^{-1}$ (200%) ^{s}

Shing et al. (2014)	10 male (HT)	~33 minutes running to fatigue at 80% VE (fed) in T_{amb} 35 °C (40% RH)	39.4	4 pg·ml^{-1} (15%) [s]
Snipe et al. (Part A) (2018a, b)	6 male, 4 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 22 °C (44% RH)	38.5	4.1 pg·ml^{-1} (5%) [ns]
Yeh et al. (Part B) (2013)	15 male, 1 female (LT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fed) in T_{amb} 33 °C (50% RH)	39.3	5 pg·ml^{-1} (54%) [s]
Antunes et al. (Part B) (2019)	19 male (MT)	45 ± 18 minutes cycling at midpoint between first and second ventilatory threshold (fasted) in 22.1 °C (55% RH)	-	5 pg·ml^{-1} (7%) [ns]
Antunes et al. (Part C) (2019)	19 male (MT)	10 ± 9 minutes cycling at midpoint between second ventilatory threshold and maximal aerobic power (fasted) in 22.1 °C (55% RH)	-	6 pg·ml^{-1} (5%) [ns]
Ashton et al. (2003)	10 males (LT)	$\dot{V}O_{2max}$ test (~15 minutes) on cycle ergometer (fasted) in T_{amb} not reported	-	9.4 pg·ml^{-1} (72%) [s]
Snipe et al. (Part B) (2018b)	6 male, 4 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (26% RH)	39.6	9.8 pg·ml^{-1} (11%) [s]
Gill et al. (2016)	8 male (MT-HT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 32 °C (34% RH)	38.6	10 pg·ml^{-1} (4%) [ns, c]
Snipe et al. (2017)	6 male, 5 female (MT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (30% RH)	39.3	10 pg·ml^{-1} (N/A %) [nb]
Selkirk et al. (Part A) (2008)	11 male (LT-MT)	To fatigue (~106 minutes) uphill walk at 4.5 km.h^{-1} (fasted) in T_{amb} 40 °C (30% RH)	39.1	~10 pg·ml^{-1} (300%) [s]
Lim et al. (Part B) (2009)	9 male (HT)	To fatigue (time not given) at 70% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (40% RH)	39.5	13 pg·ml^{-1} (92%) [s,c]
Guy et al. (2015)	8 male (LT)	10 minutes cycling at 50%, 60%, and 70% $watt_{max}$, then 5 km (fasted) in T_{amb} 35 °C (70% RH)	38.6	16 pg·ml^{-1} (9%) [ns, c, #]

Gill et al. (2015b)	13 male, 6 female (HT)	Multistage ultra-marathon stage 1 (37 km) (fed) in T_{amb} 32-40 °C (32-40% RH)	-	40 $pg \cdot ml^{-1}$ (14%) [s]
Barberio et al. (2015)	9 male (MT)	~24 minutes running at 78% $\dot{V}O_{2max}$ (fed) in T_{amb} 40 °C (40% RH) prior to heat acclimation	39.0	40 $pg \cdot ml^{-1}$ (57%) [s,c]
Moss et al. (2020)	9 male (HT)	45 minutes cycling at 40% PPO (unstated prandial state) in T_{amb} 40 °C (50% RH) prior to heat acclimation	38.9	52 $pg \cdot ml^{-1}$ (27%) [s,c]
Costa et al. (2017b)	11 male (MT-HT)	120 minutes running at 70% $\dot{V}O_{2max}$ (fed) in T_{amb} 25 °C (35% RH)	-	96 $pg \cdot ml^{-1}$ (46%) [ns, c, #]
Gill et al. (2015a)	14 male, 3 female (HT)	24 hour ultramarathon (fed) in T_{amb} 0-20 °C (54-82% RH)	-	122 $pg \cdot ml^{-1}$ (37%) [s, #]
Machado et al. (Part A) (2017)	9 male (MT)	60 minutes running at 50% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	-	130 $pg \cdot ml^{-1}$ (33%) [ns, #]
Machado et al. (Part B) (2017)	9 male (MT)	60 minutes running at 50% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported (FIO_2 = 13.5%)	-	250 $pg \cdot ml^{-1}$ (48%) [s, #]
Gaskell et al. (2019b)	10 male, 8 female (MT-HT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (25% RH)	38.6	LBP ~-2 $\mu g \cdot ml^{-1}$ (N/A%) [ns, c]
Selkirk et al. (Part A) (2008)	11 male (HT)	To fatigue (~163 minutes) uphill walk at 4.5 $km \cdot h^{-1}$ (fasted) in T_{amb} 40 °C (30% RH)	39.7	LBP ~0 $\mu g \cdot ml^{-1}$ (0%) [ns]
Moncada-Jiminez et al. (2009)	11 male (MT-HT)	135-minute laboratory duathlon at 71% $\dot{V}O_{2max}$ (15km run and 30km cycle) (fasted) in T_{amb} not reported	38.5	LBP ~0.59 $\mu g \cdot ml^{-1}$ (22%) [s, c]
Selkirk et al. (Part B) (2008)	12 male (LT-MT)	To fatigue (~106 minutes) uphill walk at 4.5 $km \cdot h^{-1}$ (fasted) in T_{amb} 40 °C (30% RH)	39.1	LBP ~1.5 $\mu g \cdot ml^{-1}$ (15%) [s]
Jonvik et al. (2019)	16 male (HT)	60 minutes cycling at 70% $watt_{max}$ (fasted) in T_{amb} not reported	-	LBP 1.6 $\mu g \cdot ml^{-1}$ (13%) [s]

Costa et al. (2019)	11 male (MT-HT)	120 minutes running at 70% $\dot{V}O_{2max}$ (fed) in T_{amb} 25 °C (35% RH)	-	sCD14-ST 0.05 µg·ml^{-1} (N/A%) ^{ns,c}
Gaskell et al. (2019b)	10 male, 8 female (MT-HT)	120 minutes running at 60% $\dot{V}O_{2max}$ (fed) in T_{amb} 35 °C (25% RH)	38.6	sCD14-ST 0.1 µg·ml^{-1} (N/A%) $^{s, c}$
Costa et al. (2020a)	11 male (MT-HT)	120 minutes running at 70% $\dot{V}O_{2max}$ (fed) in T_{amb} 25 °C (43% RH)	-	~0.1 µg·ml^{-1} (N/A%) ^{ns,c}
Stuempfle et al. (2016)	15 male, 5 female (MT)	161-km ultramarathon (26.8 ± 2.4 hours; fed) in T_{amb} 0-30 °C (N/A RH)	38.3	sCD14-ST 0.6 µg·ml^{-1} (63%) ^{s}
Pugh et al. (2019)	10 male, 2 female (MT)	42.4 km track marathon (4.1 ± 0.8 hours; fed) in T_{amb} 16-17 °C (N/A RH)	-	sCD14-ST 5.4 µg·ml^{-1} (164%) $^{s, c}$

Where: LT = Low-trained (35-49 ml·kg·min^{-1} $\dot{V}O_{2max}$); MT = Moderate-trained (50-59 ml·kg·min^{-1} $\dot{V}O_{2max}$); HT = High-trained (60+ ml·kg·min^{-1} $\dot{V}O_{2max}$). s = significant change post-exercise ($p < 0.05$); ns = non-significant change post-exercise ($p > 0.05$); nb = no baseline resting data to compare with; c = control/placebo trial of study. # Where data have been converted from EU·ml^{-1} to pg·ml^{-1} through standard conversions (1 EU·ml^{-1} = 100 pg·ml^{-1}).

2.11 Aetiology of Gastrointestinal Barrier Integrity Loss

The aetiology GI barrier integrity loss in response to exertional-heat strain is multifactorial and incompletely understood. To date, the three best supported explanations relate to the combined influences of direct hyperthermia (Dokladny et al., 2016), reactive oxygen species (Grootjans et al., 2016) and pro-inflammatory cytokines (De Punder and Pruimboom, 2015) on structural GI integrity. The evidence supporting each of these conceptual pathways is derived from research utilising both *in vitro* and *in vivo* experimental designs.

2.11.1 Hyperthermia

In vitro, hyperthermia is well-characterised to dysregulate GI TJ complex structures in a temperature- and duration- dependant manner (Dokladny et al., 2016). In comparison to the well-established temperature threshold (43 °C) for protein denaturation (Sakaguchi et al., 1995), GI TJ dysregulation can occur at temperatures *circa* 39°C. Early evidence performed by Dokladny et al. (2006, 2008) found that when human colonic adenocarcinoma (Caco-2) cells are exposed to 39 °C and 41 °C heat-stress for 4-hours, they display 10% and 20% reduction in transepithelial electrical resistance (TER), respectively. Importantly, these responses could not be attributed to enhanced heat-induced apoptosis

or necrosis. Instead, western-blot analysis revealed protein expression of the intracellular scaffolding protein, zonula occludin-1 (ZO-1), to be downregulated, concomitant with an upregulation of the TJ transmembrane protein, occludin. These findings were later confirmed by comparable studies using T84 small intestinal cells (Yang et al., 2007; Marchbank et al., 2011; Davison et al., 2016), Caco-2 cells (Xiao et al., 2013; Zuhl et al., 2014; Davison et al., 2016) and NCM460 colonic (Marchbank et al., 2011) cells. In a more extensive recent study, exposure of human ICE-6 epithelial cells for 1.5 hours to 42 °C, suppressed expression of multiple TJ proteins, including claudin's-6, 9-, 13-, 17- and 22 (Xia et al., 2019). Despite this evidence, the cellular pathways regulating GI TJ integrity responses to hyperthermia have to date been incompletely characterised. Selective molecular inhibition studies have shown heat-shock protein 70 (HSP70; Dokladny et al., 2016), sodium-dependent glucose cotransporter (SGLT; Ikari et al., 2005) and AMP-activated protein kinase (AMPK; Xia et al., 2019) to all positively regulate TJ integrity under hyperthermia, whereas protein kinase-c (PKC) and myosin light-chain kinase (MLCK) appear to have a negative regulatory function (Yang et al., 2007).

Concordant with *in vitro* cell models, hyperthermia has been consistently found to stimulate *in vivo* GI barrier integrity loss (Dokladny et al., 2016). Whilst T_{core} is often tightly associated with GI barrier integrity disturbance, the whole-body nature of this experimental model is unable to clarify whether hyperthermia directly or indirectly disturbs GI integrity. In early studies using primates (Gathiram et al., 1987a, 1987b, 1988) and rodents (Lambert et al., 2002; Oliver et al., 2012), GI permeability increased during brief hyperthermia in a temperature- and duration- dependant manner. Further evidence found GI permeability to increase proportional to histological measures of epithelial cell shedding, vacuole accumulation, loss of microvilli and reduced crypt height during progressive hyperthermia (Toth et al., 1992; Lambert et al., 2002; Liu et al., 2012). In comparison to this evidence, the influence of hyperthermia on TJ structure has been poorly characterised. In two studies where rats were passively exposed to 40 °C for 1.5-2 hours, electron microscopy of small intestinal tissues revealed intact (Lambert et al., 2002) or minor disruption (Liu et al., 2002) of TJ structures. However, more recently Xia et al. (2019) revealed using immunohistology that ZO-1 and claudin-1 protein expression were reduced in response to a comparable rodent CHS model. When collating all available human laboratory research, a recent systematic review (September 2016) reported a strong negative association (r = 0.91) between peak T_{core} and GI barrier integrity (DSAT, I-FABP or

endotoxin) in response to sub-clinical exertional-heat stress (Pires et al., 2016). Taken together, these results indicate that hyperthermia disrupts GI TJ formation, however, whether hyperthermia directly regulates TJ anatomy still requires further verification.

2.11.2 Reactive Oxygen Species

Reactive oxygen species (ROS), such as the superoxide anion, hydrogen peroxide, and hydroxyl radical are formed by the partial reduction of oxygen (Ray et al., 2012). Cellular ROS are generated endogenously during normal mitochondrial oxidative phosphorylation, granulocyte oxidative burst, or can arise from interactions with exogenous compounds, such as xenobiotics. When ROS overwhelm the cellular antioxidant defence system, oxidative damage to nucleic acids, proteins, and lipids will occur (Fulda et al., 2010). Brief hyperthermia (42-45°C) directly increases ROS production in several cell types by disruption of mitochondrial membrane integrity (Flanagan et al. 1998; Christen et al., 2018). In the small intestine, Lambert et al. (2002) found *in situ* exposure of rat intestinal sacs to 1-hour of hyperthermia (41.5-42.0°C) did not influence lipid peroxidation. Furthermore, antioxidant co-incubation (e.g. Ebselen, Tempol) did not protect paracellular permeability in this study. In opposition, Oliver et al. (2012) reported hyperthermia increased protein oxidation by ~25%, when applying a similar methodological design. Like Lambert et al. (2002), antioxidant co-incubation with both Tiron and Trolox failed to improve paracellular permeability in this study, however, *N*-acetylcysteine (a stronger antioxidant) did protect GI permeability via attenuation of protein oxidation. A major limitation of *in vitro* studies is the inability to modulate tissue perfusion, given localised ischemia is the primary cause of oxidative stress during *in vivo* hyperthermia (Lambert et al., 2002).

In humans, splanchnic vascular beds receive *circa* 20-30% of total resting cardiac output but consume only 10-20% of the supplied oxygen (ter Steege and Kolkman, 2012). This means that in response to exertional-heat stress, cardiac output is theoretically safely redistributed from the splanchnic organs, towards the peripheral tissues to support thermoregulation. Hypoperfusion of splanchnic vascular beds, measured using doppler ultrasonography, occurs proportional to exercise intensity, duration, and T_{core} (Otte et al., 2001). Reduced splanchnic perfusion causes localised tissue ischemia, which subsequently enhances ROS production through mitochondrial electron transport chain dysfunction (Wu et al., 2018). In human experimental ischemia, the appearance of subepithelial spaces occurred within the small intestine inside 15-minutes after jejunal stapling and continued

to disintegrate over time (Derikx et al., 2008; Grootjans et al., 2011). During subsequent tissue reperfusion, there may be further minor ROS induced damage caused by infiltrating leukocyte oxidative burst, however, restoration of the GI barrier usually occurs within 1-4 hours following ischemia (Grootjans et al., 2016). In comparison to the small intestine, the large intestine is less susceptible to ischemic injury, given the lower partial pressure of oxygen across this GI segment (Grootjans et al., 2013). In a sub-clinical human exercise model, splanchnic perfusion was positively associated with small intestinal epithelial injury (van Wijck et al., 2011a). In a sub-clinical rodent CHS model, ROS concentrations measured in portal blood via electron paramagnetic resonance spectroscopy increased by 100-200% upon reaching a T_{core} of 39 °C (Hall et al., 1994), however, co-administration of a xanthine oxidase antagonist (allopurinol) attenuated this response and downstream portal endotoxemia (Hall et al., 2001).

2.11.3 Cytokines

Cytokines comprise a large family of intercellular signalling molecules that perform many regulatory inflammatory functions (Turner et al., 2014). Inflammation is a physiological response to initial tissue injury, however, induction of SIRS following excessive localised inflammation can become self-destructive (Heled et al., 2013). Broadly, cytokines are categorised as either pro-inflammatory (e.g. TNF-α, IL-1β, IL-6) or anti-inflammatory (IL-1ra, IL-4, IL-10), which function in a counter-regulatory manner to maintain homeostasis. In response to strenuous exercise, systemically derived cytokines largely originate from skeletal muscle and blood leukocytes (Suzuki, 2018). The specific biological roles of individual cytokines on GI barrier integrity have been characterised *in vitro* (Al-Sadi et al., 2009). Incubation of intestinal cells (e.g. Caco-2, HT-29) with physiological concentrations of TNF-α (Marano et al., 1998; Ma et al., 2004), IL-1β (Al-Sadi et al., 2007, 2008) and IL-6 (Tazukei et al., 2003; Suzuki et al., 2011) all increase paracellular permeability in a concentration- and duration- dependant manner. In comparison, IL-10 enhanced basal GI barrier function and suppressed the influence of pro-inflammatory cytokine action (Madsen et al., 1997). The mechanism behind how cytokines disrupt GI barrier integrity is hypothesised to relate to MLCK-mediated phosphorylation of the myosin light chain (MLC) on TJ formation (De Punder and Pruinboom, 2015). In comparison, cytokines do not appear to influence GI barrier integrity via altering epithelial apoptosis or proliferation (Ma et al., 2004). A final consideration relates to the minimum duration (> 12 hours) of cytokine exposure required to influence GI barrier integrity *in vitro*, which

potentially implies cytokines have little influence on GI permeability responses to brief exertional-heat stress (Al-Sadi et al., 2009).

In humans, both passive hyperthermia (Ahlers et al., 2005) and exertional-heat stress (Rhind et al., 2004; Selkirk et al., 2008) increase systemic pro-inflammatory cytokine concentrations in a temperature- and duration- dependant manner. In a sub-clinical exertional-heat stroke model, supplementation with the anti-inflammatory curcumin, significantly blunted peak cytokine (TNF-α, IL-10) and I-FABP responses (Szymanski et al., 2018). Whilst these data suggest that blocking systemic cytokine responses protects GI barrier integrity, an alternative hypothesis is that improved GI barrier integrity conversely blunted cytokine responses by suppressing GI MT (De Punder and Pruinboom, 2015). Indeed, a follow-up study to Szymanski et al. (2018) reported no influence of curcumin supplementation on inflammatory protein expression in blood leukocytes (Falgiano et al., 2019), which favours the latter explanation. In comparison, more direct evidence has shown treatment with monoclonal anti-TNF-α antibodies to improve GI permeability in patients with chronic GI diseases (Suenaert et al., 2002; Olesen et al., 2016). Another consideration is the unclear influence of IL-6 on GI barrier integrity. For example, IL-6 gene knock-out (Yang et al., 2003) protects mice during haemorrhagic shock, however, IL-6 treatment weakened GI barrier integrity (Al-Sadi et al., 2014). Taken together, these results indicate that whilst pro-inflammatory cytokines clearly disrupt *in vitro* GI barrier integrity in non-stressed conditions, their influence during exertional-heat stress is more complex.

2.12 Nutritional Countermeasures

Nutritional countermeasures could modulate key cellular pathways involved in mitigating the influence of exertional-heat stress on GI barrier integrity disturbance. Diet regimens and nutrition supplements with evidence that they can influence GI barrier integrity following exercise and/or exertional-heat stress specifically are reviewed. Furthermore, the mechanistic basis hypothesised to underpin each nutritional intervention and recommendations for applied practise are presented.

2.12.1 Carbohydrate

Carbohydrates (CHO) are the major macronutrient of western diets and an essential nutrient for optimising endurance exercise performance. The physiological response to CHO ingestion is highly dependent on its biochemical formula, where high glycaemic index CHO (e.g. glucose, maltose) have rapid bioavailability, and low glycaemic index CHO (e.g.

fructose, galactose) have delayed bioavailability. The volume, tonicity and osmolality of CHO are all equally influential. In healthy humans, ingestion of a single CHO-rich meal (55-70% of total kcal) evokes equivocal (endotoxin [Ghanim et al., 2010; Deopurkar et al., 2010] or slightly improved (I-FABP; [Edinburgh et al., 2018; Salvador et al., 2019]) GI barrier integrity responses when examined acutely postprandial. In comparison, rodent models of acute GI distress indicate oral maltodextrin (Deniz et al. 2007) or sucrose (Ramadass et al., 2010) ingestion favourably influence GI barrier integrity. Mechanisms of action at the whole-body level are likely multifactorial, including regulation of the GI microbiota (David et al., 2014) and elevation of splanchnic perfusion (Gentilcore et al., 2014). Nevertheless, *in vitro* studies indicate that high glucose exposure reduces GI TJ stability through abnormally redistributing several TJ proteins (Lerner et al., 2015). Compared with ingestion of a single CHO-rich meal, ingestion of a single fat-rich meal results in significant acute GI MT (Ghanim et al., 2010; Deopurkar et al., 2010).

Contrary to proposed hypotheses, preliminary research did not find CHO ingestion (30-60 g·hour^{-1} glucose) to protect GI barrier integrity (utilising the DSAT) above a water placebo when ingested throughout 60-90 minutes of moderate intensity exercise (70% $\dot{V}O_{2max}$) (van Nieuwenhoven et al., 2000a; Lambert et al., 2001). However, follow-up studies reported attenuated GI barrier integrity loss (I-FABP and DSAT) with glucose ingestion (60 g·hour^{-1}) during two-hours moderate intensity running (60% $\dot{V}O_{2max}$) in the heat (35 °C and 25% RH; Snipe et al., 2017), and with sucrose ingestion (40 g·hour^{-1}) prior/during a one-hour moderate intensity cycle (70% $watt_{max}$) (Jonvik et al., 2018). However, neither intervention ameliorated the severity of GI MT. Formulations of single- and multi-transportable CHO mixtures (i.e. 1.8 g·min^{-1} glucose; 1.2 and 0.6 g·min^{-1} glucose plus fructose; 0.6 and 1.2 g·min^{-1} glucose plus sucrose) all tended to (interaction effect $p = 0.10$) reduce I-FABP concentrations (area under the curve at 30 minute intervals) to a similar extent relative to water during three hours of low-intensity cycling (50% $Watt_{max}$) (Trommelen et al., 2017). Similarly, ingestion of 60 g·hour^{-1} of either potato flesh puree or carbohydrate gel (2:1 maltodextrin/fructose) completely attenuated the rise in I-FABP observed throughout a 2.5 hour mixed-intensity cycle (2 hours 60% $\dot{V}O_{2max}$ then a 20 km time trial in temperate conditions) (Salvador et al., 2019). To date, only one study has reported an adverse effect of CHO ingestion during exercise (1 hour 70% $\dot{V}O_{2max}$ running in 35 °C and 12-20% RH) on GI barrier integrity, with ingestion of a multi-transportable CHO gel (18 g maltodextrin and 9 g fructose) 20-minutes into exercise shown to increase GI

barrier integrity (I-FABP, endotoxin) loss relative to a placebo (Sessions et al., 2016). However, in this study exertional-heat stress alone surprisingly had no influence on GI barrier integrity, whilst in the CHO condition the magnitude of GI integrity loss was minimal. Currently little is known about the influence of pre-exercise CHO availability on GI barrier integrity. One study reported a 48-hour low (20% CHO) *versus* high (60% CHO) CHO-diet had no influence on GI MT following a duathlon (Moncada-Jiminez et al., 2009).

Practical recommendations for CHO ingestion on GI barrier integrity are unable to be established at present, given the large variation in findings from seemingly comparable studies. This lack of consistency cannot be attributed to differences in prandial status, exercise intensity, CHO type/dose or participant demographic. In general, the application of traditional sports nutrition guidelines for CHO ingestion do not appear to adversely influence GI barrier integrity, and more likely appear to offer favourable benefits. Future research is required to determine the most effective CHO formulations for fuelling exercise and maintaining GI barrier integrity. Potentially important factors include: the carbohydrate source, osmolality, and delivery format (e.g. fluid, gel, solid). The impact of pre-exercise CHO status (e.g. fasted training) might also influence the GI barrier response to feeding.

2.12.2 Bovine Colostrum

Bovine colostrum (BC) is the milk produced by cows during the first 24-48 hours post-partum, and its composition markedly differs from milk produced later in lactation (Rathe et al., 2014). In humans, colostrum provides many health benefits to the neonate, including tissue development and immune defence (Uruakpa et al., 2002). BC contains a variety of growth factors (e.g. IGF-1) and immunomodulatory components (e.g. immunoglobulins, cytokines) at higher concentrations than human colostrum (Prosser et al., 2004). The use of a BC nutritional supplement (liquid and powder) to maintain GI barrier function in healthy adults has been shown to reduce GI permeability post NSAID administration (Playford et al., 1999), and can blunt systemic elevations in endotoxin following critical illness (Eslamian et al., 2019). These findings are supported by *in vitro* studies on Caco-2 cells, where BC blunted GI cell apoptosis and increased epithelial resistance during heat exposure (Marchbank et al., 2011). Mechanisms of action include: increased epithelial cell proliferation, upregulation of cytoprotective I-HSP expression and improved TJ stability (Davison et al., 2016).

In a series of experiments, 14 days of BC (20 g·d^{-1}) halved the 3-fold rise in urinary DSAT ratio and circulating I-FABP concentrations following short-duration (20 minutes) high-intensity running (80% $\dot{V}O_{2max}$) (Marchbank et al., 2011; Davison et al., 2016; March et al., 2017). Whilst these results show promise, such benefits appear attenuated by more demanding exercise protocols. Two comparable studies reported no effect of either a moderate (14 days at 20 g·d^{-1}; McKenna et al., 2017) or high (7 days at 1.7 g·kg·d^{-1}; Morrison et al., 2014) BC dosing on I-FABP concentrations following a fatiguing run in the heat (30-40 °C; 50% RH). Likewise, March et al. (2019), using their earlier BC supplementation protocol (March et al., 2017), reported only a minor (~10%) suppression of I-FABP concentration and a non-significant blunting of circulating *Bacteroides*/total 16S DNA following a 1-hour run (70% $\dot{V}O_{2max}$) in the heat (30 °C/60% RH).

Practical recommendations support a BC dose of 20 g·d^{-1} for 14 days to protect the GI tract during moderately demanding exercise, though little-to-no benefits appear likely during more arduous exertional-heat stress. Two days of BC supplementation with the same daily dose offered no protective benefits (Davison et al., 2016). Chronic low dose (500 mg·d^{-1}) BC ingestion improved resting GI permeability (DSAT ratio) in athletes during heavy training (Hałasa et al., 2017), but chronic high dose (60 g·d^{-1}) BC ingestion increased GI permeability (Buckley et al., 2009). Further work is required to determine the optimal time-course and BC dose to support GI barrier function. As there are large inter-manufacturer variations in BC formulations, future research should include accurate characterisation of the bioactive components in intervention trials, as these components are likely to have a significant bearing on study findings (Jasion et al., 2015). BC appears to be well-tolerated in healthy individuals in doses up to 60 g·d^{-1} over several weeks, and although IGF-1 is on the World Anti-Doping Agency banned substance list, it is unlikely BC can result a positive doping control (Davison et al., 2019).

2.12.3 Nitric Oxide

The free radicle gas, Nitric Oxide (NO), performs multiple signalling roles in the body. Synthesis occurs through two complementary pathways: the NO synthase (NOS) dependant L-arginine pathway; and the NOS independent nitrate (NO_3), nitrite (NO_2), NO serial reduction pathway (Lundberg et al., 2018). Supplementation with NO precursors, including L-arginine (Castillo et al., 1993), L-citrulline and inorganic NO_3 (Petersson et al., 2007), are all capable of upregulating NO bioavailability across the splanchnic organs. Rodent models show this increase in NO blunts GI histopathological damage and

subsequent MT following NSAID ingestion (Tanaka et al., 2001), small bowel obstruction (Batista et al., 2012) and ischemic-reperfusion injury (Sukhotnik et al., 2005). The vasodilatory role of NO in maintaining GI microcirculation appears to be one of the main mechanisms (van Wijck et al., 2012b), with enhanced antioxidant scavenging (Rubanyi et al., 1991), constrained neutrophil activation (Beutheu et al., 2014) and increased GI TJ protein expression (Kubes et al., 1991) as complementary pathways.

Only two studies have investigated the influence of nitric oxide precursors on exercise-induced GI barrier integrity loss. In mice, addition of 2% $_{L}$-arginine to the standard diet for 7 days prevented a rise in GI barrier loss relative to the control following 1-hour running to fatigue in the heat (34 °C) (Costa et al., 2013). Similarly in humans, van Wijck et al. (2014) reported acute $_{L}$-citrulline supplementation (10 g given 0.5 hours pre-exercise) successfully maintained splanchnic perfusion and blunted the rise in systemic I-FABP during 1 hour of moderate intensity cycling (70% $watt_{max}$). However, this intervention did not reduce peak post-exercise I-FABP concentrations, or the urinary DSAT ratio. Likewise, only 1 study has examined the influence of NO_3 supplementation on exercise-induced GI barrier integrity loss (Jonvik et al., 2019), reporting no influence of acute sodium NO_3 (800 mg given 2.5 hours pre-exercise) on circulating I-FABP or LBP concentration following 1-hour of moderate intensity (70% $watt_{max}$) cycling (Jonvik et al., 2019).

Practical recommendations regarding the use of $_{L}$-arginine, $_{L}$-citrulline or inorganic NO_3 to protect the GI tract during exercise are inconclusive. Further work is required to substantiate present findings and to verify potential benefits over a range of exercise protocols. Likewise, evidence is required to confirm whether benefits are observed in highly-trained populations (who tend not to respond to NO supplementation), and to determine which NO precursors provide the most effective GI protection. A further practical consideration is the apparent impaired thermoregulation associated with reduced cutaneous vasodilation, which might disrupt the GI barrier especially when exercising in the heat (Kuennen et al., 2015).

2.12.4 Probiotics

Probiotics are live microorganisms considered to regulate the GI microbiota, which might confer health benefits when consumed in adequate quantities (Parvez et al., 2006). They are found in low concentrations across various food sources (e.g. non-pasteurised dairy products), and regular consumption has been recommended in patients with GI

conditions since the early 1900s (Parvez et al., 2006). More recently, probiotic supplementation to support GI barrier function has received extensive examination. Whilst positive effects are reported in ~50% of human studies, these are not universal, and may reflect the large variation in dose and strains administered (Bron et al., 2012). Inconclusive effects are also reported *in vitro* on GI cellular apoptosis and epithelial integrity when Caco-2 cells are cultured with probiotics prior to insult (Hsieh et al., 2015). Mechanisms of action are incompletely understood, but are believed to include: inhibition of pathogenic bacterial overgrowth; competition with pathogenic bacteria for binding sites on mucins and/or epithelial cells; increased mucosal immunoglobulin and antimicrobial protein secretion; increased epithelial cell proliferation; upregulated I-HSP expression; suppressed GI inflammation; and increased TJ stability through upregulation of GI TJ protein expression (West et al., 2009).

Several studies have examined the influence of probiotic supplementation on GI barrier responses to exertional-heat stress. 4 weeks daily consumption of a multi-strain probiotic (45 x 10^9 colony forming units [CFU]; from 3 strains) blunted DSAT ratios (8%) and circulating endotoxin concentrations (~12%) following a ~35-minute fatiguing run (80% ventilatory threshold) in the heat (35 °C/40% RH) (Shing et al., 2013). A follow-up study reported daily ingestion of a similar multi-strain probiotic (3 x 10^9 CFU; from 9 strains) for a period of 12 weeks approximately halved basal endotoxin concentrations immediately prior to and 6-days following an ultra-triathlon (Roberts et al., 2014). In contrast, seven days high-dose single strain probiotic supplementation (45 x 10^{11} CFU·day^{-1} *Lactobacillus Casei*) was associated with an increased rise in endotoxin concentrations, compared with placebo, following two hours moderate-intensity running (60% $\dot{V}O_{2max}$) in the heat (34 °C/32% RH) (Gill et al., 2016). Likewise, 4 weeks daily supplementation with a multi-strain probiotic (25 x 10^9 CFU; from 5 strains) had no influence on either DSAT, I-FABP or sCD14 responses following a simulated 42.2 km marathon in temperate conditions (Pugh et al., 2019). Finally, 4 weeks supplementation with a single strain probiotic (2 x 10^8 CFU *Lactobacillus Salivarius*) had no influence on DSAT responses, (or faecal microbial composition), following 2 hours of moderate intensity running (60% $\dot{V}O_{2max}$) in temperate conditions (Axelrod et al., 2019). It is unlikely the final two studies were sufficiently powered to detect an influence of probiotic supplementation on GI barrier integrity.

The present data indicate that probiotic supplementation has little influence on GI barrier integrity in response to exercise. It is not possible to elucidate whether inconsistent

responses are attributable to the specific probiotic strain, duration of supplementation or another factor. Future research is required to develop effective probiotic supplementation regimes and will need to address factors such as strain(s), timing and dose. It will also be necessary to verify potential efficacy using relevant exercise (heat stress) protocols. Global metabolomics approaches have linked exercise-induced GI barrier function loss with alterations in GI microbiota composition during a 4-day military arctic training exercise (51 km ski march; (Karl et al., 2017), and such methodologies should be applied when developing probiotic supplements to support GI barrier integrity. Probiotic use is considered safe in healthy populations, when consumed acutely and chronically (Pyne et al., 2015).

2.12.5 Polyphenols

Polyphenols are natural compounds that defend plants against damage from radiation and pathogens. Over 8000 polyphenols have been identified, which are classified into 4 major categories: flavonoids; phenolic acids; stilbenes; and lignans. Quercetin is the most abundant dietary flavonoid polyphenol (De Vries et al., 1998), and in rodents' supplementation has been shown to maintain GI barrier integrity following methotrexate (MTX)-induced intestinal damage (Sukhotnik et al., 2018). However, *in vitro* evidence from human Caco-2 cells is less conclusive, with quercetin shown to both improve (Amasheh et al., 2008; Suzuki and Hara, 2009) and impair (Freedman et al., 2001; Dokladny et al., 2008) GI barrier integrity in response to heat stress. Proposed mechanisms in favourable studies include modulation of vasodilatory factors (e.g. NO; Freedman et al., 2001), elevated antioxidant scavenging (Valenzano et al., 2015) and improved TJ protein expression (Hosokawa et al., 1992). Proposed mechanisms in detrimental studies relate to reduced cytoprotective I-HSP expression (Dokladny et al., 2006) and TJ stability through disruption of occludin TJ localisation (Dokladny et al., 2008. Both positive and negative responses are reported when Caco-2 cells are supplemented *in vitro* with additional polyphenols (Oteiza et al., 2018). Human studies assessing the efficacy of polyphenol supplementation on GI barrier integrity are lacking, and where *in vitro* studies administer physiologically relevant polyphenol doses, the effects have been negligible (Sergent et al., 2010).

Only two studies have examined the influence of polyphenol supplementation on exercise-induced GI barrier integrity. Supplementation with quercetin (2 g given 1-hour pre-exercise), a plant flavanol, caused a 2-fold increase in both urinary lactulose and plasma endotoxin concentration compared to placebo in response to 100-minutes walking (1.8

m·s^{-1}) in a 46 °C (20% RH) environment (Kuennen et al., 2011). However, more promisingly, supplementation with curcumin (3 days of 0.5 g·d^{-1}), a constituent of turmeric, blunted circulating I-FABP concentrations by ~30% after 1-hour moderate intensity running (65% $\dot{V}O_{2max}$) in the heat (37 °C/25% RH) (Szymanski et al., 2017).

There are no practical recommendations supporting polyphenol use to protect the GI tract during strenuous exercise. Despite promising *in vitro* observations, more work is required to determine the optimal formulation, time-course and polyphenol dose to support GI barrier function across different exercise-modalities. No studies have successfully measured the effect of polyphenols on secondary GI MT post-exercise and future studies should attempt to control for dietary polyphenol intake.

2.12.6 Zinc-Carnosine

Zinc-Carnosine (ZnC) is a pharmaceutical chelate of zinc and $_{L}$-carnosine (Takei, 2012). It is widely used in Japan to treat gastric ulcers, and more recently has been marketed in Europe to support GI health (Mahmood, et al., 2007). Zinc is an essential trace element and a co-factor in numerous tissue regenerative and immunomodulatory enzymatic reactions (Roohani et al., 2013), whilst $_{L}$-carnosine is a cytoplasmic dipeptide of beta-alanine and $_{L}$-histidine (Sale et al., 2013). Daily ZnC ingestion improves GI barrier integrity in healthy humans following chronic GI barrier damaging NSAID ingestion (Mahmood, et al., 2007). These protective benefits are reported to be synergistic compared with consuming either ingredient individually (Omatsu et al., 2010). *In vitro* studies of rat intestinal and human Caco-2 cells support these reports, where ZnC blunts GI cellular apoptosis (Fujii et al., 2000) and increases epithelial electrical resistance (Davison et al., 2016) upon damage, in a dose-dependent fashion. Mechanisms of action appear multifactorial, including increased: epithelial cell proliferation (Mahamood et al., 2007), I-HSP concentrations (Davison et al., 2016)]; antioxidant activity (Saunders et al., 2017); and stability of TJs through blunting phosphorylated occludin and claudin-1 expression (Davison et al., 2016).

To date, only 1 study has investigated the influence of ZnC on exercise-induced GI damage. 14 days of ZnC (75 mg·d^{-1}) attenuated a 3-fold rise in GI permeability (70% reduction in DSAT ratio) after a short-duration (20 minutes) high-intensity running (80% $\dot{V}O_{2max}$) protocol (Davison et al., 2016). This effect was comparable to that observed with BC (20 g·day^{-1} for 14 days) in this study, and when the 2-treatments were combined the

benefits were synergistic (85% reduction DSAT ratio). Furthermore, the combination of ZnC and BC blunted the exercise-induced increase in DSAT ratio by 30% after only 2-days, whilst no protection was offered by either ingredient in isolation at this point.

Practical recommendations support ZnC use at a dose of 75 $mg \cdot d^{-1}$ for 14 days to protect the GI tract during moderately demanding exercise. Further work is needed to substantiate existing findings and verify the potential benefits of ZnC during more strenuous exercise. No studies have successfully measured the influence of ZnC on secondary GI MT. Research is required to determine the optimal time-course and dose of ZnC to support GI barrier function with chronic and acute supplementation. Larger doses of ZnC (150 $mg \cdot d^{-1}$) appear well-tolerated in patients with chronic GI disease over several weeks, and dose-dependent *in vitro* evidence suggests this might offer greater protection (Omatsu et al., 2010).

2.12.7 Future Research Directions

Investigation of nutritional countermeasures that support GI barrier integrity during arduous exertional-heat stress is an important and expanding area of research. Preliminary observations indicate some diet regimens and nutritional supplements could benefit populations at risk of EHS. Optimal supplementation strategies should be safe, well-tolerated, practical (e.g. affordable, low mass, widely-available), fast acting and effective in wide-ranging scenarios (e.g. exercise intensity/duration, population). It is also important that they are without secondary adverse responses, especially those relating to skeletal muscle adaptation, thermoregulation, immune function and bone health. Whilst there are numerous examples of well-conducted studies reporting beneficial effects from diet regimens and individual supplements on GI barrier integrity, it is currently not possible to provide definitive guidance. In part this is due to limitations and variations in study designs and in some instance’s incomplete characterisation of the bioactive nutrients.

Future research should address dietary regimens and nutritional supplements that satisfy the above requirements when tested in the most demanding scenarios. It would appear worthwhile to assess the synergy between ingredients that maintain GI integrity, especially if they are believed to act via separate physiological pathways. Further supplements warranting exploration include: omega-3 polyunsaturated fatty acids (Barbalho et al., 2016); vitamin C (Ashton et al., 2003); vitamin D (Raftery et al., 2015) and prebiotics (Carlson and Slavin, 2016). Research should target specific populations (e.g. sex,

training status), supplementation timings (e.g. repeat dosing, delayed/post-exercise ingestion), safety (e.g. GI symptoms) and monitor the continued efficacy of supplementation following chronic application.

2.13 Glutamine

L-glutamine (GLN) is the most abundant free amino acid in the human body. In states of good health, GLN concentrations are maintained between 600 to 800 $\mu mol \cdot l^{-1}$ in plasma, 2 to 5 $mmol \cdot l^{-1}$ in enterocytes and 5 to 20 $mmol \cdot l^{-1}$ (wet-weight) in organ tissue (Soeters and Grecu, 2012). This means that GLN contributes *circa* 20% of the total systemic- and 40-60% of the total intracellular amino acid pool (Labow et al., 2001). Traditionally, GLN was classified as a *non-essential* nutrient, however, this classification was updated in 1990 to *conditionally essential* given that whole-body GLN concentrations are depleted in response to severe catabolism (Lacey and Wilmore, 1990). This means that in non-catabolic states, endogenous GLN concentrations are largely maintained without a requirement for exogenous dietary intake, however, during severe catabolism exogenous supplementation is required to prevent deficiency, commonly defined as a plasma concentration below 420 $\mu mol \cdot l^{-1}$ (Wu, 2010). In critically ill patients entering ICU, numerous studies have associated hypoglutanemia with poor clinical outcome (Wischmeyer, 2007). Importantly, GLN supplementation (~0.2-0.5 $g \cdot kg \cdot d^{-1}$) is well substantiated to improve clinical outcome (McRae, 2017), including protecting GI barrier integrity in patients experiencing illnesses indicative of GLN deficiency, such as burns (Zhou et al., 2003; Peng et al., 2004), radiotherapy (Yoshida et al., 1998; Yao et al., 2015), and major abdominal surgery (van Der Hulst et al. 1993; Jiang et al., 1999; Shu et al., 2016). Based on the evidence from clinical settings, GLN supplementation would appear to have potential as a countermeasure to support GI barrier integrity in populations at risk of EHS. Furthermore, from an ecological standpoint, GLN has several logistical benefits for implementation in occupational settings. For example, it is widely available, inexpensive (£15-20 per kg), lightweight, non-perishable, vegetarian/vegan and non-patented (Gleeson, 2008).

2.13.1 Gastrointestinal Metabolism

The GI tract is the major location for GLN metabolism in humans (Mithieux, 2001). On average, GLN accounts for *circa* 35% of total carbon dioxide production across the GI tract, with a further 50% generated from ketone bodies (Hanson and Parsons, 1978). This means that unlike other major organs, GLN is quantitatively a more important respiratory

fuel than glucose to the GI tract (Windmueller, 1984). Two mechanisms that explain the high metabolic rate of GLN across the GI tract are the high rate of glutamine synthetase activity (3-6 $\mu mol \cdot h \cdot mg^{-1}$) and strong substrate affinity (Cruzat et al., 2018). Since germ-free rodents have comparable GLN metabolism to conventional rodents, the GI microbiota does not appear to be involved in GLN metabolism (Windmueller and Spaeth, 1974). Despite the reliance on GLN as the major respiratory fuel in GI mucosal cells, intracellular concentrations are low (0.2 $\mu mol \cdot g^{-1}$) (Windmueller, 1984). This means that the GI tract is reliant on either endogenous GLN uptake and/or exogenous delivery into the lumen from the diet. *In vivo* models using radiolabelled GLN or comparing arterial-venous GLN flux have shown the GI tract to uptake GLN at a maximal rate of 75 $\mu mol \cdot h^{-1}$, with *circa* 80% of uptake occurring in the small intestine (Windmueller and Spaeth, 1974). The uptake of GLN across the GI tract is controlled fundamentally by: (1) systemic GLN delivery via the cardiovascular system; (2) the activity of intracellular membrane transporter proteins; and (3) the rate of intracellular GLN hydrolysis (Souba, 1993). Using radiolabelled GLN tracers, 75% of luminal GLN is metabolised first-pass (Windmueller and Spaeth, 1980). The fate of the remaining 25% is distributed approximately equally between incorporation into tissue protein; and conversion into delta^1-pyrroline-5-carboxylate that enables the formation of citrulline, proline and ornithine (Felig and Wahren, 1971).

2.13.2 Exercise Metabolism

The influence of exercise on extracellular GLN metabolism is poorly characterised (Table 10). Reasons for equivocal data relate to variability in the exercise stimulus, prandial status (Gleeson et al., 1998) and analytical methodologies (Walsh et al., 1998a). In response to short-duration (<40 minutes), high-intensity (>80% $\dot{V}O_{2max}$) exercise, plasma GLN concentrations are either unchanged or marginally increase (5-20%; Table 10). In these studies, GLN concentrations later declined 10-20% below basal levels when re-assessed 2-10 hours into recovery (Walsh et al., 1998b; Robson et al., 1999). It is speculated that the initial increase in plasma GLN concentration with this form of exercise is caused by glutamate acting as a sink for enhanced intramuscular ammonia production (Sewell et al., 1994) or exercise-induced haemoconcentration (Walsh et al., 1998a).

In comparison to short-duration, high-intensity exercise, plasma GLN concentrations decline (10-25%) during and in the early hours following prolonged duration (> 2 hours), sub-maximal (50-75% $\dot{V}O_{2max}$) exercise (Table 10). The influence of heat stress on plasma GLN responses to exercise has never been directly examined, however, 60-90

minutes of moderate intensity exercise (60% $\dot{V}O_{2max}$) in T_{amb} ≥ 35°C consistently reduces plasma GLN concentrations, whereas similar exercise in temperate environments appears to have less influence (Table 10). The suppression of plasma GLN concentrations after prolonged exercise is probably caused by a combination of factors (Walsh et al., 1998a). These include increased: (1) gluconeogenesis to support metabolism and acute-phase protein production; (2) renal uptake to buffer metabolic acidosis; and (3) direct metabolism by activated leukocytes in response to stimulation by cortisol and growth hormone (Gleeson, 2008). Given that exercise-induced intramuscular glycogen utilisation (Fink et al., 1975; Febbraio et al., 1994) and stress hormone release (Niess et al., 2003; Rhind et al., 2004) are amplified by environmental temperature, there is a mechanistic basis to anticipate a greater depletion of plasma GLN in response to exertional-heat stress.

Plasma GLN reflects the net balance between intracellular utilisation and release by organ tissues. In response to exercise, most research focuses on measuring GLN kinetics in skeletal muscle given it is the major derivative of resting plasma GLN (Ruderman, 1975) and has potential to impact exercise performance (Coqueiro et al., 2019). Early research by Eriksson et al. (1985) reported an intensity dependant (35%, 50%, 80% $\dot{V}O_{2max}$) increase in lower-limb GLN release during 15 minutes of cycling, whilst Sahlin et al. (1990) reported a 2-fold increase in GLN release during exhaustive cycling at 75% $\dot{V}O_{2max}$. In Sahlin et al. (1990) sustained GLN release resulted in a *circa* 10% depletion of intramuscular GLN concentrations on exercise termination, a finding that is supported by Rennie et al. (1981) who reported a *circa* 33% reduction in intramuscular GLN concentrations following 3.75 hours low-intensity (50% $\dot{V}O_{2max}$) cycling. In rodents, plasma, skeletal muscle, hepatic and renal GLN concentrations were each suppressed by 20%, 37%, 65% and 13%, respectively, in response to 30 minutes exhaustive swimming (Christophe et al., 1971). Likewise, comparable reductions in plasma (17%), skeletal muscle (19%) and hepatic (43%) GLN concentration were shown in rodents ran to exhaustion at 28 $m \cdot min^{-1}$ (Dohm et al., 1981). The influence of exercise on GI GLN concentrations has never been examined. These findings suggest depletion of plasma GLN following prolonged strenuous exercise likely contributes to the suppression of intracellular GLN concentrations during and shortly following exercise (Walsh et al., 1998a).

Table 10. Influence of exercise-(heat) stress on systemic glutamine concentration.

Author	Subjects	Exercise Protocol	Glutamine (Δ pre-to-post exercise)
Nava et al. (2019)	7 male, 4 female (MT)	87 minutes intermittent firefighting stimulation (fed) (~60% $\dot{V}O_{2max}$) in T_{amb} 38 °C (35% RH)	-183 µmol·l^{-1} (-19%) $^{s, c, \#}$
Borgenvik et al. (2012)	9 male (HT)	24 ultra-endurance trial (running, cycling, kayaking) (fed) in 18-22 °C (RH not reported)	-128 µmol·l^{-1} (-23%) ^{s}
Walsh et al. (2000)	7 male (HT)	120 minutes cycling at 60% $\dot{V}O_{2max}$ (fasted) in T_{amb} 22 °C (66% RH)	-116 µmol·l^{-1} (-22%) $^{s, c}$
Zuhl et al. (2015)	2 male, 5 females (LT/MT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 30 °C (12-20% RH)	~-113 µmol·l^{-1} (-17%) $^{ns, c,}$
Rennie et al. (1981)	4 male (LT-HT)	3.75 hours treadmill running at 50% $\dot{V}O_{2max}$ (fed) in T_{amb} not reported	-112 µmol·l^{-1} (-19%) ^{s}
Rohde et al. (1996)	8 male (MT-HT)	2.5 km swim, 81 km cycle, 19 km run (fed) in T_{amb} not reported	~-100 µmol·l^{-1} (-20%) ^{s}
Pugh et al. (2017b)	10 male (MT)	60 minutes at 70% $\dot{V}O_{2max}$ running (fasted) in T_{amb} 30 °C (4-45% RH)	~-100 µmol·l^{-1} (-10%) $^{ns, c}$
Parry-Billings et al. (Part A) (1992)	24 male (N/A)	42.2 km marathon (fed) in T_{amb} not reported	-96 µmol·l^{-1} (16%) $^{s, c}$
Zuhl et al. (2014)	4 male, 3 female (LT/MT)	60 minutes running at 70% $\dot{V}O_{2max}$ (fasted) in T_{amb} 30 °C (12-20% RH)	~-78 µmol·l^{-1} (-10%) $^{ns, c}$
Sawaki et al. (Part A) (2004)	11 male (HT)	42.2 km marathon (fed) in T_{amb} not reported	-71 µmol·l^{-1} (-10%) ^{s}
Moore et al. (2019)	5 male and 3 female (MT)	78 minutes intermittent firefighting stimulation (~60% $\dot{V}O_{2max}$) (fed) in T_{amb} 35 °C (35% RH)	~-67 µmol·l^{-1} (-22%) $^{s, \#}$
Kargotich et al. (Part A) (2004)	8 male (HT)	15x 100 metre swimming intervals at 90% of peak freestyle time (fed) in T_{amb} not reported	-56 µmol·l^{-1} (-5%) ^{ns}
Krzywkowski et al. (2001)	8 male (MT)	120 minutes cycling at 75% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	-50 µmol·l^{-1} (-15%) ^{s}
Gleeson et al. (1998)	12 male (LT)	60 minutes cycling at 70% $\dot{V}O_{2max}$ (fed) in 18 °C (50% RH)	~-20 µmol·l^{-1} (-3%) $^{ns, c}$
Walsh et al. (1998b)	8 male (MT-HT)	20 x 1 minute cycle sprints at 100% $\dot{V}O_{2max}$ (fasted) (separated by 2 mins recovery at 30% $\dot{V}O_{2max}$) in T_{amb} not reported	-18 µmol·l^{-1} (3%) ^{ns}
Lehmann et al. (1995)	9 male (HT)	Ultra-triathlon (7.5 km swim, 360 km cycle and 85 km run) (fed) in T_{amb} not reported	-13 µmol·l^{-1} (1%) ^{ns}

Decombaz et al. (1979)	8 male (MT-HT)	100-km ultramarathon (fed) in 20 °C (RH not reported)	(-11%) [s]
Hoffman et al. (2010)	10 male (MT)	Cycling to exhaustion at 75% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	0 $\mu mol \cdot l^{-1}$ (0%) [ns]
Wilkinson et al. (2006)	8 male (MT)	90 minutes cycling at 65% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	5 $\mu mol \cdot l^{-1}$ (1%) [ns]
Robson et al. (Part A) (1999)	18 male (MT-HT)	37 ± 18 minutes cycling at 80% $\dot{V}O_{2max}$ to fatigue (fasted) in T_{amb} not reported	~10 $\mu mol \cdot l^{-1}$ (2%) [ns]
Kargotich et al. (Part B) (2004)	8 male (HT)	15x 100 metre swimming intervals at 70% of peak freestyle time (fed) in T_{amb} not reported	22 $\mu mol \cdot l^{-1}$ (2%) [ns]
McCormack et al. (2015)	12 male (MT)	60 minutes running at 75% $\dot{V}O_{2max}$ (fasted) in 23 °C (44% RH)	~30 $\mu mol \cdot l^{-1}$ (4%) [ns, c]
Sewell et al. (Part A) (1994)	7 male, 2 female (MT)	1-minute running at 20 $km \cdot h^{-1}$ (fasted) in T_{amb} not reported	35 $\mu mol \cdot l^{-1}$ (5%) [s]
Katz et al. (1986)	8 male (LT-MMT)	Cycle to exhaustion (5.2 minutes) at 97% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	41 $\mu mol \cdot l^{-1}$ (8%) [ns]
Parry-Billings et al. (Part C) (1992)	4 male (N/A)	Cycle to exhaustion at 73% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	48 $\mu mol \cdot l^{-1}$ (8%) [ns]
Parry-Billings et al. (Part B) (1992)	12 male (N/A)	30 km treadmill run (fasted) in T_{amb} not reported	53 $\mu mol \cdot l^{-1}$ (8%) [ns]
Kargotich et al. (2005)	20 male (LT)	15 x 1 minute cycle sprints at 100% $\dot{V}O_{2max}$ (fasted) (separated by 2 mins recovery at 30% $\dot{V}O_{2max}$) in T_{amb} not reported	56 $\mu mol \cdot l^{-1}$ (5%) [ns]
van Hall et al. (1998)	8 male (HT)	Alternating 2-minute cycling at 50% $watt_{max}$ and 80% $watt_{max}$ until exhaustion (59-140 minutes) in (fasted) T_{amb} not reported	~60 $\mu mol \cdot l^{-1}$ (9%) [ns]
Parry-Billings et al. (Part D) (1992)	10 male (N/A)	10 x 6 second cycle sprints with 30 seconds recovery (fased) in T_{amb} not reported	60 $\mu mol \cdot l^{-1}$ (11%) [s]
Eriksson et al. (Part A) (1985)	11 male (LT-MT)	15 minutes cycling at 55% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	64 $\mu mol \cdot l^{-1}$ (13%) [s]
Sawaki et al. (Part B) (2004)	7 male (HT)	21.1 km half-marathon (fed) in T_{amb} not reported	78 $\mu mol \cdot l^{-1}$ (13%) [s]
Eriksson et al. (Part B) (1985)	11 male (LT-MT)	15 minutes cycling at 35% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	78 $\mu mol \cdot l^{-1}$ (14%) [s]

Robson et al. (Part B) (1999)	18 male (MT-HT)	164 ± 23 minutes cycling at 55% $\dot{V}O_{2max}$ to fatigue (or 3 hours) in (fasted) T_{amb} not reported	80 µmol·l^{-1} (15%) ^{ns}
Maughan and Gleeson, (1988)	5 male (LT)	Cycling to exhaustion at 70% $\dot{V}O_{2max}$ (~120 minutes) in (fasted) T_{amb} not reported	80 µmol·l^{-1} (15%) ^{s}
Sewell et al. (Part B) (1994)	7 male, 2 female (MT)	Running to exhaustion (210 seconds) at 20 km·h^{-1} in (fasted) T_{amb} not reported	95 µmol·l^{-1} (14%) ^{s}
Eriksson et al. (Part C) (1985)	11 male (LT-MT)	15 minutes cycling at 80% $\dot{V}O_{2max}$ (fasted) in T_{amb} not reported	128 µmol·l^{-1} (19%) ^{s}
Babj et al. (1983)	8 male (LT-MT)	10 minutes incremental cycling at 25, 50 and 75% $\dot{V}O_{2max}$; then at 100% $\dot{V}O_{2max}$ until exhaustion (fasted) in T_{amb} not reported	159 µmol·l^{-1} (28%)

Where: LT = Low-trained (35-49 ml·kg·min^{-1} $\dot{V}O_{2max}$); MT = Moderate-trained (50-59 ml·kg·min^{-1} $\dot{V}O_{2max}$); HT = High-trained (60+ ml·kg·min^{-1} $\dot{V}O_{2max}$). s = significant change post-exercise ($p < 0.05$); ns = non-significant change post-exercise ($p > 0.05$); c = control trial of study. # Where data have been converted from mg·l^{-1} through standard conversions (molecular weight = 146.14 g·mol^{-1}).

2.14 Glutamine on Gastrointestinal Barrier Integrity

Over recent decades, numerous investigations have examined the efficacy of exogenous GLN supplementation during severe catabolic stress. The GI tract is the major site for GLN metabolism, whereby adequate GLN availability might be required to maintain GI barrier integrity (Wang et al., 2014). There are several potential mechanisms via which exogenous GLN supplementation is hypothesised to protect GI barrier integrity. These include: (1) increased cellular proliferation and reduced cellular apoptosis; (2) heat shock protein biosynthesis; (3) glutathione biosynthesis; and (4) stabilising GI TJ protein complexes (Ziegler et al., 2000; Rhoads and Wu, 2009).

2.14.1 Cellular Proliferation and Apoptosis

The GI epithelium is a plastic tissue that is completely regenerated every 3-5 days (Potten and Loeffler, 1990). This process is fulfilled by cellular proliferation and apoptosis (Rhoades et al., 1997; Bach et al., 2000). Proliferation defines the process by which cells divide, whereas apoptosis defines the process of programmed cell death (van Der Flier and Clevers, 2009). In the GI tract, cellular proliferation broadly involves intestinal stem cell (e.g. Lgr5, Bmi1) division in the intestinal crypts, before differentiation into cells with

specialist functions (Wang et al., 2014). Spontaneous apoptosis of mature intestinal cells is required to maintain healthy GI morphology (Watson and Prichard, 2000). Thus, during phases of tissue growth, cellular proliferation overcomes the rate of apoptosis, however, apoptosis in dominant during phases of tissue atrophy (Kim and Kim, 2017). Powerful stimuli that modulate GI epithelial proliferation and apoptosis include: luminal nutrient availability, digestion, GI microbial products and cellular adhesion (Wang et al., 2014).

GLN supplementation enhances *in vitro* cellular proliferation in intestinal cells in a dose-dependent manner (Wischmeyer et al., 1997; Wiren et al., 1998; Demarco et al., 1999). In comparison, pharmacological depletion of endogenous GLN availability blunts cellular proliferation (Papaconstantinou et al., 1998; Larson et al., 2007). Several mechanisms might explain how GLN modulates GI cellular proliferation. First, GLN is the preferential respiratory fuel for GI epithelial cells (Curi et al., 2005). Second, GLN is a precursor for purine and pyrimidine nucleotide synthesis, required for DNA synthesis (Wang et al., 2014). Third, GLN up-regulates the expression of ornithine decarboxylase, a key enzyme required for DNA and protein synthesis (Rhoades and Wu, 2009). Fourth, GLN upregulates expression of mitogenic signalling pathways for gene transcription (Rhoads et al., 1997, 2000). Finally, GLN increases both the protein abundance and/or augments the action of growth factors involved in stimulating intestinal proliferation, such as epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) (Ko et al., 1993; Booth et al., 1995; Avissar et al., 2008). Furthermore, recent evidence has shown GLN supplementation enhances intestinal stem cell differentiation via an unknown mechanism (Chen et al., 2018a, 2018b).

Concordant with the influence of GLN on *in vitro* cellular proliferation, GLN has known anti-apoptotic properties (Fuchs and Bode, 2006). Most of this research has focused on the influence of pharmacological depletion of GLN, which increases spontaneous apoptosis (Ko et al., 1993; Papaconstantinou et al., 1998; Larson et al., 2007). Similarly, GLN supplementation effectively reduced toxin-induced apoptosis (Carneiro et al., 2006), sodium laurate-induced apoptosis (Takayama et al., 2009) and importantly heat induced apoptosis (Chow and Zhang, 1998) in a dose-dependent manner (Wang et al., 2014). Several mechanisms have been proposed to explain how GLN modulates GI cellular apoptosis. First, GLN downregulates activation of the caspase family of protease enzymes, which modulate apoptosis (Deniel et al., 2007; Larson et al. 2007). Second, GLN protects the endoplasmic reticulum, which is a major signal for apoptosis (Crespo et al., 2012). Third,

GLN blocks the action of pro-inflammatory cytokines (e.g. TNF-α) on apoptosis inducing ligands (Evans et al., 2003). Fourth, GLN stimulates autophagy via influencing mechanistic target of rapamycin (mTOR) signalling (Sakiyama et al., 2009). Autophagy provides a protective response against GI stress (Kim and Kim, 2017). Finally, GLN enhances the expression of heat shock proteins and glutathione, which both modulate apoptotic cell death (Circu and Aw, 2011).

In vivo evidence in rodents, livestock and humans has repeatedly shown chronic GLN supplementation to induce positive morphological adaptations (e.g. intestinal weight, villus height, crypt depth) of GI anatomy (Wong et al., 2014). These studies include evidence in rats (Mandir and Goodlad, 1999; Potsic et al., 2002) and swine (Haynes et al., 2009), which found addition of GLN (1-4 $g \cdot kg \cdot d^{-1}$) into standard feed enhanced both intestinal weight and villus height over a 6-14 day period. In humans, the data is equally compelling in the few studies that have examined the influence of GLN supplementation (0.30-0.50 $g \cdot kg \cdot d^{-1}$) within isocaloric total parenteral nutrition on GI atrophy in the days following major abdominal surgery (van der Hulst et al., 1993; Tang et al., 2007).

2.14.2 Heat Shock Proteins

Heat Shock Proteins (HSPs) are a conserved family of stress proteins, ubiquitous across all living organisms (Feder and Hofmann, 1999). Individual HSPs are classified into seven families based on their molecular weight (10-110 kDa) and function across all major cell compartments. Broadly, these functions include the cytoprotection of stable proteins, folding and chaperoning of newly synthesised proteins, selective renaturation or degradation of denatured proteins, and antigen presentation to T-lymphocytes (Tamura et al., 2012; Saibil, 2013). In states of health, constitutive HSPs perform general cell surveillance roles, but inducible HSPs are largely inactive bound to heat shock factors (HSFs) within the cytosol (Feder and Hofmann, 1999). However, upon stress exposure (e.g. temperature, oxygen, pH), inducible HSPs are activated following HSF detachment (Santoro, 2000). This process in widely-termed the *stress response*. Concurrently, trimer formation of unbound HSFs modulates movement into the nucleus, where subsequent promoter binding to the heat shock element triggers HSP gene transcription (Feder and Hofmann, 1999). When HSP gene transcription is of significant magnitude to increase intracellular protein expression, greater cellular resilience is afforded upon subsequent stressful insult. This process is termed *stress tolerance* (Benjamin and Mcmillan, 1999).

Scientific understanding of the functions performed by HSPs have increased exponentially since discovery in *Drosophila* salivary gland cells following mild heat-stress over half-a-century ago (Ritossa, 1962). In the context of the GI tract, *in vitro* and *in vivo* evidence support the concept that HSPs (specifically HSP25 and HSP70) protect structural integrity in response to stress (Petrof et al., 2004; Dokladny et al., 2015). *In vitro*, initiation of HSP stress tolerance through acute thermal pre-conditioning, strengthens GI barrier integrity upon exposure to subsequent more severe hyperthermia (Moseley et al., 1994; Dokladny et al., 2006; Yang et al., 2007). Relevantly, the reported benefits of pre-conditioning on GI barrier integrity appear directly related to the increase in HSP expression, given that both pharmacological blockade (Ropeleski et al., 2003; Dokladny et al., 2006) and genetic knock-down (Musch et al., 1999; Liu et al., 2003) of HSP transcription attenuated this response. In humans, HSP expression displays a proximal-to-distal concentration gradient indicative of greater microbial concentrations in the large intestine (Arvans et al., 2005; Hu et al., 2010). Induction of the HSP response through thermal pre-conditioning can reduce mortality following normally lethal endotoxin injection (Ryan et al., 1992; Hotchkiss et al., 1993) and histological GI injury following ischemic shock (Stojadinovic et al., 1995, 1997; Goldhill et al., 1999).

GLN was the first discovered non-toxic pharmacological regulator of HSP expression, with seminal research reporting GLN to increase HSP expression in heat-stressed *Drosophila* Kc cells in a dose-dependent manner (Sanders and Kon, 1991, 1992). In intestinal cells, GLN upregulates HSP expression in non-stressed cells in a dose-dependent manner (1-10 mM) in some (Wischmeyer et al., 1997; Chow and Zhang, 1998; Musch et al., 1998), but not all (Lindermann et al., 2001; Zuhl et al., 2014) studies. These inconsistencies are hard to fully elucidate, but potentially relate to discrepancies in the cell line and/or HSP isoform assessed (Kallweit et al., 2012). Crucially, when intestinal cells are subject to severe hyperthermia, GLN universally enhanced HSP expression and protected GI barrier integrity. The benefits of GLN on GI barrier integrity have been directly attributed to the HSP response, given that both pharmacological blockade (Wischmeyer et al., 1997) and genetic knockdown (Musch et al., 1998) of HSP transcription attenuate the benefits GLN on GI barrier integrity. The regulatory mechanisms through which GLN increases HSP expression are poorly understood, but appear independent of GLN metabolism (Phanvijhitsiri et al., 2006). Instead, the influence of GLN on HSF-1 activity likely mediates increased HSP expression (Leite et al., 2016). Unlike GLN, supplementation with other

amino acids (e.g. alanine, arginine, glycine, histidine, proline) do not modulate HSP expression in GI epithelial cells (Phanvijhitsiri et al., 2006).

Translational studies have attempted to replicate *in vitro* evidence of GLN on GI barrier function *in vivo*. In a preliminary study, infusion of a single intravenous GLN bolus (0.15-0.75 g·kg^{-1}) in unstressed rats enhanced HSP expression in a dose dependant manner across multiple organs (Wischmeyer et al., 2001). Specific to the GI tract, increased colonic HSP expression was evident between 1-72 hours following GLN administration, though iliac HSP expression remained unchanged throughout. In the second part of this study, GLN supplementation enhanced HSP expression in the colon in response to endotoxin injection, which was negatively associated with the severity of histological tissue injury (Wischmeyer et al., 2001). In the context of HS, Singleton and Wischmeyer (2006) found 5-days of high dose (0.90 g·kg^{-1}) oral GLN enhanced HSF-1 expression in the cecum at rest, which subsequently resulted in increased HSP expression between 1-4 hours following passive hyperthermia. Importantly, in this study GLN supplementation was negatively associated with GI permeability, endotoxemia and 5-day mortality rate. In healthy humans, ethical constrains limit assessment of GLN supplementation on GI intracellular HSP expression, though acute (1x 0.90 g·kg·FFM^{-1}; Zuhl et al., 2015) and chronic (7x 0.90 g·kg·FFM·day^{-1}; Dokladny et al., 2013) GLN supplementation both enhance HSP expression in peripheral blood mononuclear cells both at rest (36% and 90%, respectively) and following exertional-heat stress (30 and 75%, respectively).

2.14.3 Glutathione

Glutathione (GSH) is a tripeptide low-molecular weight thiol present in most human cells (Meister, 1984, 1988). The GSH system protects cell membranes against oxidative damage caused by unstable ROS and oxoaldehydes. Major functions of GSH that support oxidation-reduction reactions include: antioxidant defence; storage and transport of cysteine; leukotriene and prostaglandin metabolism; and regulation of gene expression, cell proliferation, apoptosis and cytokine production (Wu et al., 2004). GSH is nonenzymatically oxidised to glutathione disulphide (GSSG) by ROS. The GSH:GSSG ratio is a primary regulator of cellular redox potential (Mates et al., 2002). During catabolic stress (e.g. sepsis), suppressed intracellular GSH (e.g. in skeletal muscle) is associated with poor clinical outcome (Hammarqvist et al., 1997; Westman et al., 2006; Rodas et al., 2012). In rodent models, strenuous aerobic exercise (25 m·min^{-1}) decreases total plasma/tissue GSH

to a similar magnitude shown with clinical trauma, which could leave GI tissue more susceptible to oxidative injury (Lew et al., 1985; Aoi et al., 2015).

GLN is an efficient precursor of GSH in the GI tract (Cao et al., 1998). During catabolic stress, GSH precursors $_{\text{L}}$-glycine and $_{\text{L}}$-cysteine are maintained intracellularly, which suggests that $_{\text{L}}$-glutamate availability is the step-limiting factor for GSH depletion (Wu et al., 2004). Given $_{\text{L}}$-glutamate is a product of GLN hydrolysis, many studies have examined the efficacy of exogenous GLN supplementation as a safe technique to increase intracellular GSH concentrations during catabolism (Amores-Sanchez and Medina, 1999). For example, in rodents, acute strenuous exercise (2 hours at 25 m·min^{-1}) and chronic exercise training (1 h·day^{-1} at 25 m·min^{-1}) both depleted intracellular $_{\text{L}}$-glutamate concentrations by 10-50% in the soleus, gastrocnemius, and liver, which could be completely abolished with 21 days prior high-dose (1 g·kg·d^{-1}) intravenous GLN supplementation (Cruzat and Tirapegui, 2009; Petry et al., 2014). Importantly, GLN supplementation also increased the GSH/GSSG ratio and reduced biomarkers of oxidative multi-organ injury (e.g. creatine kinase, myoglobin). This finding is supported by evidence in both animals (Hong et al., 1992; Yoshida et al., 1995; Roth et al., 2002) and humans (O'Riordain et al., 1996; Flaring et al., 2003; Eroglu, 2009) demonstrating acute high-dose (~0.5 g·kg·d^{-1}) GLN supplementation to enhance both intracellular GSH concentrations, and reduce oxidative tissue damage in the days following abdominal surgery, chemotherapy and sepsis.

In the small intestine, GSH concentrations are *circa* 2-fold greater than plasma, but 33% lower than in the liver, which is the primary location for GSH synthesis (Kelly, 1993). The influence of GSH supplementation on *in vitro* and *in vivo* GI barrier integrity has been the subject of direct research attention. Lash et al. (1986) demonstrated incubation of rat small intestinal epithelial cells for 20 minutes with 1 mM GSH enhanced intracellular GSH concentrations and protected against chemical oxidative injury. In support, Kelly (1993) and Rao et al. (2000) demonstrated pre-incubation of rat intestinal epithelial cells and human Caco-2 cells with GSH protected against hydrogen peroxide induced oxidative injury and reduced GI permeability. *In vivo*, injecting rats with the inflammatory agent Turpentine, reduced small intestinal villus height and crypt depth, however, simultaneous GSH treatment was able to restore GI histology (Belmonte et al., 2007). In rodents, several studies have shown GLN supplementation to increase intracellular GSH availability and reduce biomarkers of oxidative stress (e.g. lipid peroxidation) in GI tissue, when subjected

to experimental ischemia-reperfusion injury (Harward et al., 1994; Manhart et al., 2001). Whether blunted oxidative stress translates to strengthened GI barrier integrity with GLN supplementation was not confirmed in these studies.

2.14.4 Paracellular Tight Junction Structure

TJs are apical structures that regulate the paracellular passage of molecules across the cell membrane (Groschwitz and Hogan, 2009). They are dynamic structures that interact with external stimuli to modulate their tightness (Harhaj and Antoneth, 2004). Downstream regulation of the assembly, maintenance, and disassembly of TJs is influenced by protein kinase activation (Ulluvishewa et al., 2011). Broadly, the activation of protein kinase signalling pathways phosphorylates TJ proteins, which can alter GI barrier integrity (Rigor et al., 2013). For example, activation of protein kinase C upregulates protein expression of ZO-1, occludin, and claudin-1 (Koizumi et al., 2008).

GLN is a positive regulator of GI TJ protein expression (Wang et al., 2014). In human Caco-2 cells, GLN deprivation and GLN synthetase inhibition both markedly reduced TJ protein expression (ZO-1, claudin-1, occludin) and increased paracellular permeability (DeMarco et al. 2003; Li et al. 2004). In DeMarco et al. (2003), the negative influence of GLN deprivation could be rescued with subsequent GLN supplementation within 1-hour. In various stress models, GLN supplementation protected *in vitro* TJ protein expression, TER and paracellular permeability (Kim and Kim, 2017). In Seth et al. (2004), GLN maintained the expression of ZO-1, occludin and claudin-1 in Caco-2 cells in a time- and dose-dependent manner when administered prior to acetaldehyde exposure. Notably, these benefits could not be replicated in cells supplemented with matched doses of alanine, lysine, or arginine. Later studies reported similar benefits of GLN supplementation in doses > 2 mM on Caco-2 cells in response to 75-minutes of hyperthermia at 41.8 °C (Zuhl et al., 2014). Within the current literature, it is unclear whether GLN enhanced TJ protein synthesis, reduced TJ degradation, or both (Wang et al., 2014). *In vivo*, 1-4 weeks of additional GLN supplementation to standard diets of rodents and swine increased basal GI TJ protein expression (Xing et al., 2017) and protected GI integrity to chemical toxic injury (Ewaschuk et al., 2011; Beutheu et al., 2014).

2.15 Glutamine, Gastrointestinal Barrier Integrity and Exertional-Heat Stress

In the field of sports-nutrition, GLN supplementation lost popularity as an ergogenic aid after the early 2000s, given its apparent inability to improve either, body composition, exercise performance or post-exercise immune function (Gleeson, 2008). However, in recent years, scientific interest in GLN supplementation was re-established on the premise that it can protect GI barrier integrity during exertional-heat stress (Table 11).

The seminal study to examine the influence of GLN supplementation on GI barrier integrity following exercise was undertaken by Lambert et al. (2001). In this randomised, cross-over study, highly trained participants ran in thermoneutral conditions (22 °C/48% RH) for 1-hour at moderate intensity (70% $\dot{V}O_{2max}$), ingesting either water, carbohydrate (0.18 g·kg·BM^{-1}) or carbohydrate plus GLN (0.018 g·kg·BM^{-1}) every 10-minutes. However, post-exercise urine DSAT responses (~0.045 ± 0.020) were not different between the three nutritional interventions. In the second part of this study, participants repeated the experiment having ingested 1300 mg of aspirin 12-hours and immediately before exercise. Here, GI permeability was significantly increased with water ingestion (DSAT = ~ 0.185 ± 0.030), but this response was attenuated to a similar extent with both carbohydrate and carbohydrate plus GLN supplementation by approximately 32% and 38%, respectively. Unfortunately, the conclusions of this study are limited given that the severity of thermoregulatory strain was mild (T_{core} = 38.4 ± 0.2°C), GLN was not assessed in isolation and the total GLN dose was relatively small (8-12 grams).

Following the null data reported by Lambert et al. (2001), research focus moved away from low dose GLN supplementation intending to maintain endogenous GLN availability, to provision of large oral doses aimed to stimulate molecular pathways that strengthen GI barrier integrity. In Zuhl et al. (2014), the influence of 7 days high-dose oral GLN (3x 0.30 g·kg·fat free mass [FFM]·d^{-1}) or taste-matched non-calorific placebo supplementation on GI permeability were compared following 1-hour of moderate intensity (70% $\dot{V}O_{2max}$) running in the heat (35 °C/20% RH). This protocol induced more pronounced thermoregulatory strain (T_{core} peak = 39.5 °C) than in Lambert et al. (2001), which lead to a 172% increase in GI permeability above baseline in the placebo trial (5-hour urine DSAT = 0.060 ± 0.047 *versus* 0.022 ± 0.008). In comparison, GLN supplementation completely attenuated the increase in GI permeability in response to exertional-heat stress (0.027 ± 0.007). Mechanistically, basal plasma GLN concentrations increased by 128% following GLN supplementation, before returning to a similar concentration to the placebo

condition after exertional-heat stress. These findings suggest GLN is metabolised at a faster rate when bioavailability is increased, although this study was unable to assess specific molecular pathways through which GLN improved GI permeability. In follow-up, Zuhl et al. (2015) examined whether 7-days GLN supplementation was necessary to protect GI barrier integrity, or whether the same benefits could be achieved with a single acute GLN bolus (0.90 $g \cdot kg \cdot FFM^{-1}$) taken 2-hours prior to exertional-heat stress. Herein using an identical experimental design, GI permeability was increased above rest (0.02 ± 0.01) following the exertional-heat stress in the placebo condition (0.06 ± 0.01), although this response was again attenuated with GLN supplementation (0.04 ± 0.02).

Having established that acute GLN supplementation may protect GI barrier integrity in response to exertional-heat stress, replication studies have since attempted to optimise aspects of the intervention. Given that 0.90 $g \cdot kg \cdot FFM^{-1}$ of GLN is above the observed safe dose of 14 $g \cdot d^{-1}$ recommended for human consumption (Shao and Hathcock, 2008), Pugh et al. (2017b) examined whether lower GLN doses (0.25 and 0.5 $g \cdot kg \cdot FFM^{-1}$) offered efficacy. This study utilised Zuhl et al.'s (2015) methodology. Herein, plasma DSAT increased *circa* 200% following exertional-heat stress in the placebo trial. Applying magnitude-based inferences Pugh et al. (2017b) concluded GLN supplementation reduced GI permeability in a dose-dependent manner. Despite this conclusion, the effect sizes were only categorised as: *likely moderate*, *likely small* and *very likely moderate* in the 0.25, 0.50 and 0.90 $g \cdot kg \cdot FFM^{-1}$ GLN conditions, respectively. Comparable conclusions were made for plasma I-FABP responses, with a *likely small* post-EHST suppression in both the 0.50 and 0.90 $g \cdot kg \cdot FFM^{-1}$ GLN trial. The most recent contribution in this field was made by Osborne et al. (2019b) who investigated the influence of 0.90 $g \cdot kg \cdot FFM^{-1}$ GLN ingested 1-hour before a 20-km cycling time trial (~33 minutes) in the heat (35 °C/50% RH) on I-FABP. These authors found no effects of GLN, though speculated their conclusion to be underpowered, given divergence in the within-condition effect sizes favouring GLN. In this study, peak T_{core} responses were low (~38.0°C), which contributed to the small effect of exercise on I-FABP.

Practical recommendations support the use of acute high-dose (0.90 $g \cdot kg \cdot FFM^{-1}$) oral GLN supplementation 2-hours prior to sub-clinical exertional-heat stress to protect GI barrier integrity. This nutritional intervention has clear ecological relevance in occupational settings, especially in circumstances where implementation of alternative mitigation strategies is infeasible. Examples include: short-notice deployment without opportunity for heat-acclimatisation, physically unfit individuals and during field operations where cooling

interventions (e.g. cold water, electric fans) are unavailable. Despite the potential benefits of GLN supplementation on GI barrier integrity, further research is required to confirm these findings following more severe exertional-heat stress protocols and extending biomarker analysis to include secondary measures of GI MT. The oral tolerance of high-dose GLN supplementation is a safety and performance concern that requires closer examination before widespread application. Likewise, a limitation of previous research has been the performance of trials in the fasted state, whereby positive findings are potentially attributable to improvement in post-prandial splanchnic perfusion, rather than any direct benefits GLN *per se*.

Table 11. Influence of Glutamine Supplementation on GI barrier Integrity Responses to Exertional-Heat Stress.

Author	Subjects	Exercise Protocol	Glutamine Supplementation	Peak T_{core} (°C)	L/M or L/R	I-FABP Δ pre-to-post exercise (%)
Lambert et al. (2001)	12 male, 4 female (HT)	60 minutes at 70% $\dot{V}O_{2max}$ running (fasted) in T_{amb} 22 °C (48% RH). Carbohydrate (0.018 g·kg^{-1} BM) ingested every 10 minutes during exercise with or without GLN (0.018 g·kg^{-1} BM)	0.00 g·kg·BM^{-1}	38.4	0.045 ^{nb}	-
			0.11 g·kg·BM^{-1}	38.4	0.045 ^{nb}	-
Zuhl et al. (2014)	4 male, 3 female (LT/MT)	60 minutes at 70% $\dot{V}O_{2max}$ running (fasted) in T_{amb} 35 °C (20% RH). GLN ingested in 3x 0.30 g·kg·FFM·day^{-1} for 7 days in 500ml of water plus lemon squash (4:1). Final dose 2-hours pre exercise	0.00 g·kg·FFM^{-1}	39.4	0.060 ^{s}	-
			0.90 g·kg·FFM^{-1}	39.5	0.027 ^{ns}	-
Zuhl et al. (2015)	2 male, 5 female (LT/MT)	60 minutes at 70% $\dot{V}O_{2max}$ running (fasted) in T_{amb} 35 °C (10-20% RH). GLN ingested 2-hours pre exercise	0.00 g·kg·FFM^{-1}	39.5	0.060 ^{s}	-
			0.90 g·kg·FFM^{-1}	39.3	0.040 ^{ns}	-
Pugh et al. (2017b)	10 male (MT)	60 minutes at 70% $\dot{V}O_{2max}$ running (fasted) in T_{amb} 30 °C (40-45% RH). GLN ingested 2-hours pre exercise	0.00 g·kg·FFM^{-1}	38.5	~0.090	~250 pg·ml^{-1} (71%)
			0.25 g·kg·FFM^{-1}	38.5	~0.065	~280 pg·ml^{-1} (78%)
			0.50 g·kg·FFM^{-1}	38.6	~0.070	~150 pg·ml^{-1} (50%)
			0.90 g·kg·FFM^{-1}	38.4	~0.055	~200 pg·ml^{-1} (80%)
Osborne et al. (2019b)	12 male (MT-HT)	33 minutes (20 km) cycling time trial (fasted) in 35 °C (50% RH). GLN ingested in 500ml of water plus lemon squash (4:1) 1-hour pre exercise	0.00 g·kg·FFM^{-1}	38.0	-	440 pg·ml^{-1} (83%)
			0.90 g·kg·FFM^{-1}	38.0	-	205 pg·ml^{-1} (34%)

Where: LT = Low-trained (35-49 ml·kg·min^{-1} $\dot{V}O_{2max}$); MT = Moderate-trained (50-59 ml·kg·min^{-1} $\dot{V}O_{2max}$); HT = High-trained (60+ ml·kg·min^{-1} $\dot{V}O_{2max}$). s = significant change post-exercise ($p < 0.05$); ns = non-significant change post-exercise ($p > 0.05$); nb = no basal data.

2.16 Summary

As homeotherms, humans attempt to regulate T_{core} around a set-point of 37.0 ± 1.0°C. Regulation of thermal equilibrium is achieved through combined behavioural and physiological heat transfer pathways. EHS is the most severe condition along a continuum of heat-related illnesses. In recent history, the mean annual incidence of EHS is *circa* 0.75/1000 personnel·year^{-1} in the United Kingdom Armed Forces. This disease burden has negative implications on personnel health and operational performance. Current heat-illness policy documents (e.g. JSP 539) principally advocate a thermoregulatory approach to EHS management, despite growing recognition that GI MT contributes to disease pathophysiology. A variety of techniques are available to assess GI barrier integrity and MT *in vivo*, which have been applied in contemporary thermal exercise physiology research to examine the efficacy of potential EHS countermeasures. GLN is a conditionally essential amino acid, which performs important regulatory functions in the maintenance of GI barrier integrity. Recent evidence supports acute high-dose (0.90 g·kg·FFM^{-1}) oral GLN supplementation 2-hours prior to sub-clinical exertional-heat stress to protect GI barrier integrity, although confirmatory further research is required to optimise this intervention.

2.16.1 Thesis Aims

The primary aims of this thesis were to determine:

1) The reliability of gastrointestinal barrier integrity and microbial translocation biomarkers at rest and following exertional-heat stress (chapter 4)

2) The influence of aerobic fitness on gastrointestinal barrier integrity and microbial translocation in response to a military exertional-heat stress test (chapter 5)

3) The gastrointestinal tolerance of low, medium and high dose acute oral $_{L}$-glutamine supplementation in healthy adults (chapter 6)

4) The influence of low dose acute $_{L}$-glutamine supplementation on gastrointestinal barrier integrity and microbial translocation in response to low intensity (chapter 7) and high intensity (chapter 8) exertional-heat stress

Chapter 3 - General Methodology

This chapter describes the common methods and materials utilised within the experimental studies of this thesis. Where modified measures were undertaken, these are detailed in full within the methods section of the relevant experimental chapter.

3.1 Research Ethics

Prior to each investigation, full ethical approval was obtained from the Plymouth MARJON University Research Ethics Committee. Potential participants were given a full written and verbal brief of the experimental procedures, benefits and potential risks of harm using ley terms. A minimum of 24 hours was given prior to participants providing signed informed consent. Participants were notified of their right to withdraw at any time without reason. All investigations were conducted following with the revised guidelines of the *Declaration of Helsinki (2013)*.

3.2 Health and Safety

Experiments took place at the British Association of Sport and Exercise Science (BASES) accredited Exercise Physiology Laboratories at Plymouth MARJON University. Standard institutional operating procedures were followed throughout each experiment. All investigators were first aid qualified. Participants were fully supervised throughout testing. Risk Assessments were completed for all non-routine procedures. Control of Substances Hazardous to Heath (COSHH) forms were completed prior to use of chemicals.

3.2.1 Equipment Sterilisation

To prevent contamination, all reusable equipment was sterilised before and after use. Respiratory gas collection masks, heart rate belts and drink bottles were soaked in disinfectant (Milton®, Procter & Gamble, UK) for 15 minutes. This was followed by a thorough rinse in cold water. Electrical equipment contacting the body, including, heart rate monitors and skin thermistors, were cleaned using 70% isopropyl alcohol (Alcotip, Universal, London, UK). All exercise equipment and surfaces were wiped clean using disinfectant spray (Virkon® Rely+On, Du Point, Suffolk, UK). The military clothing ensemble and textile heart rate straps were machine washed at 40 °C for 1 hour using antibacterial powder.

3.2.2 Waste Disposal

Non-reusable biological waste, including rectal thermistors, medical gauze and nitrile gloves were all disposed in biohazard waste containers. Medical sharps used during phlebotomy (e.g. venepuncture needles) were disposed in a sharps bin. National guidelines were followed for the handling and incineration of biological material.

3.3 Participants

3.3.1 Demographic

Healthy males, residing in the United Kingdom were recruited for this programme of research. All participants were aged 18-45 years, non-smokers and undertook $\geq$ 4 structured exercise per week. Females were excluded given the unavailability of steroid hormone testing to confirm the phase of menstruation (De Jonge et al., 2019), which influences both thermoregulation (Shechter and Boivin, 2010) and GI barrier function (Farage et al., 2009). Inclusion criteria insured participants had a body fat percentage of between 6 to 25 % and a maximal oxygen uptake ($\dot{V}O_{2max}$) of 40 to 55 ml·kg·min^{-1} (except chapter 5). These characteristics were chosen specifically to represent the typical demographic of UK Armed Forces personnel (Table 12).

3.3.2 Recruitment

Participants were recruited from the general university population and local sports clubs using advertisement posters and on social media (e.g. Twitter™, Facebook™). No financial compensation was provided for participation.

3.3.3 Medical Criteria

A general medical questionnaire was completed to confirm all participants were in good health prior to involvement. Exclusion criteria included a previous history of thermoregulatory, nervous, cardiovascular, respiratory, renal, liver, skeletal and chronic infectious (e.g. hepatitis) illness. Participants with specific conditions of the GI tract, such as coeliac disease, irritable bowel syndrome and abdominal surgery were excluded. Specific conditions of the rectum (e.g. rectal bleeding, haemorrhoids) were screened for to ensure no additional risk of rectal thermistor insertion. Participants with a history of nausea/dizziness upon exposure to medical equipment (e.g. needles) were not recruited. Postponements were made if participants reported an upper respiratory or GI infection within 14 days prior to commencing main trials.

Table 12. Demographic Measurements of Male British Armed Forces Defence Personnel.

Reference	Population	Body Mass (kg)	Body Fat (%)	$\dot{V}O_{2max}$ (ml·kg·min^{-1})
Legg and Duggan, 1996	62 British Army Recruits (Pre-Basic Training)	67 ± 9	N/A	52 ± 5*
Williams et al., 1999	47 British Army Recruits (Pre-Basic Training)	69 ± 9	11 ± 4	50 ± 5*
Bilzon et al. 2001	34 Royal Navy Personnel	76 ± 11	17 ± 4	53 ± 5
Williams et al. 2005	19 British Army Recruits (Pre-Basic Training)	68 ± 5	12 ± 4	45 ± 5*
Lunt et al. 2013	156 Male and 44 Female. 15 British Army and 162 Royal Navy Personnel	78 ± 13	N/A	48 ± 8
Fallowfield et al. 2014	249 British Royal Marines (Pre-Deployment)	82 ± 9	17 ± 5	53 ± 4*
Fallowfield et al. 2018	990 Royal Airforce Recruits (Pre-Basic Training)	73 ± 11	N/A	49 ± 5*
Fallowfield et al. 2019	98 British Army Personnel (Pre- Deployment)	82 ± 9	17 ± 5	53 ± 4*

** $\dot{V}O_{2max}$ predicted from 1.5 mile run time.*

3.4 Anthropometry

3.4.1 Height

Height was measured barefoot using a fixed stadiometer (Marsden HM-200, Rotherham, UK) with participants stood vertically in the anatomical position. The stadiometer arm was lowered until it rested horizontally on the most superior aspect of the head. Following deep inhalation, the scale was read to the nearest 0.1cm.

3.4.2 Body Mass

Body mass was measured using an electronic scale (MC-180MA, Tanita, Tokyo, Japan). This measure was taken having voided and > 2 hours post prandial. Participants wore a t-shirt and shorts. A recording was taken once the digital display had stabilised to the nearest 0.05 kg.

3.4.3 Body Composition

Skinfold thicknesses were measured using callipers (Harpenden, Holtain Ltd, Crymych, UK) in accordance with the International Society for the Advancement of

Kinanthropometry (ISAK) guidelines (Marfell-Jones et al. 2006). Participants stood in the anatomical position and assessments were made on the right side of the body. The sum of skinfolds was determined from four sites: bicep, tricep, subscapular and iliac crest. Each skinfold thickness was taken in duplicate to the nearest 1 mm and the mean value recorded. One researcher performed all skin-fold measurements. Predictions of body density were calculated in accordance with Durin and Womersley (1974) using age related equations for males (Equation 1). Body density was subsequently converted to percentage body fat using the formulae of Siri (Siri, 1954; Equation 2).

Equation 1. Calculation of Body Density for adult males.

Body density (males 18-19) = 1.1620 – (0.0630 x Log SF)

Body density (males 20-29) = 1.1631 – (0.0632 x Log SF)

Body density (males 30-39) = 1.1422 – (0.0544 x Log SF)

SF = Sum of Bicep, Tricep, Subscapular and Iliac Crest skinfold (mm)

Equation 2. Calculation of body fat (%) in humans.

Body Fat (%) = [(4.95/body density) – 4.50] x 100

To assess the reliability of the skinfold thickness body composition assessment method, 12 participants were measured in duplicate (Table 13). Technical error was calculated using a battery of reliability statistics (Section 3.14.8).

Table 13. Reliability of the skin fold thickness body composition assessment (n = 12).

Body Fat (%)	Data 1	Data 2
Mean ± SD	13.7 ± 4.9	13.9 ± 4.8
r	0.99	
TEM	0.2	
CV (%)	1.7	
B-A (95% LoA)	-0.2 (0.6)	

r = Pearson's Correlation; TEM = standard error of the mean; CV% = coefficient of variation; and B-A 95% LOA = Bland-Altman Mean Bias and 95% limits of agreement.

3.5 Pre-Trial Standardisation

3.5.1 Lifestyle

Ambient temperature (T_{amb}) recorded by the nearest meteorological station (Camborne, United Kingdom; latitude: 50.218 ° N) remained below 20 °C throughout data collection (Met Office, 2020). Participants were excluded if they were regularly (> 1 per week) exposed to prolonged thermal exposures (e.g. sun beds, saunas) or had visited a warm climate (~ 7 days; > 25°C) within the two months preceding enrolment. Main trials were separated by ≥ 7 to ≤ 14 days. This was to minimise the likelihood of alterations in both participants lifestyle and potential carry-over effects caused by testing (Barnett and Maughan, 1993; Mee et al., 2015). Strenuous physical activity was prohibited for 48 hours prior to main trials and participants were asked to maintain their regular exercise programme throughout the data collection period. Non-steroidal anti-inflammatory drugs (e.g. aspirin, Lambert et al., 2007; ibuprofen, van Wijck et al., 2012b) were prohibited for 48 hours before main trials. The start time for main trails was standardised (08:00-10:00 hours) to control for the influence of circadian variation (Atkinson and Reilly, 1996).

3.5.2 Diet

Habitual diets were maintained throughout the data collection period. Macro- and micro-nutrient analysis of food record diaries was not performed, to minimise the burden placed on participants with the aim of improving overall compliance. To this point, short-term (1-7 days) manipulation of dietary intake, for example macronutrient composition (Moncada-Jimenez et al., 2009), Fermentable Oligo-, Di-, Mono-saccharides And Polyols (FODMAP) composition (Gaskell et al., 2019b), and fresh food *versus* sterile meal rations (Karl et al., 2019), do not influence GI barrier integrity in response to exercise. Nutritional supplementation (e.g. whey protein, probiotics, polyphenols) were prohibited for 14 days prior to and until the end of the experimental period. Alcohol was excluded for 48-hours (Robinson et al., 1981; Bala et al., 2014) and caffeine for 24-hours (Roti et al., 2006; Millard-Stafford et al., 2007) before each trial. Main experimental trials were undertaken following an overnight fast that was a minimum 10-hours in duration (Edinburgh et al., 2018). Diet was uncontrolled prior to baseline fitness tests, though participants were advised to arrive at the laboratory a minimum of two-hours post prandial.

3.5.3 Hydration

Participants were asked to consume 500 ml of water 2 hours before laboratory visits. This hydration control was selected in line with American College of Sports Medicine guidance (Casa et al., 2005). Hydration status was determined through the assessment of plasma osmolality (Section 3.12.6), urine osmolality (Section 3.12.7) and urine specific gravity (Section 3.12.8). Criteria for euhydration were: plasma osmolality ≤ 290 $mOsm.kg^{-1}$; urine osmolality ≤ 700 $mOsm.kg^{-1}$; and urine specific gravity ≤ 1.020 AU (Casa et al., 2005).

3.6 L-Glutamine Supplementation

Informed-Sport certified unflavoured L-glutamine (GLN) crystalline powder (Myprotein, Northwich, UK) was suspended in 500 ml of water/lemon flavour sugar-free cordial (Fruit Squash – no added sugar, Robinsons, UK) in a 4:1 ratio. The beverage was freshly prepared by an individual independent of the study within 1-hour of consumption (Ziegler et al., 1990). The placebo (PLA) supplement consisted of the identical water/lemon flavour sugar-free cordial alone (Zuhl et al., 2014). The GLN and PLA supplements were administered in an opaque bottle to match visual appearance. A standard blinding questionnaire was completed by participants at the end of trials. Participants consumed the entire bolus within a 5-to-10-minute period after agitation for ~30 secs. The ingestion timing was 1 hour prior to commencing the exertional-heat test, to allow time for GLN uptake to saturate the small intestine (Windmueller and Spaeth, 1974). Dosage was measured on an electric scale (E12000, Sartorius, Epsom, UK) to the nearest 0.01 g.

3.7 Ambient Environment

Baseline anthropometric and aerobic fitness testing was performed in normothermic laboratory conditions (18-22°C; 40-60% RH), confirmed using a portal weather station (Technoline WS-6730, TechnoTrade GmbH, Germany).

Main trials were performed in an environmental chamber (201003-1, TIS Services, Hampshire, UK). Neither convective fanning or direct heating were implemented. T_{amb} (EUS-UU-VL3-O, Grant, Cambridge, UK) and RH (Technoline WS-6730, TechnoTrade GmbH, Germany) were measured at 15-sec and 5-min intervals, respectively.

WGBT was calculated as the international standard for heat stress (ISO 7243). WGBT is the meteorological index utilised by various militaries worldwide (Military Headquarters

of the Surgeon General, 2019). WBGT was indirectly estimated from T_{amb} and RH for an indoor environment based upon standard equations (Liljegren et al., 2008). This technique has been validated with a standard error <0.6°C; Patel et al., 2013; Lemke and Kjellstrom, 2012). This analysis was undertaken using an online open-access software (http://www.climatechip.org/heat-stress-index-calculation).

3.8 Exercise

All exercise testing was performed on a motorised treadmill (Desmo HP, Woodway GmbH, Weil am Rhein, Germany). Participants were blinded from physiological feedback throughout. The treadmill was calibrated annually by the manufacturer.

3.8.1 Clothing

A standard warm weather military jacket (8415-99-597-0428) and trousers (8415-99-317-8270) were worn throughout main trials. This control was implemented to minimise the influence of clothing on heat storage (Havenith, 1999) and for ecological validity (Military Headquarters of the Surgeon General, 2019). Clothing sizes were standardised between trials and fitted based on baseline anthropometric measurements. The jacket neckline was fully un-zipped and sleeves were rolled to the wrist. During baseline assessments participants wore a sports t-shirt and shorts.

3.8.2 Fluid Replacement

Plain water was administered throughout main trials in accordance with worldwide military guidelines (Spitz et al., 2012). A brief modification of this guidance was to prescribe fluid volume relative to body mass, rather than as an absolute volume (e.g. 1 $l \cdot h^{-1}$ = 12 $ml \cdot kg \cdot h^{-1}$). Water was served at T_{amb} temperature (28-30°C) to minimise the influence on thermoregulation (Bongers et al., 2015) and GI barrier integrity (Snipe and Costa, 2018b).

3.8.3 Lactate Threshold

An incremental running test was performed to determine anaerobic lactate threshold (LT) (Chalmers et al., 2015). Participants arrived ≥ 2 hours post prandial. The test began at a speed corresponding to a rate of perceived exertion (RPE; Section 3.12.1) of 11 on a fixed 1% inclination. Belt speed was increased by 1 $km \cdot h^{-1}$ every 4-minutes, until rating of perceived exertion ≥ 18. Between stages participants dismounted the treadmill for 1 minute.

Capillary blood was collected between test stages. After sterilising the finger with 70% isopropyl alcohol, the skin was punctured using a disposable lancet. Blood (20 µl) was drawn into a plain capillary tube and immediately inverted into a lysing stabilizing agent. $_{L}$-lactate concentration was determined ≤ 2 hours (Davison et al., 2000) using an automated biochemical analyser (Biosen C-Line, EKF Diagnostics GmbH, Magdeburg, Germany). Prior to each use, manufacturer recommended calibrations were performed. The biochemical analyser had a resolution of 0.01 mmol·l^{-1}.

To assess the reliability of the automated $_{L}$-lactate analyser, 12 basal and 12 post-LT samples were analysed in duplicate (Table 14). Technical error was calculated using a battery of reliability statistics (Section 3.14.8).

Table 14. Reliability of the Biosen C-Line automated L-lactate analyser in resting and post lactate-threshold blood samples (*n* = 12).

Blood Lactate Concentration (mmol·l^{-1})	Data 1		Data 2
Mean ± SD	1.49 ± 0.39		1.51 ± 0.37
r		1.00	
TEM		0.03	
CV (%)		1.7	
B-A (95% LoA)		-0.02 (0.08)	
Mean ± SD	8.19 ± 1.60		8.27 ± 1.63
r		1.00	
TEM		0.10	
CV (%)		1.0	
B-A (95% LoA)		-0.07 (0.27)	

r = Pearson's Correlation; TEM = standard error of the mean; CV% = coefficient of variation; and B-A 95% LOA = Bland-Altman Mean Bias and 95% limits of agreement.

Anaerobic LT was classified using the modified D-max method (Bishop et al., 1998). A third order polynomial regression curve was plotted between incremental lactate measurements. LT was defined as the maximal perpendicular distance on a straight line formed by the workload one bout preceding a ≥ 0.40 mmol·l^{-1} rise in blood lactate above baseline and the final lactate point. This analysis was undertaken using the open-access *Lactate-E 2.0* extension for Microsoft Excel (Newell et al., 2007).

This LT test has previously been shown to offer strong re-test reliability (ICC = 0.96; TEM = 0.27 $km.h^{-1}$; CV = 1.9%) and validity in predicting 10 km (-0.1 $km \cdot h^{-1}$) running time trial performance (Chalmers et al., 2015). Likewise, a recent study comparing 56 potential LT techniques, found the combination of a four-minute stage length and the modified D-max technique to be most accurate (-1.1 watts, r = 0.96, effect size = 0.03) method for predicting maximal lactate steady state (Jamnick et al., 2018).

3.8.4 Maximal Oxygen Uptake

An incremental running test (Taylor et al. 1955) was performed to determine maximal oxygen uptake ($\dot{V}O_{2max}$). Following a five-minute warm-up at 6 $km \cdot h^{-1}$, the test began at a speed of 10 $km \cdot h^{-1}$ on a 1% inclination. The treadmill speed was then increased at 1 $km \cdot h^{-1}$ increments every three minutes until reaching 13 $km \cdot h^{-1}$, when inclination was then increased by 2% every two minutes. The test was terminated when the participant reached volitional exhaustion. Expired gas was measured using a breath-by-breath metabolic cart (Section 3.10.1) and mean values recorded at 15 second intervals. Heart rate (Section 3.10.2) and rating of perceived exertion (Section 3.12.1) were measuring during the final 10 seconds of each stage. The criteria used to establish a true $\dot{V}O_{2max}$ included three from: (1) a plateau in $\dot{V}O_2$ (an increase ≤ 2 $ml \cdot kg \cdot min^{-1}$) despite increasing exercise intensity; (2) a respiratory exchange ratio ≥ 1.15; (3) a heart rate ≤ 10 $b \cdot min^{-1}$ of the age-predicted maximum (220-age); and (4) a rating of perceived exertion of 20 (Winter et al. 2007). The highest 30 second average $\dot{V}O_2$ was taken to be $\dot{V}O_{2max}$.

3.8.5 Military Exertional-Heat Stress Test

Upon entering the environmental chamber, participants sat for 20-minutes to allow thermoregulatory systems to stabilise, before resting physiological measures were collected (Marins et al., 2014). This protocol involved two bouts of 40-mins fixed-intensity treadmill walking at 6 $km \cdot h^{-1}$ on a 7% incline. Exercise was interspersed by 20 minutes seated recovery. The ambient environment was regulated at 35 °C and 30 % RH (WBGT = 26°C). At the beginning of the 20 minutes seated recovery period, participants undertook 4 minutes forearm cold-water immersion to help relieve thermal strain (Section 3.8.5). Fluid consumption (Section 3.8.1) took place during the final 16-minutes of seated recovery.

The military EHST was designed in accordance with international military work/rest (~2:1 ratio) schedule guidelines for physically fit, non-acclimated service personnel, undertaking moderate-to-high intensity exercise (Tables 15, 16 and 17). Considerations are given to the conservative nature of UK *versus* NATO Armed Forces guidelines (Table 17).

Table 15. UK work/rest guidelines (Military Headquarters of the Surgeon General, 2019).

MAXIMUM WORK RATE	Maritime	Land	Air
	1-hour activity followed by 30 minutes rest		
LOW	Working seated	Lying, guard duty	RW pilots (light) routine flight, FJ pilots aspects of routine flight (climb, cruise, descent and circuits), Rear crew transit
MEDIUM	Rounds, manning upper deck/gangway duty	Marching 3.6 kph (2.3 mph) 30 kg load	Rear crew walking to the aircraft, RW pilots (medium) routine flight, RW pilots (light) complex flight phases.
HIGH	Transiting around ship/ boat	Marching 5.6 kph (3.5 mph) with a 20kg load, patrolling, digging, field assaults	Rear crew tasks (e.g. flight preparations, landing and low level transit tasks, trooping, under-slung loads, refuelling,
VERY HIGH	No example available	Marching 8 kph (5 mph) no load, marching 5.6 kph (3.5 mph) 30 kg load	Heavy aircraft repair
EXTREME	Fire-fighting	Running in sports kit, speed marching 9.7 kph (6 mph) 15kg load	Loading/unloading heavy cargo (23 kg aircraft strops)

Table 16. UK upper WBGT index for unacclimated service personnel (Military Headquarters of the Surgeon General, 2019).

Unacclimatised	
Maximum Work Rate	**Upper WBGT Index (°C)**
1 LOW	32
2 MEDIUM	26
3 HIGH	24
4 VERY HIGH	22
5 EXTREME	20

NOTES

1. Adherence to the WBGT Index Limits will minimise the risk of heat illness, for 95% of normal, healthy Service Personnel. As the WBGT Index Upper Limit increases, the maximum work rate should be decreased in order to minimise the risk of heat illness. Heat illness can still occur, and all personnel should remain vigilant to this risk.
2. The WBGT Index SI unit is °C WBGT. The readings are in °C but these should not be confused with ambient temperatures as reported on weather forecasts.
3. If the operational imperative requires that the activity should be undertaken outside the guidance levels, then consideration should be given to reducing the duration of work to less than an hour and increasing frequency and duration of rest periods. Conduct commanders risk assessment. Medical advice should be sought, as needed.
4. Values in the table are applicable to: Service Personnel wearing a single layer uniform with sleeves rolled up and without helmets. Men and women equally, given comparable levels of physical fitness.
5. For unfit Service Personnel (ie those who cannot pass their mandatory fitness test): the WBGT Index Upper Limit should be lowered.
6. Commanders should anticipate changes to WBGT during the course of a duty period
7. Activities shown in the Maritime and Air domains are evidence-based examples. Examples of activities shown in the Land domain have been adapted from other sources.

Table 17. NATO work/rest guidelines (Shawtz et al., 2016).

WGBT Index (°C)	Heat Category	Unacclimatised		
		Easy Work	Moderate Work	Hard Work
		Work/Rest (min)	Work/Rest (min)	Work/Rest (min)
25.5-27.7	White	No Limit	No Limit	40/20
27.8-29.4	Green	No Limit	50/10	30/30
29.5-31.0	Yellow	No Limit	40/20	30/30
31.1-32.2	Red	No Limit	30/30	20/40
32.3+	Black	50/10	20/40	10/50

Work Rate Examples

Easy Work (250 watts)

- Walking Hard Surface 4 km/h <14 kg load
- Weapon Maintenance
- Marksmanship Training
- Drill and ceremony

Moderate Work (~425 watts)

- Walking hard surface at 5.5 km/h <18 kg load
- Patrolling
- Defence position construction
- Field assaults
- Conditioning exercise, calisthenics, light / mod effort

Hard Work (~600 watts)

- Walking hard surface 5.5 km/h > 18 kg load
- Running, 8 km/h
- Loading / unloading truck
- Pushing heavy objects (>35 kg)
- Conditioning exercise, calisthenics heavy effort

3.8.6 High Intensity Exertional-Heat Stress Test

This protocol involved treadmill running at a speed corresponding to LT intensity (1% gradient) for 30 minutes or until volitional exhaustion. The ambient environment was regulated at 40 °C and 40 % RH (WBGT = 31.9 °C). Upon entering the environmental chamber, participants stood for 20-minutes to allow thermoregulatory systems to stabilise. Resting physiological measures were taken during the final minute of this period (Marins et al., 2014). Fluid replenishment (Section 3.8.1) was implemented immediately following exercise termination (Section 3.8).

Relative exercise intensity was prescribed based on % LT given this technique induces more homogenous inter-individual responses (e.g. heart rate, T_{core}) than alternative prescription methods (% $\dot{V}O_{2max}$) (Meyer et al., 1999; Baldwin et al., 2000). Likewise, in recent years, some investigators have recommended relative intensity EHST prescription based upon metabolic heat production (MHP) per unit body mass ($W \cdot kg^{-1}$), with the aim to better control inter-individual variation in T_{core} (Cramer and Jay, 2015). However, this technique leads to large inter-individual variation in metabolic, cardiovascular, and perceptual responses (Smoljanic et al., 2014). The relative intensity (anaerobic LT) set during the test was anticipated to increase T_{rec} (Section 3.9.1) to *circa* 39.5 °C in ~25-30 minutes (Potter et al., 2017). In tests were participants reached volitional exhaustion prior to completing the EHST, the duration was matched during the subsequent experimental trial.

3.8.7 Forearm Cold-Water Immersion

Forearm immersion cooling is a simple and effective technique to decrease T_{core} that is endorsed by militaries worldwide (De Groot et al., 2013). This technique was applied within the military EHST (Section 3.8.5) to rapidly cool participants between repeat exercise bouts. A temperature-regulated water bath (Clifton NE4-28, Bennett Scientific Ltd, UK) was maintained at a target temperature of 15 °C (Comark KM20REF, RS Calibration, UK). Participants immersed their forearms to a depth of the antecubital fossa for 4 minutes. The 15 °C water temperature was selected as the central value (10-20°C) advised by NATO medical guidelines (Spitz et al., 2012). Anticipated T_{core} cooling rates are *circa* 0.03-0.05°C·min^{-1} (De Groot et al., 2013).

3.8.8 Termination Criteria

End-point EHST termination criteria were: (1) trial completion; (2) T_{core} > 39.8°C; (3) central nervous system abnormalities (e.g. confusion, aggression, collapse); (4) volitional exhaustion; (5) participant request; or (6) upon discretion of the principal investigator. Following kit de-instrumentation, participants were removed from the environmental chamber into a normothermic room and cooled via whole-body fanning. Participants were permitted to leave the laboratory once all post exercise measurements had been recorded and T_{core} had recovered below 38.6 °C (Belval et al., 2019).

3.9 Thermoregulation

3.9.1 Core Temperature

Core (rectal) temperature (T_{core}) was measured using a flexible, sterile, disposable thermistor probe (Phillips 21090A, Guildford, UK). The probe was self-inserted 12 cm beyond the entrance of the anal sphincter. This depth was chosen as a central value within the range (8-16 cm) where rectal tissue temperature is considered most consistent (Lee et al., 2010; Miller et al., 2017) and representative of overall T_{core} (Byrne and Lim, 2007). T_{rec} was measured continuously every 15 seconds throughout main trials using a temperature logger recording to a sensitivity of 0.01 °C (Squirrel SQ2010, Grant Instruments, Cambridge, UK). All data were averaged over 1-minute intervals.

The selection of rectum as the anatomical site to assess T_{core} was chosen based on several rationale. These include: participant comfort, acceptable day-to-day reliability (Mee et al., 2015; Willmott et al., 2015; Watkins et al., 2018) and uniformity with previous

GI barrier integrity research (Pires et al., 2017). In comparison, oesophageal (T_{oso}) probe insertion is uncomfortable (i.e. pharyngeal reflex) and confounded by fluid ingestion, whilst the GI telemetric pill (T_{GI}) is subject to large intra- and inter- individual variance in pill motility across the GI tract (Byrne and Lim, 2007). Forehead, sublingual, temporal, aural and axillary devices all offer poor validity and/or reliability (Ryan-Wenger et al., 2018). Previous exertional-heat stress studies show T_{rec} and T_{GI} to respond comparably following device calibration (Gant et al., 2006; Travers et al., 2016), though in some cases T_{rec} has been shown to systematically underpredict T_{GI} beyond the limit of clinical accuracy (> 0.1°C; Byrne and Lim, 2007).

3.9.2 Skin Temperature

Skin temperature (T_{skin}) was measured using hard-wired stainless-steel contact thermistors (EUS-UU-VL3-O, Grant, Cambridge, UK). These were attached to 4 sites on the left side of the body using a ~2.5 (W) x 5 (L) cm strip of porous surgical tape (Transpore™, 3M, MN, USA) and then covered by a ~5 (W) x 5 (L) cm square of elasticated 100% cotton tape (KT Tape®, KT Health, UT, USA; Psikuta et al., 2014). These sites were the chest (T_{chest}; midpoint of the pectoralis major), arm (T_{arm}; midpoint of the triceps brachii lateral head), thigh (T_{thigh}; midpoint of the rectus femoris), and calf (T_{calf}; gastrocnemius lateral head). Specific anatomical positions were estimated given thermistor positioning across a 5 x 5 cm regional location does not influence calculation of mean T_{skin} (Maniar et al., 2015). T_{skin} was measured continuously every 15 seconds throughout main trials using a temperature logger recording to a sensitivity of 0.01 °C (Squirrel SQ2010, Grant Instruments, Cambridge, UK). All data were averaged over 1-minute intervals. Mean T_{skin} was calculated using a 4-point weighted equation (Equation 3).

Equation 3. Calculation of mean skin temperature (Ramanathan, 1964).

$$T_{skin}\ (°C) = 0.3\ (T_{chest} + T_{arm}) + 0.2\ (T_{thigh} + T_{calf})$$

The selection of hard-wired thermistors to assess mean T_{skin} was chosen based on several rationale. These include: strong accuracy during physical modelling (Smith et al., 2010), reliability during *in vivo* assessment (James et al., 2014) and practicality to position thermistors below clothing (Macrae et al., 2018). Limitations of this method include: a small skin surface contact area (Bach et al., 2015) and artefact caused by adhesive tape insulation (Tyler, 2011; Psikuta et al., 2014). In comparison, infrared contact sensors and thermal

cameras both offer weaker validity and/or reliability when applied during exercise (Fernandes et al., 2014; James et al., 2014; Bach et al., 2015). The 4-site equation of Ramanathan (1964) was selected to allow a full range of movement without wire entanglement during exercise (Lui et al., 2011).

3.9.3 Mean Whole-Body Temperature

Mean whole-body temperature (T_{body}) was calculated using a 2-compartmental thermometry model from T_{rec} and T_{skin} (Equation 4). The selected weighting for the core/shell compartments together with a correction factor was chosen to account for the systematic underestimation of T_{body} during steady-state exercise (Jay et al., 2007a). Improvements to this model can made by accounting for muscle temperature in a 3-compartmental model (Jay et al., 2007b) or by applying an individualised correction factor based on MHP, body surface area and environmental conditions (Jay et al., 2010).

Equation 4. Calculation of whole-body temperature (Jay et al., 2007a).

$$T_b\ (°C) = (0.8 \times T_{rec}) + (0.2 \times T_{skin}) + 0.4$$

3.10 Cardiopulmonary

3.10.1 Expired Respiratory Gas

Expired respiratory gas was measured using an online breath-by-breath gas analysis system (Cortex Metalyser 3B, Cortex, Leipzig, Germany). A fitted face mask was worn for all sample collections. The analyser recorded oxygen (O_2) and carbon dioxide (CO_2) fractions of expired air and determined respiratory volume (VE) from the flow rate through a transducer (Triple® V, Cortex, Leipzig, Germany). Mean respiratory gas exchange values were calculated every 15-seconds. Recorded respiratory parameters were used to calculate oxygen uptake ($\dot{V}O_2$) and the respiratory exchange ratio (RER) using an inbuilt gas analysis software package (Metasoft, Cortex, Leipzig, Germany).

The gas analysis system was calibrated for air pressure, volume, and composition prior to use. Barometric pressure was measured using a portable weather station (Technoline WS-6730, TechnoTrade GmbH, Germany). The volume calibration was performed using a 3 litre syringe (Hans Rudolph, KA, USA) through a systems transducer. According to the manufacturer guidance, the flow sensor measured values to the accuracy of ± 2% with a range of 0.05-20 $l \cdot s^{-1}$. A two-point calibration was then undertaken for gas

concentrations to represent inspired and expired air using laboratory ambient gas (O_2 = ~20.95%; CO_2 = ~0.04%) and concentrated gas from a cylinder (O_2 = 15.00%; CO_2 = 5.00%; British Oxygen Company, Guildford, UK).

3.10.2 Heart Rate

Heart rate was measured using a dual-channel textile-based Electrocardiogram (ECG) and recorded on a Sensor Electronics Module (SEM) unit (EQ02, Equivital™, Cambridge UK). The ECG belt was securely fastened around the chest. The SEM unit was configured to continuously sample heart rate at 256 Hz at 15 second intervals throughout trials. All data were averaged over 1-minute intervals. The accuracy of the Equivital™ monitor has previously been validated against the Holter monitor (SEER MC Holter monitor, GE Healthcare, USA) over 24-hours free-living (LoA = 2.8 $b \cdot min^{-1}$; Akintola et al., 2016).

3.11 Sudomotor

3.11.1 Whole-Body Sweat Rate

Whole-body sweat rate (SWR) was calculated from the Δ nude body mass assessed immediately prior and following main trials. Residual sweat was removed through towel drying prior to measurements being taken on an electric scale (Section 3.4.2) to the nearest 0.05 kg. Corrections were made to account for fluid intake, blood withdrawal and urine output. However, no corrections were made for respiratory water vapour and metabolic weight loss since these variables can be assumed comparable between trials (Baker, 2017). SWR was calculated relative to time (Equation 5).

Equation 5. Calculation of whole-body sweat rate (SWR).

SWR ($g \cdot hr^{-1}$) = [body mass pre (kg) – (body mass post (kg) + fluid intake (kg) - urine output (kg) - blood withdrawal (kg)] / exercise duration (hr)

3.12 Perception

3.12.1 Rating of Perceived Exertion

Subjective perception of physical exertion was measured using the rating of perceived exertion (RPE) scale (Borg, et al., 1970). RPE ranges from 6 (very, very light), through 13 (somewhat hard), to 20 (very, very hard) along a 15-point scale. The standardisation of instructions included giving a clear understanding of anchoring the top

and bottom ratings to previous experiences of no exertion (RPE = 6) to maximum exertion (RPE = 20) during physical activity (Mauger et al., 2013).

3.12.2 Thermal Sensation

Subjective perception of thermal sensation was measured using the thermal sensation (TS) scale (Toner et al., 1986). TS ranges from 0.0 (unbearably cold), through 4.0 (comfortable), to 8.0 (unbearably hot) along a 17-point scale. The standardisation of instructions included giving a clear understanding of anchoring the top and bottom ratings to previous experiences of being unbearably cold (TS = 0) and unbearably hot (TS = 8).

3.12.3 Gastrointestinal Symptoms

Subjective perception of gastrointestinal symptoms (GIS) were measured using a modified visual analogue scale (mVAS; Gaskell et al., 2019a). The mVAS is a 20-item questionnaire of common GI symptoms that range from 0 (absent), through 1-4 (mild GIS), then 5-9 (serve GIS) to 10 (extremely severe GIS) along a 10-point scale. Regurgitation and defaecation will result in temporary or complete cessation of exercise, therefore are presented as 0 and 10 ratings only. The VAS gives a clear explanation of each symptom and has been validated against face-to-face interviews (Bengtsson et al., 2013). The standardisation of instructions included giving a clear understanding of anchoring the top and bottom ratings to previous perceptions and/or experiences of each specific GI symptom. For analysis, symptoms were grouped as: total-, upper- and lower- GI symptoms, based on previous guidance (Gaskell et al., 2019a). Symptom incidence was indicative of scores rated ≥ 1 and severity indicative of the accumulated score of all reported symptoms. Symptom severity are presented as mean ± range of accumulated scores for consistency with published guidance (Gaskell et al., 2019a).

3.13 Phlebotomy and Haematology

3.13.1 Phlebotomy

Venous blood samples were collected via venepuncture. Participants stood vertical in the anatomical position for 20 minutes prior to sampling, to allow changes in capillary filtration pressure to stabilise (Shirreffs and Maughan, 1994). To assist selection of an appropriate forearm antecubital vein, a tourniquet was applied above the elbow. The duration of stasis was minimised to as short as feasibly possible (< 1 minute). The sample site was sterilised with 70% isopropyl alcohol (Alcotip, Universal, London, UK) and the

needle (Beckton Dickson 22-guage eclipse needle, Plymouth, UK) advanced into the chosen vein. Following each blood collection, the needle was removed from the vein and compression applied to the site using medical gauze until bleeding ceased.

Blood was drawn into serum-separator (SST II) and Ethylenediaminetetraacetic acid (K_2EDTA) vacutainers® (Becton Dickinson and Company, Plymouth, UK). The SST II tube was allowed to clot for 30 minutes at room temperature before centrifugation.

3.13.2 Centrifugation and Biofluid Storage

Whole venous blood samples were centrifuged (BR 401, Denley, UK) at 1300*g* at 4 °C for 15 minutes to separate serum (i.e. SST II vacutainer®) and plasma (i.e. K_2EDTA vacutainer®). Aliquots were pipetted in quadruple (~0.5 ml) using pyrogen-free pipette tips (FischerBrand™, UK) into 1.5 ml polypropylene microtubes (FischerBrand™, UK). These tubes were certified pyrogen, DNAase and RNAase free. All samples were stored in a -80 °C freezer (Ultra Low Temperature Freezer, New Brunswick Scientific, UK) for long-term storage. Multiple freeze-thaw cycles were avoided.

3.13.3 Haemoglobin

Haemoglobin was measured in duplicate using a portable photometric analyser (Hemocue® Hb 201+, EFK Diagnostics, Madeburg, Germany). Fresh whole venous blood (10uL) was transferred into a microcuvette (Hemocue®, EFK Diagnostics, Madeburg, Germany). Inside the microcuvette the blood mixes with dry reagents and this stimulates an azide methaemoglobin chemical reaction. The analyser is factory calibrated against the hemiglobincyanide (HiCN) method and is sensitive to 1 $g \cdot l^{-1}$ (von Schenck et al., 1986). The mean value was recorded from the duplicate assessment of each sample. Any sample with an intra-sample CV greater than 10% was repeated.

To assess the reliability of the photometric analyser, 12 samples were analysed in duplicate (Table 18). Technical error was calculated using a battery of reliability statistics (Section 3.14.8).

Table 18. Reliability of the Hemocue® Hb 201+ portable photometric analyser (*n* = 12).

Haemoglobin (g·L^{-1})	Data 1	Data 2
Mean ± SD	15.7 ± 1.1	15.7 ± 1.1
r	0.99	
TEM	0.1	
CV (%)	0.4	
B-A (95% LoA)	0.0 (0.2)	

r = Pearson's Correlation; TEM = standard error of the mean; CV% = coefficient of variation; and B-A 95% LOA = Bland-Altman Mean Bias and 95% limits of agreement.

3.13.4 Haematocrit

Haematocrit was measured in duplicate using the microcapillary technique. Fresh venous blood was transferred into a lithium heparin microcapillary tubes until ~75% full (Hawksley and Sons Ltd, Lancing, England). Haematocrit was separated through centrifugation at 14,000g for 4 minutes at room temperature (Haematospin 1400, Hawksley and Sons Ltd, Lancing, England). The ratio between red blood cells and plasma where measured using a microcapillary reader (Hawksley and Sons Ltd, Lancing, England). Any sample with a duplicate CV greater than 10% was repeated.

To assess the reliability of microcapillary assessment, 12 samples were analysed in duplicate (Table 19). Technical error was calculated using a battery of reliability statistics (Section 3.14.8).

Table 19. Reliability of the haematocrit microcapillary technique (*n* = 12).

Haematocrit (%)	Data 1	Data 2
Mean ± SD	46 ± 3	46 ± 3
r	0.98	
TEM	0	
CV (%)	0.5	
B-A (95% LoA)	0 (1)	

r = Pearson's Correlation; TEM = standard error of the mean; CV% = coefficient of variation; and B-A 95% LOA = Bland-Altman Mean Bias and 95% limits of agreement.

3.13.5 Plasma Volume

Blood and plasma volumes were calculated using standard equations (Equation 6). Haematological concentrations were uncorrected for plasma volume where analytes had a molecular weight below 80 kDa.

Equation 6. Calculation of plasma volume change (Dill and Costill, 1974).

$BV_{Post} = BV_{Pre} ([Hb]_{Pre} / [Hb]_{Post})$

$CV_{Post} = BV_{Post} (Hct_{Post})$

$PV_{Post} = BV_{Post} – CV_{Post}$

$\Delta PV (\%) = 100 (PV_{Post} – PV_{Pre})/PV_{Pre}$

Where: BV = blood volume, CV = red cell volume, PV = plasma volume, Hb = Haemoglobin, Hct = Haematocrit.

3.13.6 Plasma Osmolality

Plasma osmolality was assessed in duplicate fresh 50µl EDTA plasma samples using a freeze point osmometer (Osmomat 3000, Gonotec GmbH, Berlin, Germany). The osmometer has a resolution of ` $mOsmkg^{-1}$ and an accuracy of ± 2 $mOsm·kg^{-1}$. Prior to use, the accuracy of the device was assessed in duplicate using a reference standard (290 $mOsmkg^{-1}$). If accuracy was outside of the specified range, a 3-point calibration was performed using water (0 $mOsmkg^{-1}$) and calibration standards (300 and 850 $mOsmkg^{-1}$).

To assess the reliability of the Gonotec 3000 freeze-point osmometer, 12 EDTA plasma samples were analysed in duplicate 2 hours following ingestion of 500 ml of plain water (Table 20). Technical error was calculated using a battery of reliability statistics (Section 3.14.8).

Table 20. Reliability of the Gonotec 3000 freeze-point osmometer in plasma (n = 12).

Osmolality ($mOsm·kg^{-1}$)	Data 1	Data 2
Mean ± SD	298 ± 3	298 ± 3
r	0.93	
TEM	1	
CV (%)	0.2	
B-A (95% LoA)	0 (2)	

r = Pearson's Correlation; TEM = standard error of the mean; CV% = coefficient of variation; and B-A 95% LOA = Bland-Altman Mean Bias and 95% limits of agreement.

3.13.7 Urine Osmolality

Urine osmolality was assessed in duplicate fresh 50µl samples using a freeze point osmometer (Osmomat 3000, Gonotec GmbH, Berlin, Germany). Urine was collected mid-stream into a 50 ml sterile polypropylene container (Sterilin™, Thermofischer Scientific, Waltham, USA). The osmometer has a resolution of 1 $mOsmkg^{-1}$ and an accuracy of ± 2 $mOsm \cdot kg^{-1}$. Prior to each use, the accuracy of the device was assessed in duplicate using a reference standard (290 $mOsmkg^{-1}$). If accuracy was outside of the specified range, then a three-point calibration was undertaken using deionised water (0 $mOsmkg^{-1}$) and 2 calibration standards (i.e. 300 and 850 $mOsmkg^{-1}$).

To assess the reliability of the Gonotec 3000 freeze-point osmometer, 12 urine samples were analysed in duplicate 2 hours following ingestion of 500 ml of plain water (Table 21). Technical error was calculated using a battery of reliability statistics. Technical error was calculated using a battery of reliability statistics (Section 3.14.8).

Table 21. Reliability of the Gonotec 3000 freeze-point osmometer in urine (n = 12).

Osmolality ($mOsm \cdot kg^{-1}$)	Data 1	Data 2
Mean ± SD	260 ± 242	262 ± 243
r	1.00	
TEM	1	
CV (%)	0.6	
B-A (95% LoA)	1 (4)	

r = Pearson's Correlation; TEM = standard error of the mean; CV% = coefficient of variation; and B-A 95% LOA = Bland-Altman Mean Bias and 95% limits of agreement.

3.13.8 Urine Specific Gravity

Urine specific gravity was assessed in duplicate using a digital handheld refractometer (3741 Pen-Urine S.G, Atago Co. Ltd, Tokyo, Japan). Urine was collected mid-stream into a 50 ml sterile polypropylene container (Sterilin™, Thermofischer Scientific, Waltham, USA). The sampling device has a resolution of 0.0001 units and a specified manufacturer accuracy of ± 0.0010. Prior to use, the accuracy of the device was assessed in duplicate using deionised water (1.000 units). If accuracy was outside of the specified range then a 1-point calibration was undertaken using deionised water.

To assess the reliability of the Atago handheld refractometer, 12 urine samples were analysed in duplicate 2 hours following ingestion of 500 ml of plain water (Table 22). Technical error was calculated using a battery of reliability statistics (Section 3.14.8).

Table 22. Reliability of the Atago 3741 Pen-Urine S.G handheld refractometer (n = 12).

Specific Gravity (AU)	Data 1	Data 2
Mean ± SD	1.008 ± 0.007	1.008 ± 0.007
r	1.00	
TEM	0	
CV (%)	0.0	
B-A (95% LoA)	0 (0)	

r = Pearson's Correlation; TEM = standard error of the mean; CV% = coefficient of variation; and B-A 95% LOA = Bland-Altman Mean Bias and 95% limits of agreement.

3.13.9 Dual-Sugar Absorption Test

The serum Dual-Sugar Absorption Test (DSAT) was selected to assess GI permeability. The probe drink contained 5 g lactulose (Lactulose Oral Solution, Sandoz, Holzkirchen, Germany) and 2 g L-rhamnose (L-Rhamnose, 99% pure, Sigma Aldrich, Missouri, USA). These sugars were dissolved in 50 ml of plain water (osmolality = ~750 $mOsm \cdot kg^{-1}$). Both sugars were sold as food-grade products. Dosages were measured using an electric scale to the nearest 0.001 g (E12000, Sartorius, Epsom, UK). These quantities were based on previous recommendations (van Wijck et al., 2013). The probe drink was ingested at the beginning (Chapter 8) or 10-minutes (Chapters 4, 5, 7) into the EHST (van Wijck et al., 2012c). Analysis was undertaken in serum 90 minutes post-ingestion.

Analysis of serum probe concentrations was undertaken using high performance liquid chromatography (HPLC) with pulsed electrochemical detection (Fleming et al., 1996). Duplicate samples (200 µl) were diluted 1:2 with an internal standard (200 µl of 250 $mg \cdot l^{-1}$ melibiose). To precipitate plasma proteins, 200 µl of 5-sulfosalicylic acid (35 $g \cdot l^{-1}$) was added and after standing for 20 minutes the samples were centrifuged at 900 *g* for 5 minutes. Supernatant was then removed and desalted with the mixed ion-exchange resin. The samples were re-centrifuged at 900 *g* for 5-minutes and supernatant injected into the column. The 2 sugars were separated via HPLC and quantified using pulsed electrochemical detection using a gold working electrode and silver/silver chloride reference electrode. The

detection potential of the pulsed electrochemical detector was -0.01 V (0-0.5 s), the oxidation potential was +0.75 V (0.51-0.64 seconds), the reduction potential was -0.75 V (0.65-0.75 seconds), and the integration period was 0.05 to 0.5 seconds. Peak heights were measured with internal standardization. The columns were washed for 4 minutes with 1 mol·l^{-1} sodium hydroxide between sample to maintain retention time stability. The technique has a sensitivity of 0.1 mg·l^{-1}. Sugar recovery was determined per litre serum (mg·l^{-1}), which was corrected relative (%) to the dose of sugar consumed (Equation 7).

Equation 7. Calculation of the lactulose to L-rhamnose recovery ratio.

$$L/R \text{ ratio} = (L_s/L_i)/(R_s/R_i)$$

Where: L_i = ingested lactulose, L_s = serum lactulose, R_i = ingested L-rhamnose, R_s = serum L-rhamnose.

3.13.10 Intestinal Fatty Acid Binding Protein

Intestinal Fatty Acid Binding Protein (I-FABP) concentration (ng·ml^{-1}) was measured in duplicate plasma samples using a pre-coated solid-phase sandwich ELISA (ELH-FABP2-1, Raybiotech, Norcross, GA, USA; Chapter 4; DY3078, DuoSet, R&D systems, Minneapolis, USA; Chapters 5, 7, 8) in line with manufacturer's instructions. Samples were diluted 1:4. Optical density was measured at 450 nm using a microplate reader (Spectrostar Nano, BMG, Ortenburg, Germany). Both kits had a sensitivity of 0.025 ng·ml^{-1} and measurable working range of 0.024 to 100 ng·ml^{-1}.

To assess the agreement between the ELH-FABP-1 and DY3078 ELISA kits, 12 samples were analysed in duplicate (Table 23). Agreement was calculated using a battery of validity statistics.

Table 23. Agreement between the ELH-FABP-2 and DY3078 I-FABP ELISA kits (n = 12).

Concentration (ng·ml^{-1})	ELH-FABP-1	DY3078
Mean ± SD	1.53 ± 0.39	1.39 ± 0.37
r	0.66	
TEM	0.15	
CV (%)	10.4	
B-A (95% LoA)	-0.05 (0.79)	

r = Pearson's Correlation; TEM = standard error of the mean; CV% = coefficient of variation; and B-A 95% LOA = Bland-Altman Mean Bias and 95% limits of agreement.

3.13.11 Claudin-3

Claudin-3 (CLDN-3) concentration ($ng \cdot ml^{-1}$) was measured in duplicate undiluted serum samples using a pre-coated solid-phase sandwich ELISA (EH1342, Finetest, Wuhan, China) in line with manufacturer's instructions. Samples were brought to room temperature overnight. Optical density was measured at 450 nm using a microplate reader (Spectrostar Nano, BMG, Ortenburg, Germany). The kit has a sensitivity of 0.188 $ng \cdot ml^{-1}$ and measurable working range of 0.313 to 20 $ng \cdot ml^{-1}$.

3.13.12 Lipopolysaccharide Binding Protein

Lipopolysaccharide Binding Protein (LBP) concentration ($\mu g \cdot ml^{-1}$) was measured in duplicate 1:200 diluted serum samples using a pre-coated solid-phase sandwich ELISA (RK01764, ABcloncal, Wuburn, USA) in line with manufacturer's instructions. Optical density was measured at 450 nm using a microplate reader (Spectrostar Nano, BMG, Ortenburg, Germany). The kit has a sensitivity of 1.29 $ng \cdot ml^{-1}$ and measurable working range of 3.12 to 200 $ng \cdot ml^{-1}$.

3.13.13 Bacterial DNA

Bacterial DNA concentrations were measured in duplicate plasma samples using a quantitative real-time polymerase chain reaction (qPCR) assay on a LightCycler 96 instrument (LightCycler 96, Roche, Basel, Switzerland). DNA was isolated from plasma using a Quick-DNA Mini Prep Plus kit (D4068, Zymo Research, Irvine, CA, USA) following manufacturer's instructions. The elution buffer was heated to 65 °C before use. Total 16S bacterial DNA was quantified in line with previously outlined methodologies (March et al., 2019) using a University library probe, with standards (E2006-2, Zymo Research, Irvine, CA, USA) and primers (Eurogentec, Liège, Belgium) specific to a 16S region of the bacterial genome. *Bacteroides* species DNA were quantified using a double-dye probe/primer kit (Path-Bacteroides-spp, Genesig, Primerdesign Ltd, Chandler's Ford, UK). Negative controls (PCR grade water) for the entire extraction process were below the limit of detection for both measures. The total 16S analysis was sensitive to 0.1 $pg \cdot \mu l^{-1}$, whilst *Bacteroides* analysis was sensitive to 1 $copy \cdot \mu l^{-1}$. Ratio data are presented as *Bacteroides*/total bacterial DNA.

3.14 Statistical Analysis

All anonymised data was stored using spreadsheet software (Microsoft Excel V.2016, Washington USA). Data analysis and graphical representation were undertaken using Graphpad Prism software (Graphpad Prism V.8, Graphpad Software Inc., CA, USA).

3.14.1 Sample Size Estimation

Sample size estimations were calculated *a priori* for each chapter using G*Power analysis software (V.3.1, Kiel, Germany). Effect sizes relating to the primary dependent variable were determined from previously published data with similar experimental designs (Jones et al. 2003). The number of participants was calculated based on achieving 80% power to determine differences between trials at an alpha level of $p \leq 0.05$.

3.14.2 Randomisation

When a randomised, counter-balanced, repeated-measures, cross-over design was applied, trial-order was determined using a computer-generated random-number generator (*www.randomizer.com*). Trial order blinding was held by an individual independent of the research and only unlocked upon commencing data analysis.

3.14.3 Central Tendency

The mean was selected to describe the central tendency of each data set. This measure was selected as it incorporates all individual data points and can draw comparisons with previous research.

3.14.4 Variation

Standard deviation (SD) was selected to describe the variance of each data set. This measure was selected as it incorporates all individual data points (describing 68.8% of the normal distribution) and can draw comparisons with previous research.

3.14.5 Normality and Sphericity

Normal distribution was assessed using the Shapiro-Wilk test to determine whether the spread of data was comparable to a set of normally distributed data with an identical mean and SD. This test was selected over the Kolmogorov-Smirnov Test given that it is more robust for small sample sizes (Field, 2013).

Sphericity was assessed using Mauchly's Test to determine whether the variance in differences was equal between trials. In cases of aspherical data, the Greenhouse-Geisser corrections were applied for epsilon < 0.75, whilst the Huynh-Feldt correction epsilon > 0.75.

3.14.6 Outliers

Outliers are data points that deviate markedly from the mean and exert disproportionate influence on conclusion regarding relationships amongst variables. Classifications for *error* outliers, followed recommended cut-offs of ± 2.24 SD units (97.5% of total data) for normally distributed data and 4 SD units (94% of total data) for non-normally distributed data (Aguinis et al., 2013). All identified outliers were removed from the categorical analysis where they were contained.

3.14.7 Tests of Statistical Difference

Statistical significance (α level) was set at $p < 0.05$. This level gives 95% confidence that there was a meaningful difference and 5% confidence that the results occurred due to random chance.

3.14.7.1 Independent and Dependant T-Test

2-tailed t-tests were calculated to determine statistically significant mean differences of normally distributed data between independent (independent t-test) or repeated (dependent t-test) samples (Field, 2013). The T-ratio produced shows the relationship between the group differences to the variability in the data and the sample size.

3.14.7.2 Mann-Whitney U and Wilcoxon Signed-Rank Tests

Non-normally distributed data were analysed using a non-parametric Mann-Whitney U test (independent samples) or Wilcoxon signed-rank test (repeated samples). The Mann-Whitney U test rank orders data to govern whether 2 samples have likely come from the same population. The Wilcoxon signed-rank test rank orders differences in magnitude order and the sum of positive and negative ranks assessed for deviation from a median of 0.

3.14.7.3 Analysis of Variance

Analysis of Variance (ANOVA) comprise a collection of statistical models that are utilised to analyse differences between multiple group means and the variation within/between-groups. 1-way ANOVA were used to compare group means of 3 or more data sets. A Friedman test was applied if data was non-normally distributed. 2-way ANOVA were used to examine the influence of 2 independent variables (i.e. time * trial) and 1dependent variable (i.e. heart rate), to assess for main and interaction effects. The calculated F statistic for each ANOVA comparison was referenced to the corresponding degrees of freedom relevant to the pre-defined statistical significance level. 2-way ANOVAs were performed irrespective of normal distribution, given their robustness to this violation (Maxwell, 1990).

3.14.7.4 Post-Hoc Analysis

A significant main or interaction effect with ANOVA testing was followed-up using Holm-Bonferroni Stepwise Adjusted multiple t-tests or Wilcoxon signed-ranks test to determine the location of variance (Field, 2013). This was conducted to conservatively combat the familywise error rate that can occur with multiple comparisons. Consequently, this method is robust to violations of sphericity and maintain the type 1 error rate around 5%.

3.14.8 Tests of Reliability

3.14.8.1 Correlation

A Pearson's correlation coefficient was applied for two independent groups of normally distributed data, whilst a Spearman's rank correlation coefficient was applied for two independent groups of non-normally distributed data. For repeated measures analysis, associations were assessed using repeated-measures correlation (rmcorr) analysis undertaken in the rmcorr R package developed by Bakdash and Marusich (2017) (https://cran.r-project.org/web/packages/rmcorr/). This analysis accounts for non-independence among observations using analysis of covariance (ANCOVA) to statistically adjust for inter-individual variability. By removing measured variance between-participants, rmcorr provides the best linear fit for each participant using parallel regression lines (the same slope) with varying intercept. Correlational analysis provides a dimensionless value that can detect random error trends. Pre-defined limits of acceptance

were considered to be: low (≤0.69), moderate (0.70-0.89) and high (≥0.90; Vincent, 1995). Limitations of correlational analysis include: a lack of sensitivity to systematic bias, oversensitivity to heterogeneity and small sample size bias (Atkinson and Neville, 1998).

3.14.8.2 Coefficient of Variation

Coefficient of Variation (CV), as a dimensionless unit, allows direct comparison of measures irrespective of calibration between studies or equipment (Hopkins, 2000). For biochemical analysis, this calculation was performed between each duplicate sample on a single assay plate (intra-assay CV). Pre-defined mean limits of acceptance were considered to be: very good (≤ 10%) and acceptable (≤ 20%) (Hopkins, 2000). Limitations of mean CV include: the assumption of normal distribution, representation of only a fraction of a dataset (e.g. 68% if normally distributed), inappropriateness for negative data values and bias for heteroscedastic, small sample or low-magnitude datasets (Atkinson and Nevill, 1998).

3.14.8.3 Typical Error of Measurement

Typical Error of the Measurement (TEM), expressed in the measures actual unit of measurement is appropriate for homoscedastic data; and is less susceptible to bias than alternative absolute reliability approaches when the samples size is small (Hopkins, 2000). Limitations of TEM include an assumption of normal distribution, a lack of universally rationalised limits of acceptance and bias for heteroscedastic data (Hopkins, 2000).

3.14.8.4 Bland-Altman Mean Bias and Limits of Agreement

Bland-Altman Mean Bias (B-A) and Limits of Agreement (LoA) display both systematic bias and random error, whereby the direction (mean bias) and 95% confidence limits are examined (Bland and Altman, 1986). Limitations of the B-A LoA include a lack of universally rationalised limits of acceptance; and bias for small sample and heteroscedastic data sets (Hopkins, 2000).

3.14.8.5 Heteroscedasticity

Heteroscedasticity was examined through Bland-Altman Mean Bias and Limits of Agreement analysis, then calculating the Spearman's rank non-parametric correlation coefficient between the absolute differences and individual means of repeat data points. Heteroscedasticity was assumed in the presence of a statistically significant correlation.

3.14.9 Randomisation Blinding

A general blinding questionnaire (e.g. “Placebo”or “GLN” or “don’t know” options) was administered to participants at the end of main trials The Bang Blinding Index (BI) was used to estimate the successfulness of trial blinding (Bang et al., 2010). The BI value ranges from -1 to 1. A value of 0 represents random guessing, 1 represents complete unblinding (e.g. all answers correct), and −1 represents the opposite of guessing (e.g. all responses incorrect). Unblinding can be claimed if one-side of the 95% confidence interval does not cross 0 (Bang et al., 2010).

Chapter 4 - Reliability of Gastrointestinal Barrier Integrity and Microbial Translocation Biomarkers in Response to Exertional-Heat Stress

4.1 Abstract

Purpose: Exertional-heat stress adversely disrupts gastrointestinal (GI) barrier integrity, whereby subsequent microbial translocation (MT) is believed an important even in the pathophysiology of exertional-heat stroke (EHS). Despite widespread application, the temporal reliability of popular GI barrier integity and MT biomarkers is poorly characterised.

Method: Fourteen moderately trained males completed two 80-minute exertional-heat stress tests (EHST) separated by 7-14 days. Venous blood was drawn pre, immediately- and 1-hour post both EHSTs. GI barrier integrity was assessed using the serum Dual-Sugar Absorption Test (DSAT), Intestinal Fatty-Acid Binding Protein (I-FABP) and Claudin-3 (CLDN-3). MT was assessed using plasma Lipopolysaccharide Binding Protein (LBP), total 16S bacterial DNA and *Bacteroides* DNA.

Results: No GI barrier integrity or MT biomarker, except absolute *Bacteroides* concentration, displayed systematic trial order bias ($p \geq 0.05$). I-FABP (trial 1 = Δ 0.834 ± 0.445 ng·ml^{-1}; trial 2 = Δ 0.776 ± 0.489 ng·ml^{-1}) and CLDN-3 (trial 1 = Δ 0.317 ± 0.586 ng·ml^{-1}; trial 2 = Δ 0.371 ± 0.508 ng·ml^{-1}) were increased post-EHST ($p \leq 0.01$). All MT biomarkers were unchanged post-EHST. Coefficient of variation and typical error of measurement post-EHST were: 11.5% and 0.004 (ratio) for the DSAT 90-minutes post probe ingestion; 12.2% and 0.004 (ratio) at 150-minutes post probe ingestion; 12.1% and 0.376 ng·ml^{-1} for I-FABP; 4.9% and 0.342 ng·ml^{-1} for CLDN-3; 9.2% and 0.420 µg·ml^{-1} for LBP; 9.5% and 0.15 pg·µl^{-1} for total 16S DNA; and 54.7% and 0.032 for *Bacteroides*/total 16S DNA ratio.

Conclusion: Each GI barrier integrity and MT translocation biomarker, except *Bacteroides*/total 16S ratio, had acceptable reliability at rest and post exertional-heat stress.

4.2 Introduction

The gastrointestinal (GI) microbiota is a complex microbial ecosystem, which perform numerous symbiotic functions to human health (Cani, 2018). However, to prevent immune activation the microbiota must remain contained within the GI lumen, a process that is tightly regulated by the multi-layered GI barrier (Wells et al., 2017). Exertional-heat stress is a stimulus that disrupts GI barrier integrity and in a manner broadly associated with the severity of thermal strain (Pires et al., 2017). In severe cases, luminal microbes can transverse into the systemic circulation, a response now considered to underlie multiple common athletic health conditions (Costa et al., 2017). The most concerning of these include exercise-induced anaphylaxis (Christensen et al., 2019) and exertional heatstroke (Lim, 2018). Thus, reliable biomarkers of GI barrier integrity and/or MT appear important in the surveillance, diagnosis and treatment of these conditions. To date, there is little evidence documenting the reliability of most commonplace biomarkers, which limits interpretation of their application in both laboratory and field settings.

GI barrier integrity can be assessed *in vivo* using several biomarkers of intestinal permeability, epithelial injury or tight junction integrity (Wells et al., 2017). The Dual-Sugar Absorption Test (DSAT) is the gold-standard GI permeability technique (Bischoff et al., 2014). The traditional endpoint of the DSAT is the 5-hour urinary recovery of pre-ingested lactulose-to-L-rhamnose (L/R; Bischoff et al., 2014) and offers good test-retest reliability when applied at rest (Marchbank et al., 2011). Analytical improvements have recently validated a serum DSAT over a reduced (i.e. 1-3 hours) time course (van Wijck et al., 2013) and with improved sensitivity (JanssenDuijghuijsen et al., 2016; Pugh et al., 2017a). However, given the transient appearance of sugar probes within the blood (Fleming et al, 1996), potentially due to wide heterogeneity in gastric emptying rate following exercise (Costa et al., 2017), the reliability of this technique requires verification. Intestinal Fatty Acid-Binding Protein (I-FABP) is a cytosolic protein expressed exclusively in small intestinal enterocytes and has a half-life of 11-minutes in the systemic circulation following epithelial injury (van de Poll et al. 2007). These characteristics have popularised I-FABP as a prominent biomarker of small intestinal epithelial injury (Wells et al., 2017), with serum concentrations predictive of histological injury (Schellekens et al., 2014). The temporal reliability of I-FABP has never been directly assessed and requires interrogation given its high sensitivity to sub-clinical small intestinal injury. Claudin-3 (CLDN-3) is a conserved GI

epithelial transmembrane protein, which performs an integral role in GI paracellular homeostasis (Zeissig et al., 2007). As a biomarker of GI tight junction (TJ) integrity, preliminary research has shown a strong relationship between urinary CLDN-3 concentration and histological GI CLDN-3 breakdown (Thuijls et al., 2010a, 2010b). Like I-FABP, the temporal reliability of plasma CLDN-3 is currently unknown.

GI MT can be assessed *in vivo* through several indirect biomarkers considered to be indicative of systemic microbial exposure (Wells et al., 2017). Endotoxin, a form of lipopolysaccharide located on the outer membrane of gram-negative bacteria, has traditionally been utilised for this purpose (Costa et al., 2017). However, the search for improved GI MT biomarkers is ongoing, given endotoxin analysis is susceptible to both false-positive (e.g. from exogenous contamination) and false-negative (e.g. from rapid hepatic clearance) results (Dullah and Ongkudon, 2017). Lipopolysaccharide binding protein (LBP) is a type-1 acute phase protein, secreted hepatically following systemic exposure to numerous microbial-associated molecular patterns (Schumann, 2011). However, as an acute-phase protein, its temporal reliability is likely highly subject to influence from numerous co-variates (e.g. infection) (Citronberg et al., 2016). Bacterial DNA, through conserved 16S gene sequencing, is an emerging biomarker of GI MT (Paisse et al., 2016). In comparison with alternative MT measures, one major advantage of bacterial 16S DNA is an apparent independence of hepatic clearance (Mortensen et al., 2013). One innovative study recently proposed a bacterial DNA methodology aimed to improve analytical specificity and reliability through targeting a predominant GI bacterial genus (*Bacteroides*) and correcting for total 16S DNA concentration (March et al., 2019).

The aim of the present study was to determine the reliability of biomarkers of GI barrier integrity (DSAT, I-FABP, CLDN-3) and MT (LBP, total 16S bacterial DNA, *Bacteroides* DNA) at rest and following exertional-heat stress. These data should inform prospective study design, including biomarker selection and statistical power. It is hypothesised that each biomarker of GI barrier integrity and MT would display acceptable test-retest reliability in response to exertional-heat stress.

4.3 Methods

Participants and Ethical Approval

14 healthy males volunteered to take part in this study (Table 24). All participants met the demographic (Section 3.3.1) and health (Section 3.3.3) criteria for inclusion. Informed consent was obtained for each participant following explanation of the experimental procedures (Section 3.1). The study protocol was approved by Plymouth MARJON University Research Ethics Committee (Approval Code: EP040) and conducted in accordance with the principles outlined in the *Declaration of Helsinki (2013)*.

Table 24. Participant demographic characteristics.

Measure	Mean ± SD
Age (years)	26 ± 5
Height (m)	1.78 ± 0.06
Body Mass (kg)	83.4 ± 12.6
Ecercise (h·week^{-1})	6 ± 3
Body Fat (%)	16.1 ± 4.0
$\dot{V}O_{2max}$ (ml·kg^{-1}·min^{-1})	49 ± 4

Experimental Overview

Participants visited the laboratory on 3 occasions. During the first visit, baseline anthropometrics and maximal oxygen uptake ($\dot{V}O_{2max}$) were assessed. The second and third visits were the main experimental trials, which were separated by between 7-14 days (Section 3.5.1). During both main experimental trials, participants completed a 100-minute fixed-intensity military exertional-heat stress test (EHST) (Section 3.8.5). The exercise bouts were separated by 20-minutes seated recovery, including 4-minutes forearm cold water immersion (Section 3.6.7). A pilot investigation found a ~ 2-fold elevation in dual-sugar absorption test (DSAT) responses relative to rest in response to this protocol (n = 6; DSAT 90-minute post probe ingestion; [rest] = 0.014 ± 0.006, [post EHST] = 0.028 ± 0.005; p = 0.02). Data collection coincided cooler annual periods in Plymouth, United Kingdom (Section 3.5.1). A schematic illustration of the protocol is shown in Figure 8.

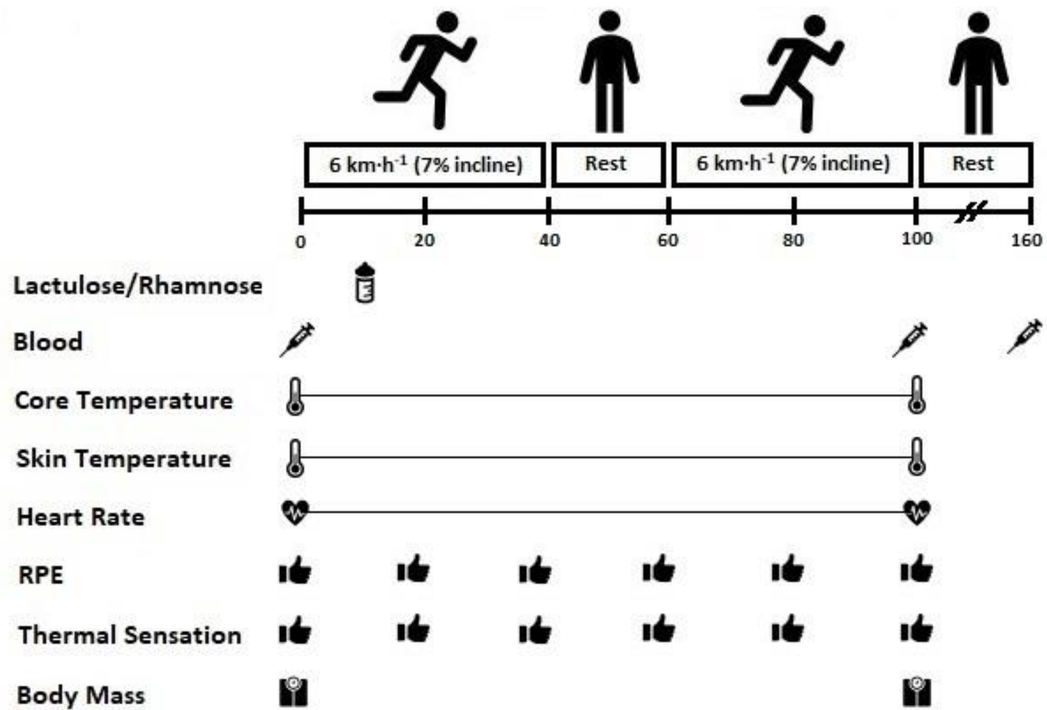

Figure 8. Schematic illustration of the experimental measurement timings.

Dietary and Lifestyle Controls

Trials were conducted following lifestyle (Section 3.5.1), dietary (Section 3.5.2) and hydration (Section 3.5.3) standardisation. Conformation was self-attested using a pre-trial control questionnaire. Participants remained fasted throughout all main experimental trials, but were permitted water (28-30°C) to drink over 20 minutes following both 40-minute EHST bouts (Section 3.8.2).

Anthropometrics

Height (Section 3.4.1), mass (Section 3.4.2) and body fat (Section 2.4.3) were measured following the International Society for the Advancement of Kinanthropometry (ISAK) guidelines (Marfell-Jones et al. 2006). The duplicate coefficient of variation (CV) for skinfold thicknesses at 4-sites was 1.5%.

Maximal Oxygen Uptake

Maximal oxygen uptake ($\dot{V}O_{2max}$) was determined using an incremental treadmill test to volitional exhaustion (Section 3.8.4) in normothermic laboratory conditions (Section 3.7). Expired metabolic gases were measured continuously using a breath-by-breath metabolic cart (3.10.1). Heart rate (HR; Section 3.10.2) and rating of perceived exertion (RPE; Section 3.12.1) were measuring during the final 10 seconds of each stage.

Exertional-Heat Stress Test

The EHST commenced at 08:30 ± 1 hour (Section 3.5.1). Upon laboratory arrival, a capillary blood sample was collected for (CV = 0.3%) plasma osmolality assessment (Section 3.13.6). Participants then measured their own nude body mass (Section 3.4.2), inserted a single use rectal thermistor (T_{core}; Section 3.9.1) and positioned a HR monitor (Section 3.10.2). Hard-wired thermistors were affixed to assess mean skin temperature (T_{skin}) (Section 3.9.2). Participants then dressed in standardised summer-military clothing (Section 3.8.1) and entered the environment chamber that was regulated at ~35 °C (Trial 1: 35.2 ± 0.3 °C; Trial 2: 35.4 ± 0.4 °C; p= 0.15) and ~30% RH (Trial 1: 28 ± 4%; Trial 2: 28 ± 2%; p= 0.25) (Section 3.7).

Following ~20 minutes seated rest, participants undertook a 80-minute fixed-intensity military EHST (Section 3.8.5). T_{core} (Section 3.9.1), T_{skin} (Section 3.9.2), mean body temperature (T_{body}; Section 3.9.3) and HR (Section 3.10.2) were continuously recorded.= throughout the EHST. RPE (Section 3.12.1) and thermal sensation (TS; Section 3.12.2), were reported at 20-minute intervals. Between the two exercise bouts, participants immersed their forearms in a cold-water bath (Trial 1: 15.4 ± 0.8°C, Trial 2: 15.3 ± 0.7°C; p = 0.39) (Section 3.6.7). Post-EHST nude mass was recorded for estimation of whole-body sweat rate (Section 3.11.1).

Blood Collection and Analysis

Venous blood samples (12 ml) were drawn immediately pre, post and one-hour post EHST (Section 3.13.1). Samples were centrifuged at 1300*g* for 15 minutes at 4 °C to separate serum and plasma. Aliquots were frozen at -80 °C until analyses. All blood handling was performed with sterile (pyrogen, DNA free) pipette tips and microtubes (Section 3.13.2).

Haematology

Haemoglobin (Section 3.13.3; CV = 0.7%) and haematocrit (Section 3.13.4; CV = 0.8%) were analysed in fresh whole blood for plasma volume estimation (Section 3.13.5). Post-exercise analyte concentrations were uncorrected for plasma volume change, given the similarity of between-trial response and low molecular weights of quantified analytes.

Dual-Sugar Absorption Test

Participants orally ingested a standard dual-sugar probe solution (Section 3.13.9)

10-minutes into the EHST. Probe concentrations were determined from serum samples collected immediately pre-, 90-minutes (i.e. post-EHST) and 150-minutes (i.e. 1-hour post-EHST) post probe ingestion using high performance liquid chromatography (Section 3.13.9). The laboratory duplicate CV was 1.8-8.5% for both probes (Fleming et al., 1996).

Enzyme Linked Immunosorbent Assays

I-FABP (Section 3.13.10), CLDN-3 (Section 3.13.11) and LBP (Section 3.13.12) were all measured immediately pre- and post- the EHST using solid-phase sandwich ELISAs. The duplicate intra-assay CV were 5.0% (I-FABP), 1.5% (CLDN-3) and 2.6% (LBP), respectively.

Quantitative Real-Time Polymerase Chain Reaction

Total 16S and *Bacteroides* DNA were measured immediately pre- and post- the EHST using quantitative real-time polymerase chain reaction assays (Section 3.13.13). The duplicate intra-assay CV were 6.3% (total 16S) and 17.5% (*Bacteroides*).

Statistics

All statistical analyses were performed using Prism Graphpad software (Section 3.14). Comparisons were made after determining normal distribution using a Shapiro-Wilk test (Section 3.14.5*).* A 2-way analysis of variance (ANOVA) with repeated measures (time x trial) was used to identify differences between the two trials for whole-body physiological, GI barrier integrity and MT data (Section 3.14.7.3, 3.14.7.4). When there was only a single comparison, a paired t-test or non-parametric Wilcoxon signed-ranks test was used to determine between-trial differences (Section 3.14.7.1, 3.14.7.2). Relationships were assessed using repeated-measures correlation analysis undertaken in the rmcorr R package (Section 3.14.8.1). Data are presented as mean ± standard deviation (SD).

A composite *a priori* battery of statistical tests was conducted to determine inter-trial reliability (Atkinson and Nevill, 1998). The DSAT was compared at each 90- and 150-minutes following sugar-probe ingestion, whilst each GI biomarker was compared at rest-, post-EHST and the delta (Δ). Systematic bias was assessed using a paired t-test (Section 3.14.7.1) or non-parametric Wilcoxon signed-ranks test (Section 3.14.7.2). Relative reliability was assessed using a Pearson's product-moment correlation coefficient or non-parametric Spearman's rank correlation coefficient (Section 3.14.8.1). Correlations were classified as small (≤0.69), moderate (0.70-0.89) and high (≥0.90). Absolute reliability was

assessed from: coefficient of variation (Section), typical error of the measurement (TEM; Section 3.14.8.3) and Bland-Altman (B-A) plots with mean difference (bias) and 95 % Limits of Agreement (Section 3.14.8.4). CVs were classified as very good (≤ 10%) and acceptable (≤ 20%). Heteroscedasticity was examined from the non-parametric correlational coefficient between absolute differences and individual means presented on B-A plots (3.14.8.5). Outliers were removed (Section 3.14.6).

Power Analysis

Given the novelty of the dependent variables being evaluated and statistical approach to undertake a battery of reliability statistical tests, it was determined infeasible to perform an *a priori* sample size calculation (Section 3.14.1). Instead, general guidance on appropriate sample sizes (n = 12) for pilot studies were followed, whilst accounting for a ~20% anticipated participant drop-out rate (Julious, 2005).

4.4 Results

Thermoregulatory, Cardiovascular and Perceptual Strain

T_{core} (Figure 9A; time x trial p = 0.63), T_{skin} (Figure 9B; time x trial p = 0.13) and T_{body} (Figure 9C; time x trial p = 0.43) all increased over time by a comparable magnitude between trial 1 and 2. The reliability of peak, mean and Δ in T_{core}, T_{skin} and T_{body} were all good (Table 25). Pre-trial plasma osmolality (trial 1: 293 ± 7 mOsmol·kg^{-1}, trial 2: 294 ± 7 mOsmol·kg^{-1}; p = 0.67), Δ plasma volume (trial 1: -0.61 ± 5.15%, trial 2: -0.02 ± 3.69%; p = 0.67), mean sweat rate (trial 1: 1.53 ± 0.38 l·h^{-1}, trial 2: 1.56 ± 0.45 l·h^{-1}; p = 0.61) and percentage body mass loss (trial 1: 1.15 ± 0.48%; trial 2: 1.21 ± 0.52%; p = 0.31) were all similar between trial 1 and 2. HR (Figure 9D; time x trial p = 0.11), RPE (Figure 9E; time x trial p = 0.38) and TS (Figure 9F; time x trial p = 0.56) all increased over time to a comparable magnitude between trial one and two. The reliability of peak, mean and Δ HR, RPE and TS were all good (Table 25).

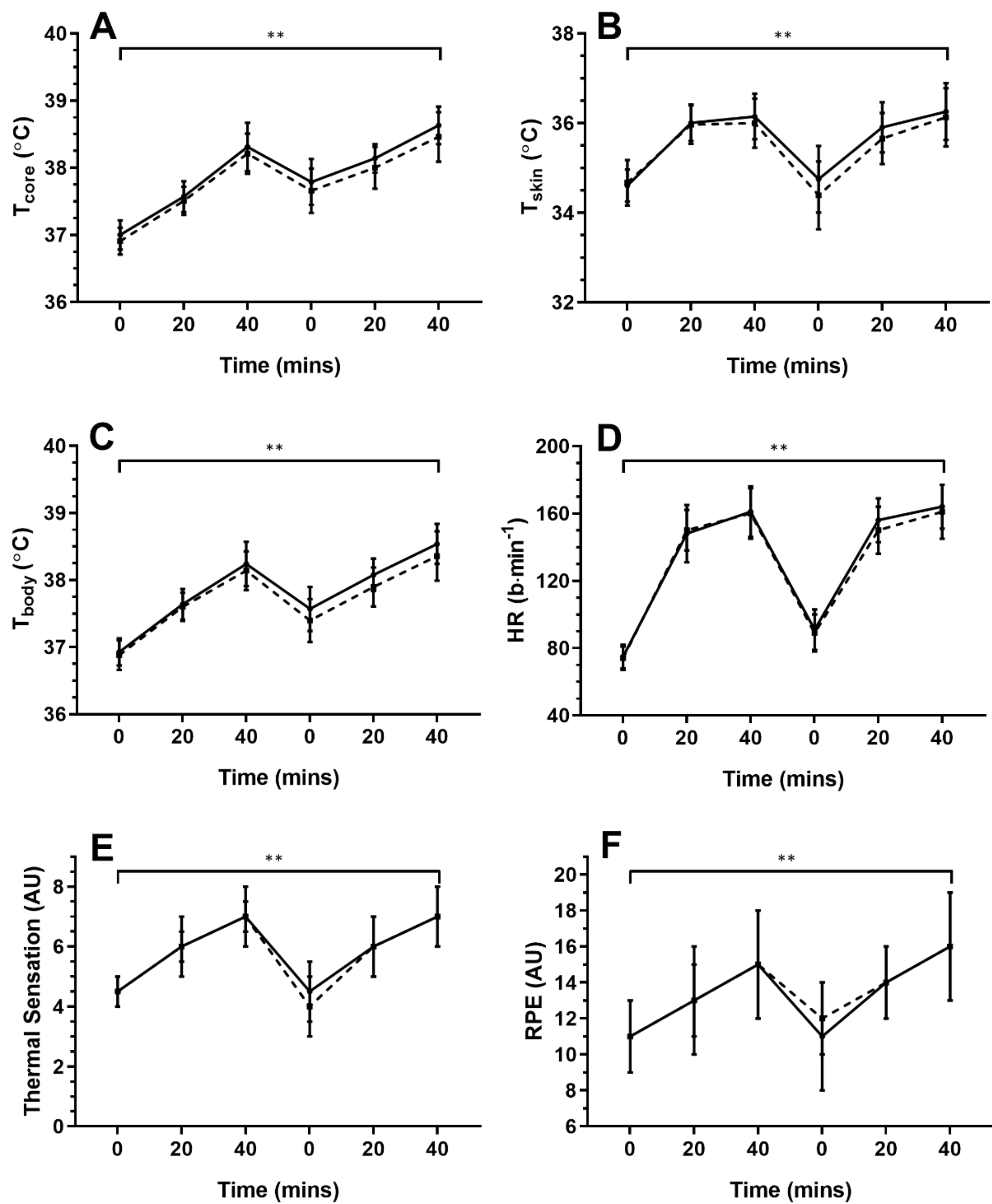

Figure 9. Whole-body physiological responses to repeated EHSTs: (A) = core temperature; (B) = mean skin temperature (*n* = 13); (C) = mean body temperature (*n* = 13); (D) = heart rate; (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = trial 1, broken line = trial 2. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01).

Table 25. Relative and absolute reliability of whole-body physiological responses.

	Trial 1 (SD)	Trial 2 (SD)	*p*	*r*	CV	TEM	Bias (LoA)
T_{core} (°C) Peak	38.63 ± 0.28	38.46 ± 0.37	0.02	0.78**	0.5	0.14	0.17 ± 0.46
T_{core} (°C) Mean	37.87 ± 0.19	37.78 ± 0.23	0.09	0.68**	0.3	0.13	0.09 ± 0.43
T_{core} (°C) Δ	1.62 ± 0.29	1.55 ± 0.44	0.59	0.48	-	0.26	0.07 ± 0.77
T_{skin} (°C) Peak	36.27 ± 0.63	36.13 ± 0.65	0.23	0.83**	0.6	0.27	0.13 ± 0.74
T_{skin} (°C) Mean	35.75 ± 0.44	35.64 ± 0.49	0.13	0.83**	0.4	0.17	0.11 ± 0.46
T_{skin} (°C) Δ	1.65 ± 0.63	1.46 ± 0.65	0.18	0.71**	-	0.35	0.20 ± 0.97
T_{body} (°C) Peak	38.54 ± 0.30	38.36 ± 0.37	0.01	0.79**	0.4	0.16	0.18 ± 0.44
T_{body} (°C) Mean	37.83 ± 0.20	37.74 ± 0.23	0.08	0.70**	0.3	0.12	0.09 ± 0.33
T_{body} (°C) Δ	1.62 ± 0.30	1.49 ± 0.40	0.18	0.60*	-	0.23	0.13 ± 0.63
HR ($b \cdot min^{-1}$) Peak	164 ± 13	162 ± 14	0.19	0.93**	2.0	4	2 ± 11
HR ($b \cdot min^{-1}$) Mean	150 ± 14	148 ± 13	0.29	0.84**	2.8	5	2 ± 15
HR ($b \cdot min^{-1}$) Δ	90 ± 11	90 ± 13	0.99	0.88**	-	4	0 ± 12
RPE (AU) Peak	16 ± 3	16 ± 3	0.99	0.92**	4.1	1	0 ± 3
RPE (AU) Mean	13 ± 2	13 ± 2	0.88	0.80**	5.2	1	0 ± 2
RPE (AU) Δ	6 ± 5	5 ± 3	0.95	0.72**	-	2	0 ± 4
TS (AU) Peak	7.0 ± 0.5	7.0 ± 0.5	0.99	0.84**	4.8	0.5	0.0 ± 1.0
TS (AU) Mean	6.0 ± 0.5	5.5 ± 0.5	0.02	0.88**	3.2	0.0	0.5 ± 0.5
TS (AU) Δ	2.5 ± 1.0	2.5 ± 0.5	0.12	0.61*	-	0.5	-0.5 ± 1.0

** = significant correlation (p ≤ 0.05); ** = significant correlation (p ≤ 0.01).*

Dual-Sugar Absorption Test

Lactulose and L-rhamnose were both undetectable in all participants' basal samples prior to probe ingestion. Inter-trial DSAT responses displayed no systematic bias between trials at both 90- (Figure 10A) and 150-minutes (Figure 10C). There was moderate relative reliability and acceptable absolute reliability at both the 90- and 150-minute time-points. B-A plots displayed bias for both the 90- (Figure 10B) and 150-minute (Figure 10D) time-points. Individual Lactulose and L-rhamnose concentrations had worse reliability than the combined L/R ratio (Table 26). Heteroscedasticity was not present for any analyses.

Intestinal Fatty Acid Binding Protein

I-FABP displayed no trial order systematic bias at either rest, post- or the Δ time-point (Figure 10E). Following EHSTs, I-FABP was elevated above rest (trial 1: Δ = 0.83 ± 0.46 $ng{\cdot}ml^{-1}$ [56 ± 31%]; trial 2: Δ = 0.78 ± 0.49 $ng{\cdot}ml^{-1}$ [46 ± 26%]; $p \leq 0.01$; Figure 10E). At all time-points, I-FABP displayed moderate relative and acceptable absolute reliability (Table 26). B-A plots are presented to illustrate bias for post EHST concentrations (Figure 10F). Heteroscedasticity was not present for any analyses. One participant was excluded as an outlier. Two participants' I-FABP responses displayed unexplainably poor reliability both at rest and post EHSTs. These data were retained given where verbal adherence to pre-trial controls was verbally confirmed. However, removal of these data would have notably improved the reliability of I-FABP both at rest (r = 0.97; CV = 6.1%; TEM = 0.20 $ng{\cdot}ml^{-1}$; B-A ± LoA = -0.05 ± 0.31 $ng{\cdot}ml^{-1}$) and post the EHST (r = 0.97; CV = 7.2%; TEM = 0.22 $ng{\cdot}ml^{-1}$; B-A ± LoA = 0.08 ± 0.47 $ng{\cdot}ml^{-1}$).

Claudin-3

CLDN-3 displayed no trial order systematic bias at either rest, post- or the Δ time-point (Figure 10G). Following EHSTs CLDN-3 was elevated above rest (trial 1: Δ = 0.32 ± 0.59 $ng{\cdot}ml^{-1}$ [5 ± 9%]; trial 2: Δ = 0.37 ± 0.51 $ng{\cdot}ml^{-1}$ [6 ± 7%]; $p \leq 0.01$; Figure 10G). At all time-points, CLDN-3 displayed high relative and very good absolute reliability (Table 26). B-A plots are presented to illustrate bias for post EHST concentrations (Figure 10H). Heteroscedasticity was not present for any analyses.

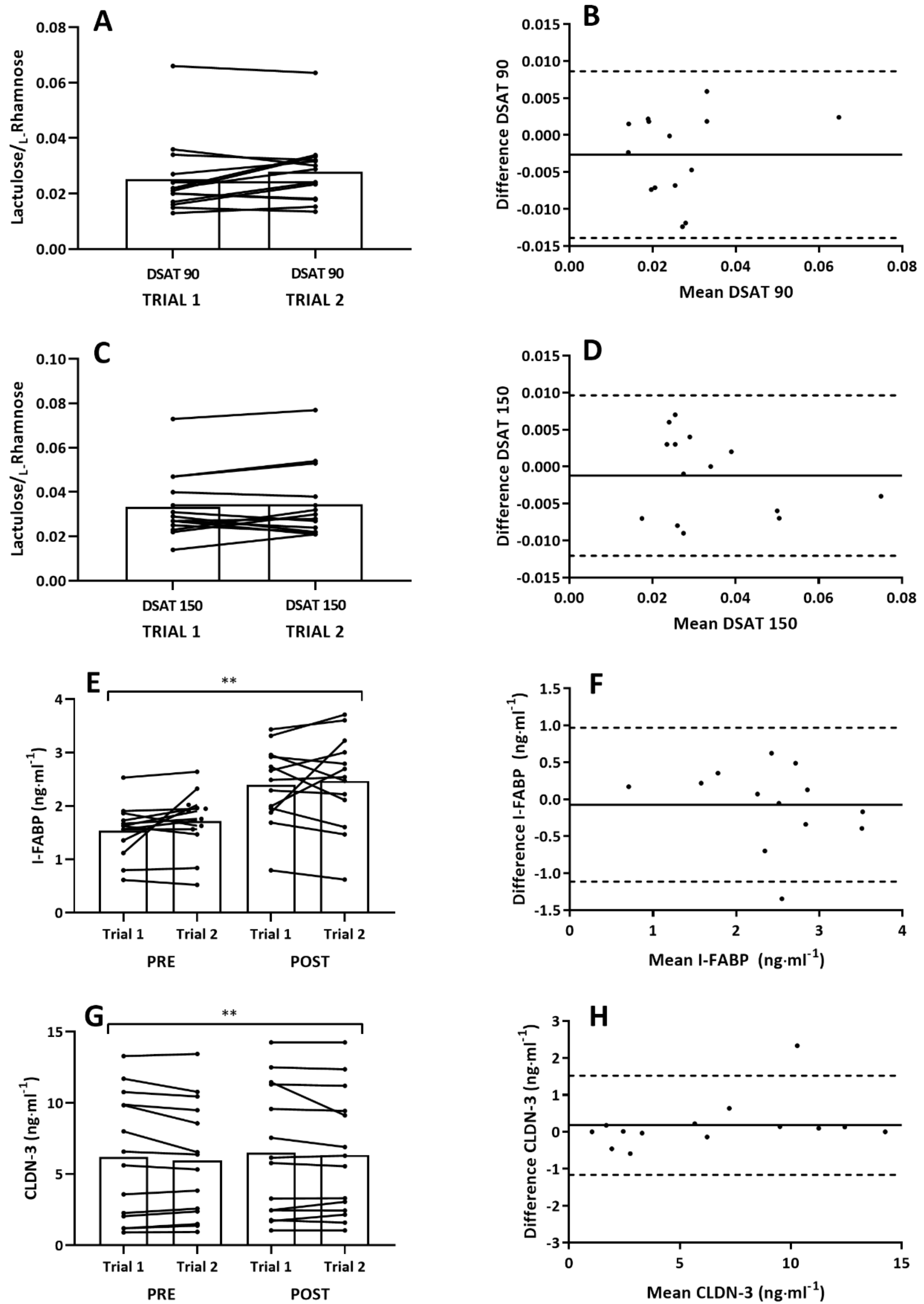

Figure 10. GI barrier integrity responses to EHST trial 1 and trial 2: (A) = L/R ratio at 90 minutes; (C) = L/R ratio at 150 minutes; (E) I-FABP (*n* = 13); and (G) = CLDN-3. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). Bland-Altman mean bias and 95% LoA between post EHST trial 1 and trial 2: (B) = L/R ratio at 90- and (D) 150- minutes; (F) I-FABP (*n* = 13); and (H) = CLDN-3.

Table 26. Relative and absolute reliability of all GI barrier integrity biomarkers.

	Trial 1 (SD)	Trial 2 (SD)	*p*	*r*	CV	TEM	Bias (LoA)
Lactulose (mg·l^{-1}) 90	1.06 ± 0.38	0.90 ± 0.43	0.33	0.60*	21.3	0.26	0.15 ± 0.73
L-Rhamnose (mg·l^{-1}) 90	15.89 ± 3.91	15.85 ± 3.13	0.29	0.53	12.9	2.60	1.04 ± 6.93
DSAT (L/R) 90	0.028 ± 0.012	0.025 ± 0.014	0.17	0.77**	11.5	0.004	-0.000 ± 0.010
Lactulose (mg·l^{-1}) 150	0.97 ± 0.48	0.95 ± 0.52	0.53	0.71**	13.0	0.13	0.02 ± 0.36
L-Rhamnose (mg·l^{-1}) 150	12.01 ± 2.95	11.24 ± 2.96	0.09	0.86**	7.6	1.15	0.77 ± 3.06
DSAT (L/R) 150	0.033 ± 0.015	0.034 ± 0.016	0.37	0.69**	12.2	0.004	0.000 ± 0.010
I-FABP (ng·ml^{-1}) Rest	1.56 ± 0.51	1.69 ± 0.56	0.11	0.75**	11.1	0.30	-0.18 ± 0.75
I-FABP (ng·ml^{-1}) Post	2.39 ± 0.73	2.47 ± 0.88	0.63	0.80**	12.1	0.38	-0.07 ± 1.04
I-FABP (ng·ml^{-1}) Δ	0.83 ± 0.45	0.78 ± 0.49	0.65	0.65**	-	0.28	0.06 ± 0.77
CLDN-3 (ng·ml^{-1}) Rest	6.21 ± 4.38	5.97 ± 4.06	0.17	0.99**	6.8	0.42	0.23 ± 1.17
CLDN-3 (ng·ml^{-1}) Post	6.59 ± 4.77	6.32 ± 4.27	0.34	0.99**	4.9	0.49	0.18 ± 1.34
CLDN-3 (ng·ml^{-1}) Δ	0.31 ± 0.59	0.37 ± 0.51	0.68	0.62*	-	0.34	-0.06 ± 0.95

** = significant correlation (p ≤ 0.05); ** = significant correlation (p ≤ 0.01).*

Lipopolysaccharide Binding Protein

LBP displayed no trial order systematic bias at either rest, post EHSTs or the Δ time-point (Figure 11A). There was no influence of the EHST on LBP concentration ($p = 0.41$). At all-time-points, LBP displayed moderate relative and very good absolute reliability (Table 27). B-A plots are presented to illustrate bias for post EHST concentrations (Figure 11B). Heteroscedasticity was not present for any analyses.

Bacterial DNA

Total 16S (Figure 11C) and *Bacteroides*/16S (Figure 11G) DNA displayed no systematic bias at either rest, post EHSTs or the Δ. *Bacteroides* DNA concentrations (Figure

11E) were systematically lower in trial 2 *versus* trial 1 ($p = 0.04$). At rest, total 16S DNA displayed moderate relative and very good absolute reliability, whereas *Bacteroides* DNA displayed poor relative and absolute reliability. The combined *Bacteroides*/16S DNA ratio subsequently showed poor relative and absolute reliability at rest (Table 27). There was no influence of the EHST on either total 16S ($p = 0.39$), *Bacteroides* ($p = 0.33$) or *Bacteroides*/16S ($p = 0.18$) DNA responses. B-A plots are presented to illustrate bias for post EHST concentrations (Figure 11D, 11F and 11H). Heteroscedasticity was not present for any analyses. One participant was excluded from all bacterial DNA analysis as an outlier.

Association between Biomarkers

Associations between GI integrity and MT biomarkers were conducted for the entire data-set ($n = 28$). Validation of the DSAT at the 90 and 150- minutes time-points found responses to be systematically greater at 150- (0.034 ± 0.015) compared with 90-minutes (0.027 ± 0.013; $p = 0.05$, ES 0.50). There was poor relative ($r = 0.07$) and absolute (CV = 31.8%, TEM = 0.014) reliability between the sample time-points, suggestive of inter-individual variability in sugar probe kinetics. Few statistically significant correlations were reported when comparing GI barrier integrity and MT biomarkers. A small positive correlation was reported between absolute post EHST I-FABP concentrations and the DSAT 150 ($r = 0.60$, $p = 0.02$). When displayed as a Δ response, small positive correlations were evident between: DSAT 90 and total 16S DNA ($r = 0.56$, $p = 0.04$), DSAT 150 and I-FABP ($r = 0.59$, $p = 0.02$) and CLDN-3 and *Bacteroides*/total 16S DNA ($r = 0.71$, $p < 0.01$). No further correlations between either peak or Δ responses for GI barrier integrity and MT biomarkers were evident.

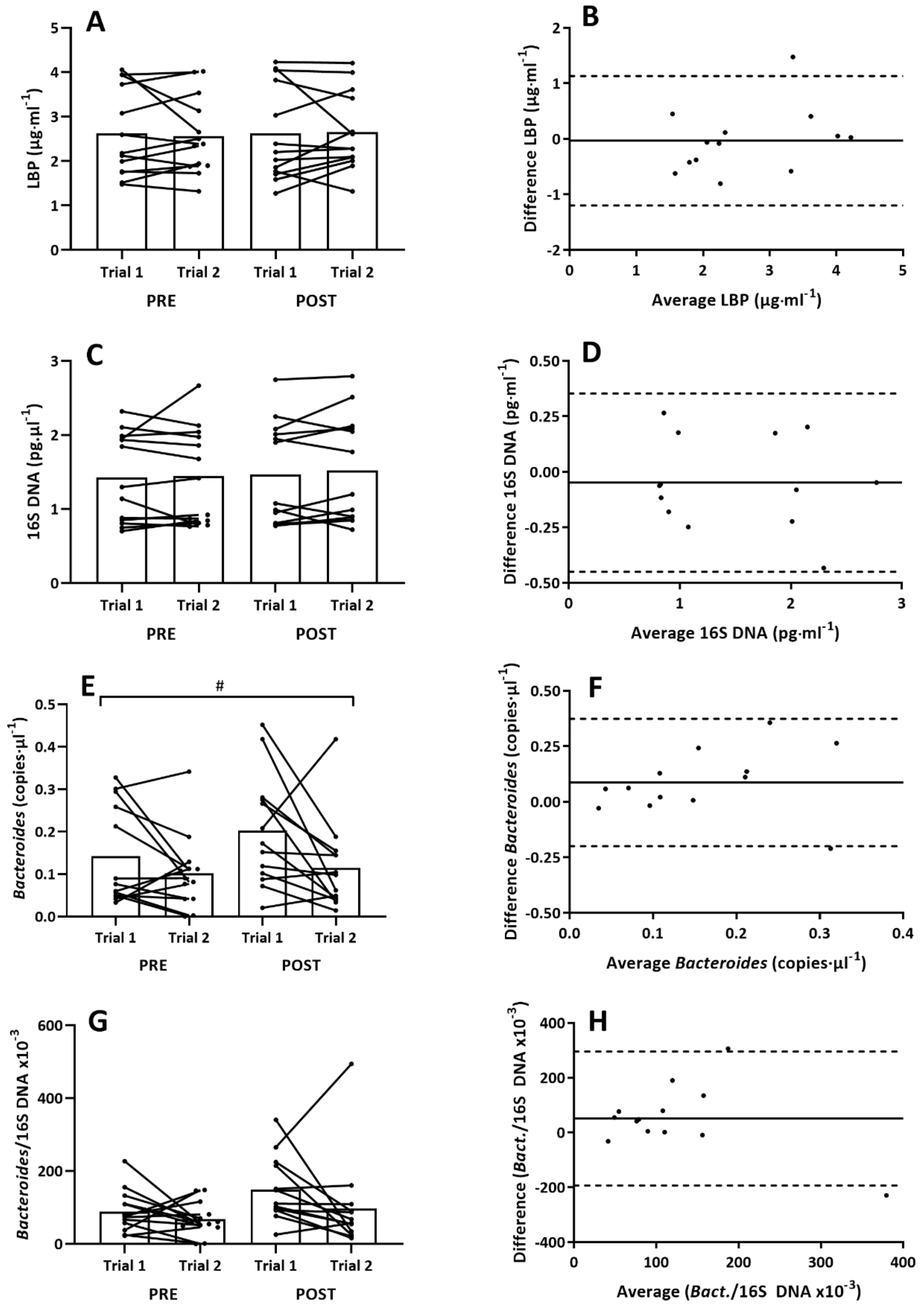

Figure 11. GI MT responses to EHST trial 1 and trial 2: (A) = LBP; (C) total 16S bacterial DNA (*n* = 13); (E) = *Bacteroides* DNA; and (G) = *Bacteroides*/total 16s bacterial DNA (*n* = 13). Significant overall effect of trial (# $p \leq 0.05$). Bland-Altman mean bias and 95% LoA between post EHST trial 1 and trial 2: (B) = LBP; (D) = total 16S bacterial DNA (*n* = 13); (F) *Bacteroides* DNA (*n* = 13); and (H) = *Bacteroides*/total 16s bacterial DNA (*n* = 13).

Table 27. Relative and absolute reliability of all GI barrier integrity biomarkers.

	Trial 1 (SD)	Trial 2 (SD)	*p*	*r*	CV	TEM	Bias (LoA)
LBP (µg·ml^{-1}) Rest	2.63 ± 0.99	2.56 ± 0.87	0.79	0.85**	10.0	0.39	0.061 ± 1.05
LBP (µg·ml^{-1}) Post	2.62 ± 1.08	2.65 ± 0.89	0.59	0.85**	9.2	0.42	-0.03 ± 1.17
LBP (µg·ml^{-1}) Δ	-0.01 ± 0.25	0.09 ± 0.23	0.38	-0.16	-	0.26	-0.09 ± 0.72
16S DNA (pg·µl^{-1}) Rest	1.43 ± 0.60	1.44 ± 0.65	0.79	0.87**	8.1	0.19	-0.02 ± 0.49
16S DNA (pg·µl^{-1}) Post	1.47 ± 0.70	1.52 ± 0.73	0.45	0.82**	9.5	0.15	-0.05 ± 0.40
16S DNA (pg·µl^{-1}) Δ	0.043 ± 0.28	0.08 ± 0.40	0.72	0.56*	-	0.24	-0.03 ± 0.65
Bacteroides DNA (copies·µl^{-1}) Rest	0.14 ± 0.12	0.10 ± 0.09	0.19	0.14	55.0	0.07	0.040 ± 0.19
Bacteroides DNA (copies·µl^{-1}) Post	0.20 ± 0.13	0.12 ± 0.11	0.04*	0.14	56.3	0.10	0.08 ± 0.29
Bacteroides DNA (copies·µl^{-1}) Δ	0.060 ± 0.15	0.01 ± 0.14	0.22	0.61*	-	0.09	0.05 ± 0.25
Bact./16S DNA Rest	0.096 ± 0.060	0.077 ± 0.053	0.31	0.13	60.2	0.052	0.022 ± 0.145
Bact./16S DNA Post	0.143 ± 0.092	0.092± 0.125	0.07	0.20	54.7	0.088	0.052 ± 0.244
Bact./16S DNA Δ	0.047 ± 0.096	0.018 ± 0.138	0.35	0.69*	-	0.077	0.030 ± 0.213

** = significant correlation (p ≤ 0.05); ** = significant correlation (p ≤ 0.01).*

4.5 Discussion

The aim of this study was to determine the short-term (1 to 2 weeks) temporal reliability of several empirical biomarkers of GI barrier integrity (DSAT, I-FABP, CLDN-3) and MT (LBP, total 16S bacterial DNA, *Bacteroides* DNA) following exertional-heat stress. The main findings of this study were that the serum DSAT, I-FABP, CLDN-3, LBP and total 16S bacterial DNA all displayed moderate-to-strong relative and acceptable absolute reliability

between repeat EHSTs. In comparison, absolute *Bacteroides* DNA and *Bacteroides/*total 16S DNA ratio displayed weak relative and unacceptable absolute reliability between repeat EHSTs.

The serum DSAT is a valid alternative to the traditional urine DSAT (Fleming et al, 1996; van Wijck et al., 2011a), whilst offers improved sensitivity to detect transient losses in GI barrier integrity following exertional-heat stress (JanssenDuijghuijsen et al., 2016; Pugh et al., 2017a). Despite this, the temporal reliability of the serum DSAT has never been previously assessed. Potential sources of variability with the serum DSAT include the transient time course of sugar probes in the blood and low absolute lactulose concentrations that challenge the detection limits of common analytical techniques (Fleming et al., 1996; van Wijck et al., 2013). In this study, the serum DSAT was demonstrated to have acceptable reliability, which was comparable to that previously reported with the urine DSAT over both a three-day (van Elburg et al. 1995) and two-week period (Marchbank et al., 2011). The optimal time-point for blood collection with the serum DSAT is an unresolved issue that concerns the methodological implementation of this measure. Herein, blood was collected at both 90-minutes post probe ingestion as this provides the most valid estimate of the urine DSAT in basal conditions (Fleming et al., 1996), and at 150-minutes post as this is where peak responses arose in previous exercise research (van Wijck et al., 2011a). Remarkedly, the temporal reliability of both time-points assessed was almost identical, though given large inter-individual variation in probe kinetics, the magnitude of responses at the two time-points had poor validity. Together, these findings advocate the use of the serum DSAT at either 90- or 150- minutes following probe ingestion (where logistically most convenient) as a reliable alternative to the urine DSAT. There appears little requirement to correct for basal sugar probe concentrations (pre-probe ingestion) following a $\geq$ 10 hour overnight fast given that all participants samples were returned negative.

I-FABP is the principal biomarker of small-intestinal epithelial injury (Wells et al., 2017). Despite growing popularity, the temporal reliability of circulating I-FABP has never been previously assessed. In the present study, resting I-FABP concentrations were consistently at the upper end of the general healthy reference range for studies utilising commercial ELISAs (0.1-2.0 ng·ml^{-1}; Treskes et al., 2017). These concentrations must be considered when evaluating the absolute reliability thresholds reported herein. The

rationale for large between-study discrepancies in absolute I-FABP concentrations are poorly understood, though are more likely attributable to analytical discrepancies (e.g. ELISA antibody, ELISA wash procedure, sample storage), than participant demographic (Treskes et al., 2017). The reliability of I-FABP at rest displayed moderate relative and acceptable absolute reliability. Following both EHSTs, I-FABP increased by approximately 50% or 0.800 ng·ml^{-1}. This response is comparable to numerous similar duration/intensity exercise protocols, such as: 45-to-60 minutes of ~70% $watt_{max}$ normothermic cycling (van Wijck et al., 2011a, [61%, Δ 0.306 ng·ml^{-1}] 2012, [61%; Δ 0.179 ng·ml^{-1}] and 20-30 minutes of ~80% $\dot{V}O_{2max}$ running (Barberio et al., 2015 [46%, Δ 0.297 ng·ml^{-1}]; March et al., 2017 [72%; Δ 0.350 ng·ml^{-1}]). In comparison, greater elevations in I-FABP have been reported following 90-120 minutes of moderate-intensity running in the heat (30°C; Morrison et al., 2014 [663%; Δ 0.203-0.806 ng·ml^{-1}]; Snipe et al., 2017 [288%, Δ 0.897 ng·ml^{-1}]; 2018 [432%, Δ 1.230 ng·ml^{-1}]. Given the high sensitivity of I-FABP to even minor GI injury, it is vital that known extraneous variables (e.g. prandial/hydration status, prior exercise) are tightly controlled prior to investigation. Whilst participants in the present study provided written conformity to all pre-trial controls, 2 participants' resting I-FABP concentrations appeared suspect to prior GI injury in 1 trial, which interestingly was unable to be detected by any other analyte.

CLDN-3 is a novel biomarker of GI TJ integrity (Wells et al., 2017). Despite introduction as a TJ biomarker almost a decade ago, the biological relevance of elevated circulating CLDN-3 remains poorly understood. This includes unknown temporal reliability. In the present study, resting CLDN-3 concentrations were consistent with previous publications (0.5-15 ng·ml^{-1}) in healthy populations (Yeh et al., 2013; Typpo et al., 2015). At rest, large inter-individual variation in CLDN-3 concentration was evident, meaning that relative reliability was almost uniform. Following both EHSTs, plasma CLDN-3 increased consistently by approximately 5-10%. This finding compares well to the only previous exercise study, where concentrations increased directly following a 1 hour moderate-intensity (70% $\dot{V}O_{2max}$) run in both a temperate (22°C; 6.7 > 7.6 ng·ml^{-1}) and hot (33°C; 6.6 > 8.2 ng·ml^{-1}) ambient environment (Yeh et al., 2013). The clinical relevance of this small, transient increase in CLDN-3 following exercise is poorly understood, though is modest in comparison with the magnitude of increase (4-20 fold) shown acutely following major non-abdominal surgery (Typpo et al., 2015; Habes et al., 2017). Promisingly, of all the GI barrier

integrity biomarkers compared, CLDN-3 displayed the strongest relative and absolute reliability.

LBP is a type-1 acute phase protein that responds to a wide-variety of microbial-associated molecular patterns and is considered a stable indirect biomarker of bacterial endotoxemia (Dullah and Ongkudon, 2017). In the present study, resting LBP concentrations displayed moderate relative and good absolute reliability. These results support previous research demonstrating basal LBP concentrations to display moderate short-term (≤ 7 day) relative reliability (intraclass correlation coefficient = 0.61) (Citronberg et al., 2016). In comparison, endotoxin has weak reliability (intra-individual CV = 22%) when examined basally over a 7-day period (Guy et al., 2017). Following the EHST, LBP was unchanged in both trials, with concentrations offering comparable levels of reliability to at rest. Whilst the evidence is sparse regarding LBP responses to exercise, previous evidence has shown minor elevations in LBP *circa* 10-15% immediately following a fatiguing treadmill walk (4.5 $km \cdot h^{-1}$) in the heat (40v°C; 106 minutes; Selkirk et al., 2008), and 1 hour of moderate intensity (70% $\dot{V}O_{2max}$) treadmill running (Jonvik et al., 2019). A potential explanation for these discrepant findings might relates to a greater severity of thermoregulatory and/or cardiovascular strain in previous studies.

Bacterial DNA is an emerging biomarker of GI MT, given the recent characterisation of the blood microbiome and improvements in analytical 16S PCR sensitivity (Paisse et al., 2016). In the present study, resting total 16S DNA concentrations displayed moderate relative and good absolute reliability. This finding is promising, given previous concerns that plasma bacterial DNA concentrations are susceptible to background sample contamination (Glassing et al., 2016). Quantification of total plasma 16S bacterial DNA in exercise settings has never been previously examined, though consistent with other MT biomarkers, the present results show total 16S bacterial DNA to be stable following moderate intensity exertional-heat stress. One criticism of total 16S bacterial DNA assessment, particularly in exercise settings, is a lack of GI specificity, with absolute concentrations like to be influenced by factors including: DNase concentration (Velders et al., 2014) and 16S DNA contamination from other body/blood compartments (Paisse et al., 2016). To account for this error, one hypothetically improved method involves targeting highly abundant GI genus, such as *Bacteroides* (~30% of GI microbiota) and applying a ratio correction for total 16S concentration (March et al., 2019). This method is particularly favourable given that

the phyla *Firmicutes* and *Bacteroidetes* comprise >90% of the GI microbiome (*Bacteroides*; Huttenhower et al., 2012) and <5% of the plasma microbiome (Paisse et al., 2016). Utilising this hypothesis, March *et al* (2019) reported that the plasma *Bacteroides*/16S DNA ratio tended to increase (~25%; p = 0.07) following a 1-hour moderate intensity (70% $\dot{V}O_{2max}$) run in the heat (30 °C), though large inter-individual variability in responses were evident. In the present study, the *Bacteroides*/16S DNA ratio was unchanged following the EHST and appeared to be systematically lower post the EHST in the second trial (but not the Δ). This systematic bias was unexpected given the uniformity of all other analytes examined and the poor analytical reliability of this biomarker (e.g. mean duplicate CV = 17.5%). It is presently unclear whether the poor reliability of this measure obscured a true effect of the EHST and/or the meaningfulness of this result in relation to more severe MT.

Evidence examining associations GI barrier integrity and/or MT biomarkers in response to exercise is limited. Given general logistical constraints of the urine/plasma DSAT, most relevant evidence has attempted to validate (correlate) this method against more practical GI barrier integrity biomarkers. These studies have generally demonstrated significant, though weak correlations (r = 0.4-0.6) between basal corrected (Δ) DSAT (urine 5 hour) and I-FABP responses in response to mild exercise-induced GI barrier integrity loss (van Wijck et al., 2011a, 2012b; March et al., 2017). In the present study, the DSAT was positively associated with total 16S DNA at 90 minutes and I-FABP at 150 minutes. These associations were however small, whilst the DSAT did not correlate with any other biomarker. A potential explanation for these weak findings might result from the lack of basal DSAT correction or the mild overall severity of GI barrier integrity loss. In general no GI barrier integrity and MT biomarkers, except for CLDN-3 and *Bacteroides*/total 16S DNA were found to be associated. Previous exercise gastroenterology research found various biomarker - combinations to be weakly- (r = 0.1-0.6; Yeh et al., 2013; Sessions et al., 2016; March et al., 2019) or un-associated (Karhu et al., 2017; Snipe et al., 2018a). Several physiological (e.g. hepatic and immune microbial clearance, transcellular MT, GI microbial density) and analytical (e.g. exogenous sample contamination, inconsistent biomarker kinetics, regional tissue distribution) factors all likely weaken the relationship between biomarkers (Wells et al, 2017).

4.6 Limitations

Despite implementation of a tightly controlled methodological design, accounting for most extraneous variables, the present results were not without some limitations. First, the EHST was only able to evoke moderate GI barrier integrity loss and did not influence MT. A previous systematic review highlighted an exercise induced T_{core} threshold of 38.6 °C for GI barrier integrity loss (DSAT, I-FABP and endotoxin) to be common (>50% incidence) and 39.0 °C for GI barrier integrity loss to be universal (100% incidence; Pires et al., 2017). Positively, no GI barrier integrity or MT biomarker displayed statistical heteroscedasticity in the present study, suggestive that absolute reliability was not dependent upon the magnitude of biomarker response. Second, biomarker analysis was limited to a single time-point after the EHST (at termination), though this is justifiable given that peak responses consistently occur at this instance during comparable exertional-heat stress interventions (e.g. *I-FABP*, Snipe et al., 2017; *CLDN-3*, Yeh et al., 2013; *LBP*, Moncada-Jimenez et al., 2010; *Bacteroides*/total 16S, March et al., 2019). Third, there was statistically significant systematic bias for peak T_{core} and T_{body} responses, which were lower (0.17 °C and 0.18 °C) following implementation of trial 2. This result was not anticipated, given numerous previous studies show a 1 week washout period to be sufficient in preventing carry-over (heat acclimation) effects in response to acute exertional-heat stress (Barrett and Maughan, 1993; Willmott et al., 2015). Fourth, given neither a basal DSAT or urinary DSAT were performed, it was not possible to directly determine either than impact of the EHST on DSAT results or make comparisons between DSAT responses between biofluids. This decision was made to facilitate participant retention by minimising the experimental time burden. Finally, females were excluded from participation due to unavailability of menstruation hormone testing. Previous evidence has shown no influence of sex on GI barrier integrity responses to exertional-heat stress when women were examined in the follicular phase of the menstrual cycle (Snipe and Costa, 2018a).

4.7 Conclusion

This is the first study to comprehensively assess the reliability of GI barrier integrity and/or microbial translocation biomarkers both at rest and following exertional (-heat) stress. Quantifying biomarker reliability is a vital step required to inform biomarker selection for application in laboratory and field settings. Each of the GI barrier integrity biomarkers assessed displayed moderate-to-good relative and acceptable absolute

reliability, both at rest and post the EHST. Serum DSAT responses had comparable reliability at 2 individual time-points following sugar-probe ingestion (90- and 150-minutes), though response kinetics displayed inconsistent time courses. I-FABP and CLDN-3 both increased following the EHST and their responses were found to be weakly associated. None of the selected MT biomarkers responded to the EHST, suggestive that a greater severity of GI barrier integrity loss is required to induce MT. LBP and total 16S DNA both demonstrated moderate-to-good relative and acceptable absolute reliability at both time-points. There was a weak association between LBP and total 16S post-EHST responses. Despite offering superior methodological rationale, *Bacteroides* DNA had unacceptable reliability. The findings of the present study have direct relevance for evaluating the efficacy of interventions to attenuate the rise in GI barrier integrity/MT when exercising in the heat. Such interventions might include exercise training, heat acclimatisation and nutritional supplementation.

Chapter 5 - Influence of Aerobic Fitness on Gastrointestinal Barrier Integrity and Microbial Translocation In Response to a Military Exertional Heat Stress Test

5.1 Abstract

Purpose: Exertional-heat stress adversely disrupts gastrointestinal (GI) barrier integrity, whereby subsequent microbial translocation (MT) is believed an important even in the pathophysiology of exertional-heat stroke (EHS). To date, the influence of aerobic fitness on GI barrier integrity and MT following exertional-heat stress is poorly characterised.

Method: Ten untrained (UT; $\dot{V}O_{2max}$ = 45 ± 3 $ml \cdot kg^{-1} \cdot min^{-1}$) and ten highly trained (HT; $\dot{V}O_{2max}$ = 64 ± 4 $ml \cdot kg^{-1} \cdot min^{-1}$) males completed an ecologically valid (military) 80-minute fixed-intensity exertional-heat stress test (EHST). Venous blood was drawn immediately pre- and post-EHST. GI barrier integrity was assessed using the serum dual-sugar absorption test (DSAT) and plasma Intestinal Fatty-Acid Binding Protein (I-FABP). MT was assessed using plasma *Bacteroides*/total 16S DNA.

Results: UT experienced greater thermoregulatory, cardiovascular and perceptual strain ($p < 0.05$) than HT during the EHST. Serum DSAT responses were similar between the 2 groups ($p = 0.59$), although Δ I-FABP was greater ($p = 0.04$) in the UT (1.14 ± 1.36 $ng \cdot ml^{-1}$) *versus* HT (0.20 ± 0.29 $ng \cdot ml^{-1}$) group. *Bacteroides*/Total 16S DNA ratio was unchanged (Δ; -0.04 ± 0.18) following the EHST in the HT group, but increased (Δ; 0.19 ± 0.25) in the UT group ($p = 0.05$). Weekly aerobic training hours had a weak, negative correlation with Δ I-FABP and *Bacteroides*/Total 16S DNA responses.

Conclusion: When exercising at the same absolute workload, UT individuals are more susceptible to small intestinal epithelial injury and MT than HT individuals. These responses appear partially attributable to greater thermoregulatory, cardiovascular, and perceptual strain.

5.2 Introduction

The gastrointestinal (GI) microbiota is a complex microbial ecosystem, which performs numerous symbiotic functions to human health (Cani, 2018). However, to prevent systemic immune activation, the microbiota must remain contained to the GI lumen, a function that is tightly regulated by the multi-layered GI barrier (Wells et al., 2017). Exertional heat stress negatively disrupts the integrity of the GI barrier (Costa et al., 2017) and in a manner broadly associated with the severity of thermal strain (Pires et al., 2017). Though poorly characterised, the mechanisms driving this response are believed attributable to the combined influence of localised ischemic injury following hypoperfusion, and paracellular tight junction breakdown following hyperthermia-mediated cytotoxicity (Dokladny et al., 2016). In cases of severe GI barrier integrity loss, subsequent systemic microbial translocation (MT) can trigger a sequela of pro-inflammatory responses via activation of NF-κβ (Deitch, 2012). These responses may underpin several potentially serious health conditions that affect physically active populations (e.g. military personnel, firefighters, athletes), most notably including exertional heatstroke (Lim, 2018).

Exertional Heat Stroke (EHS) is the most severe condition along a continuum of heat-related illnesses (Leon and Bouchama, 2011). It is medically defined by: a pathological rise in core body temperature (T_{core}; >40°C); central nervous system dysfunction (e.g. delirium, coma); and multiple organ failure (Bouchama and Knochel, 2002). In military settings, EHS poses a significant threat to operational readiness and can have long-term career/health implications for incapacitated personnel (Epstein et al., 2012). The incidence of EHS in the armed forces is presently estimated to be *circa* 0.75/1000 cases per person-year in both the United Kingdom (Stacey et al., 2016) and the United States (Army Forces Health Surveillance Centre, 2020). This prevalence is said to be primarily attributable to the widespread exposure of highly-motivated individuals to strenuous physical activity, often whilst wearing encapsulating clothing and/or when deployed to hot ambient environments (Epstein et al., 2012). Given these issues, various occupational policy reports have been published to provide guidance on effective EHS management (Belval et al., 2018; Military Headquarters of the Surgeon General, 2019). Despite this, until recently little consideration had been given to the relevance of GI-MT within the pathophysiology of EHS, which warrants future research attention (Lim, 2018).

Various intrinsic (e.g. age) and extrinsic (e.g. clothing) risk factors may predispose military personnel to EHS (Westwood et al., 2020). Aerobic fitness is one well-characterised intrinsic risk factor, whereby failure of a recent mandatory fitness test, is associated with a 2-12 fold increased odds-ratio of EHS in comparison to a successful test (Wallace et al., 2006; Moore et al., 2016; Nelson et al., 2018). Improved cardiovascular stability (e.g. plasma volume), cellular thermotolerance (e.g. intracellular heat shock protein [I-HSP] expression) and improved anti-endotoxin neutralisation (Selkirk et al., 2008) are key mechanisms that appear to explain the benefits afforded by improved aerobic fitness (Selkirk and McLellan, 2001; Kazman et al., 2013). During self-paced physical activity, modification of thermoregulatory behaviour (e.g. slower pacing) can help mitigate the enhanced risk of EHS experienced by less trained personnel virtue of poorer thermoregulation (Selkirk and McLellan, 2001). However, these behavioural modifications are often unattainable during group-paced physical activities, which accounted for 79% of EHI hospitalisations in the UK Armed Forces between 2007 - 2014 (Stacey et al., 2015). Despite this knowledge, the influence of aerobic fitness on GI barrier integrity, MT and subsequent EHS have not been adequately characterised.

In a pioneering study, untrained individuals ($\dot{V}O_{2max}$ 37-44 ml·kg·min^{-1}) experienced a *circa* 100% increase in GI MT (plasma lipopolysaccharide [LPS]) when assessed at fixed 0.5 °C T_{core} increments above 39.0 °C during a low-intensity (4.5 km·h^{-1}, 2% incline) EHST in a 40 °C ambient environment (Selkirk et al., 2008). In comparison, endotoxin and LBP concentrations were unchanged from rest throughout this protocol in highly trained individuals ($\dot{V}O_{2max}$ 54-73 ml·kg·min^{-1}), despite this group presenting an increase in both exercise and thermal capacity. The notion that increased aerobic fitness protects GI barrier integrity during exertional-heat stress was inconsistent with Morrison et al. (2014), who conversely demonstrated trained individuals ($\dot{V}O_{2max}$ 64 $\pm$ 4 ml·kg·min^{-1}) to have greater intestinal injury (plasma intestinal fatty-acid binding protein [I-FABP]) than untrained individuals ($\dot{V}O_{2max}$ 46 $\pm$ 4 ml·kg·min^{-1}) during a 90-minute self-paced EHST. In this study, mean and peak thermoregulatory strain were not different between the two fitness groups. Together, these findings suggest that elevated aerobic fitness causes more pronounced GI barrier integrity loss during relative intensity exercise, potentially caused via greater splanchnic hypoperfusion, whilst GI MT is conversely blunted, potentially caused via an improved capacity for systemic microbial neutralisation (Lim et al., 2019).

The aim of the present study was to determine the influence of aerobic fitness on GI barrier integrity (dual-sugar absorption test, I-FABP) and MT (*Bacteroides/*total 16S bacterial DNA) biomarkers in response to a fixed-intensity exertional-heat stress test. This protocol has ecological relevance to worldwide military work-guidance doctrine for physical activity in the heat (Spitz et al., 2012). It is hypothesised that highly trained individuals would experience elevated GI barrier integrity loss, but reduced GI MT in comparison to untrained individuals.

5.3 Methods

Participants and Ethical Approval

20 healthy males volunteered to participate in the present study (Table 28). Participants were classified as untrained (UT; n = 10; ≤ 50 $ml\cdot kg^{-1}\cdot min^{-1}$; ≤ 3 $h\cdot week^{-1}$) or highly trained (HT; n = 10; ≥ 60 $ml\cdot kg^{-1}\cdot min^{-1}$; ≥ 6 $h\cdot week^{-1}$) based upon *a priori* criteria for maximal oxygen uptake and weekly self-attested involvement in cardiovascular training (Morrison et al., 2014). All participants met the demographic (Section 3.3.1) and health (Section 3.3.3) criteria for inclusion. Informed consent was obtained for each participant following explanation of the experimental procedures (Section 3.1). The study protocol was approved by Plymouth MARJON University Research Ethics Committee (Approval Code: EP096) and conducted in accordance with the principles outlined in the *Declaration of Helsinki (2013)*.

Table 28. Participant demographic characteristics.

Measure	**Untrained (*n* = 10)**	**Highly trained (*n* = 10)**
Age (years)	27 ± 5	32 ± 4**
Height (m)	1.78 ± 0.04	1.77 ± 0.03
Body Mass (kg)	79.5 ± 14.0	71.4 ± 5.1
Total Exercise ($h\cdot week^{-1}$)	5 ± 1	10 ± 1**
Aerobic Training ($h\cdot week^{-1}$)	2 ± 1	9 ± 1**
Body Fat (%)	16.3 ± 3.7	9.0 ± 2.3**
$\dot{V}O_{2max}$ ($ml\cdot kg^{-1}\cdot min^{-1}$)	45 ± 3	64 ± 4**

*Significant between-group difference (*p ≤ 0.05; ** p ≤ 0.01).*

Experimental Overview

This study applied a 2-way independent groups design. Participants visited the laboratory on 2 occasions. During the first visit, baseline anthropometrics and maximal oxygen uptake ($\dot{V}O_{2max}$) were assessed. In the second visit, participants completed a 100-minute fixed-intensity military exertional-heat stress test (EHST) (Section 3.8.5). The exercise bouts were separated by 20-minutes seated recovery, including 4-minutes forearm cold water immersion (Section 3.6.7). Data collection was undertaken in Plymouth, United Kingdom, where mean daily T_{amb} at a local meteorological station (Camborne, United Kingdom; latitude: 50.218 ° N) remained below 20 °C (Met Office, 2019). A schematic representation of the experimental protocol is provided in Figure 12.

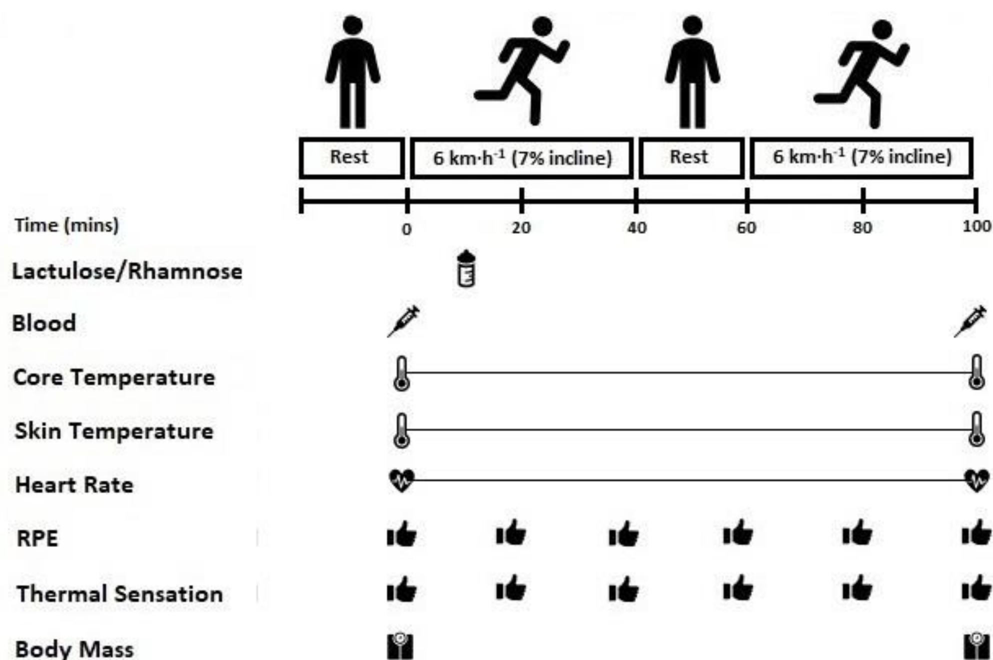

Figure 12. Schematic illustration of the experimental measurement timings.

Dietary and Lifestyle Controls

Trials were conducted following lifestyle (Section 3.5.1), dietary (Section 3.5.2) and hydration (Section 3.5.3) standardisation. Conformation was self-attested using a pre-trial control questionnaire. Participants remained fasted throughout main experimental trials, but were permitted water (28-30°C) to drink over 20 minutes following both 40-minute EHST bouts (Section 3.8.2).

Anthropometrics

Height (Section 3.4.1), mass (Section 3.4.2) and body fat (Section 2.4.3) were measured following the International Society for the Advancement of Kinanthropometry (ISAK) guidelines (Marfell-Jones et al. 2006). The duplicate coefficient of variation (CV) for skinfold thicknesses at 4-sites was 2.1%.

Maximal Oxygen Uptake

Maximal oxygen uptake ($\dot{V}O_{2max}$) was determined using an incremental treadmill test to volitional exhaustion (Section 3.8.4) in normothermic laboratory conditions (Section 3.7). Expired metabolic gases were measured continuously using a breath-by-breath metabolic cart (3.10.1). Heart rate (HR; Section 3.10.2) and rating of perceived exertion (RPE; Section 3.12.1) were measuring during the final 10 seconds of each stage.

Exercise-Heat Stress Test

The EHST commenced at 08:30 ± 1 hour (Section 3.5.1). Upon laboratory arrival, participants provided a mid-flow urine sample to assess hydration status via urine osmolality (Section 3.12.7; CV = 0.4%) and urine specific gravity (Section 3.12.8; CV = <0.1%). A capillary blood sample was also collected for (CV = 0.2%) plasma osmolality assessment (Section 3.13.6). Participants then measured their own nude body mass (Section 3.4.2), inserted a single use rectal thermistor (T_{core}; Section 3.9.1) and positioned a HR monitor (Section 3.10.2). Hard-wired thermistors were affixed to assess mean skin temperature (T_{skin}) (Section 3.9.2). Participants then dressed in standardised summer-military clothing (Section 3.8.1) and entered the environment chamber that was regulated at ~35 °C (UT: 35.0 ± 0.2 °C; HT: 35.1 ± 0.3 °C; p = 0.54) and ~30% RH (UT: 32 ± 5%; HT: 30 ± 3%; p = 0.27) (Section 3.7).

Following 20 minutes seated rest, participants undertook a 100-minute fixed-intensity military EHST (Section 3.8.5). T_{core} (Section 3.9.1), T_{skin} (Section 3.9.2), mean body temperature (T_{body}; Section 3.9.3) and HR (Section 3.10.2) were continuously recorded throughout the EHST. RPE (Section 3.12.1) and thermal sensation (TS; Section 3.12.2), were reported at 20-minute intervals. Between the two exercise bouts, participants immersed their forearms in a cold-water bath (UT: 15.6 ± 1.3°C, HT: 16.1 ± 0.7°C; p = 0.17) (Section 3.6.7). Post-EHST nude mass was recorded for estimation of whole-body sweat rate (Section 3.11.1).

Blood Collection and Analysis

Venous blood samples (12 ml) were drawn immediately pre and post the EHST (Section 3.13.1). Samples were centrifuged at 1300*g* for 15 minutes at 4 °C to separate serum and plasma. Aliquots were frozen at -80 °C until analyses. All blood handling was performed with sterile (pyrogen, DNA free) pipette tips and microtubes (Section 3.13.2).

Haematology

Haemoglobin (Section 3.13.3; CV = 0.5%) and haematocrit (Section 3.13.4; CV = 0.4%) were analysed in fresh whole blood for plasma volume estimation (Section 3.13.5). Post-exercise analyte concentrations were uncorrected for plasma volume change, given the similarity of between-trial response and low molecular weights of quantified analytes.

Dual-Sugar Absorption Test (DSAT)

Participants orally ingested a standard dual-sugar probe solution (Section 3.13.9) 10-minutes into the EHST. Probe concentrations were determined from serum samples collected 90-minutes (i.e. post-EHST) post probe ingestion using high performance liquid chromatography (Section 3.13.9). The duplicate CV for lactulose/$_L$-rhamnose was 8.8%.

Intestinal Fatty-Acid Binding Protein

I-FABP was measured immediately pre- and post- the EHST using a solid-phase sandwich ELISA (Section 3.13.10). The intra-assay CV was 4.0%.

Bacterial DNA

Total 16S and *Bacteroides* DNA were measured immediately pre- and post- the EHST using quantitative real-time polymerase chain reaction assays (Section 3.13.13). The duplicate intra-assay CV were 9.8% (total 16S) and 18.8% (*Bacteroides*).

Statistical Analyses

All statistical analyses were performed using Prism Graphpad software (Section 3.14). Comparisons were made after determining normal distribution using a Shapiro-Wilk test (Section 3.14.5*).* A 2-way analysis of variance (ANOVA) with repeated measures (time x trial) was used to identify differences between the 2 trials for whole-body physiological, GI barrier integrity and MT data (Section 3.14.7.3, 3.14.7.4). When there was only a single comparison, an independent t-test or non-parametric Mann-Whitney u test was used to

determine between-trial differences (Section 3.14.7.1, 3.14.7.2). Relationships were assessed by correlational analysis (Section 3.14.8.1). Data are presented as mean ± standard deviation (SD).

Power Analysis

An *a priori* sample size estimation was calculated *based on a*nticipated effect sizes derived from previous studies comparing I-FABP (Morrison et al., 2014) and endotoxin (Selkirk et al., 2008) responses between individuals of low and high aerobic fitness following exertional-heat stress (Section 3.14.1). In total, ≥ 9 (I-FABP) and ≥ 4 (endotoxin) participants per group were calculated necessary to detect a significant interaction effect using a two-way ANOVA with standard alpha (0.05) and beta (0.8) values.

5.4 Results

Thermoregulatory

T_{core} increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group (Figure 13A; time x group interaction; $p < 0.01$). Peak (UT: 38.88 ± 0.32 °C, HT: 38.21 ± 0.30 °C; $p < 0.01$), mean (UT: 37.99 ± 0.29 °C, HT: 37.63 ± 0.19 °C; $p < 0.01$) and Δ (UT: 1.97 ± 0.31 °C, HT: 1.35 ± 0.38 °C; $p < 0.01$) T_{core} were all greater in the UT group. T_{skin} was increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group (Figure 13B; time x group interaction; $p < 0.01$). Peak (UT: 36.23 ± 0.53 °C, HT: 35.56 ± 0.59 °C; $p = 0.02$) and Δ (UT: 1.84 ± 0.61 °C, HT: 0.81 ± 0.52 °C; $p < 0.01$) T_{skin} were greater in the UT group, but mean (UT: 35.55 ± 0.36 °C, HT: 35.56 ± 0.33 °C; $p = 0.26$) T_{skin} was similar. T_{body} was increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group (Figure 13C; time x group interaction; $p < 0.01$). Peak (UT: 38.75 ± 0.28 °C, HT: 38.08 ± 0.32 °C; $p < 0.01$), mean (UT: 37.98 ± 0.29 °C, HT: 37.63 ± 0.19 °C; $p < 0.01$) and Δ (UT: 1.95 ± 0.32 °C, HT: 1.24 ± 0.34 °C; $p < 0.01$) T_{body} were greater in the UT group. Mean sweat rate (UT: 1.52 ± 0.23 $l \cdot h^{-1}$; HT: 1.30 ± 0.25 $l \cdot h^{-1}$; $p = 0.07$) and % body mass loss (UT: 1.23 ± 0.26%; HT: 1.13 ± 0.32%; $p = 0.39$) were similar between groups.

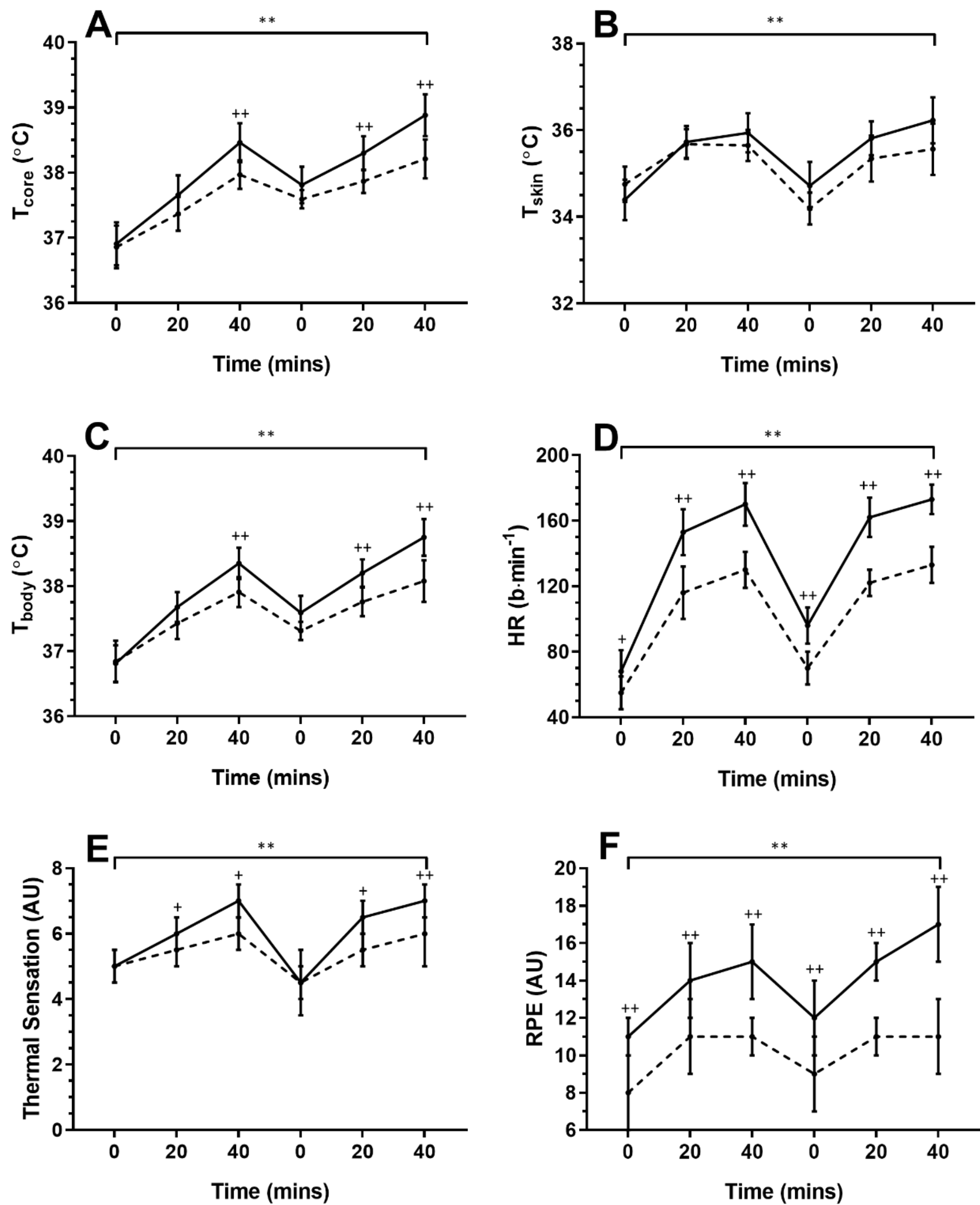

Figure 13. Whole-body physiological responses to EHSTs: (A) = core temperature; (B) = mean skin temperature; (C) = mean body temperature; (D) = heart rate; (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = UT, broken line = HT. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). Significant group * time interaction (+ p ≤ 0.05; ++ p ≤ 0.01).

Hydration and Cardiovascular

Basal urine osmolality (UT: 273 ± 109 mOsmol·kg^{-1}, HT: 261 ± 164 mOsmol·kg^{-1}; p = 0.87), urine specific gravity (UT: 1.007 ± 0.005 AU, HT: 1.005 ± 0.006 AU; p = 0.56) and plasma osmolality (UT: 296 ± 5 mOsmol·kg^{-1}, HT: 295 ± 3 mOsmol·kg^{-1}; p = 0.65) were similar

between groups. The Δ plasma volume following the EHST was similar (UT: 0.22 ± 2.59%, HT: 0.89 ± 2.49%; $p = 0.59$). HR was increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group (Figure 13D; time x group interaction; $p < 0.01$). Peak (UT: 173 ± 9 bpm; HT: 133 ± 11 bpm; $p < 0.01$), mean (UT: 156 ± 10 bpm; HT: 119 ± 6 bpm; $p < 0.01$) and Δ (UT: 105 ± 17 bpm; HT: 78 ± 13 bpm; $p < 0.01$) HR were all greater in the UT group.

Perception

RPE was increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group (Figure 13E; time x group interaction $p < 0.01$). Peak (UT: 17 ± 2 AU; HT: 11 ± 2 AU; $p < 0.01$), mean (UT: 14 ± 1 AU; HT: 10 ± 1 AU; $p < 0.01$) and Δ (UT: 6 ± 3 AU; HT: 2 ± 2 AU; $p < 0.01$) RPE were all higher in the UT group. TS was increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group (Figure 13F; time x group interaction; $p < 0.01$). Peak (UT: 7.0 ± 0.5 AU; HT: 6.0 ± 1.0 AU; $p < 0.01$), mean (UT: 6.0 ± 0.5 AU; HT: 5.5 ± 0.5 AU; $p < 0.01$) and Δ (UT: 2.5 ± 1.0 AU; HT: 1.0 ± 1.0 AU; $p < 0.01$) TS were all higher in the UT group.

Gastrointestinal Barrier Integrity

The DSAT (lactulose/$_{L}$-rhamnose ratio) was similar between the UT (0.039 ± 0.030) and HT (0.027 ± 0.011) groups (Figure 14A; $p = 0.59$). I-FABP concentration increased (time; $p = 0.01$) from pre- (UT: 1.17 ± 0.35 ng·ml^{-1}; HT: 1.81 ± 1.10 ng·ml^{-1}) to post-EHST (UT: 2.31 ± 1.34 ng·ml^{-1}; HT: 2.01 ± 1.03 ng·ml^{-1}), and to a greater extent in the UT group (Figure 14B; time x group interaction; $p = 0.05$). This interaction effect was not visible at either time point following post-hoc correction. The Δ I-FABP response was however greater in the UT (1.14 ± 1.35 ng·ml^{-1} [119 ± 77%]) *versus* the HT (0.20 ± 0.29 ng·ml^{-1} [16 ± 27%]) group ($p = 0.02$).

Microbial Translocation

Total 16S DNA was unchanged (time; $p = 0.34$) from pre- (UT = 5.50 ± 1.38 µg·ml^{-1}; HT = 5.54 ± 0.74 µg·ml^{-1}) to post-EHST (UT = 5.60 ± 0.82 pg·µl^{-1}; HT = 5.94 ± 0.94 pg·µl^{-1}) in both groups (Figure 15A; time x group interaction; $p = 0.56$). There was no difference in the Δ total 16S DNA between the UT (0.10 ± 1.16 µg·ml^{-1}) and HT (0.40 ± 1.13 µg·ml^{-1}) groups ($p = 0.56$). *Bacteroides*/total 16S DNA ratio displayed a significant time x group interaction (Figure 15B; $p = 0.04$). However, there was no significant difference in *Bacteroides*/total

16S DNA ratio between groups at either pre- (UT = 0.14 ± 0.10; HT = 0.20 ± 0.21; p = 0.44) or post- (UT = 0.32 ± 0.26; HT = 0.16 ± 0.08; p = 0.13) the EHST following *post hoc* adjustment. The Δ *Bacteroides*/total 16S DNA ratio was greater in the UT (0.18 ± 0.25) *versus* HT (-0.04 ± 0.18) group (p < 0.01). Unfortunately, *Bacteroides* concentrations were below the limit of detection in 7/40 samples (in these cases ratio data are presented as zero).

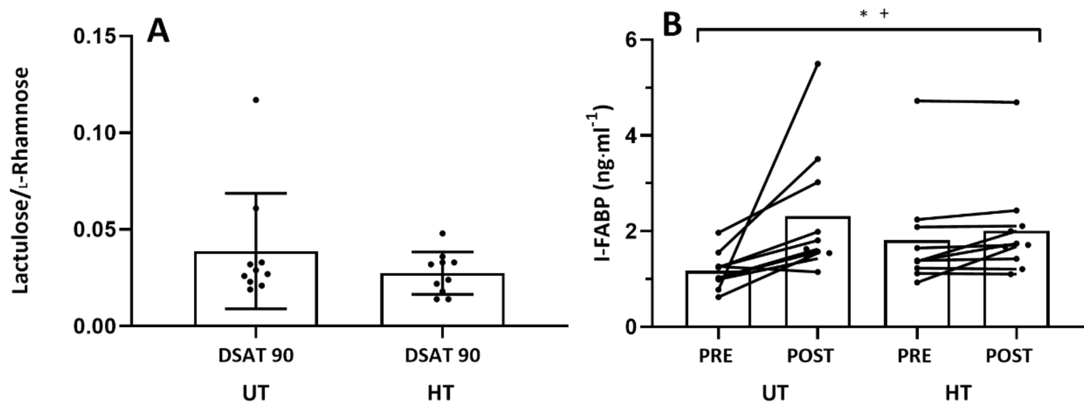

Figure 14. Gastrointestinal barrier integrity responses pre- and immediately post- the exertional-heat stress tests: (A) = L/R ratio (DSAT) at 90 minutes; (B) I-FABP. UT = untrained group, HT = highly trained group. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). Significant group * time interaction (+ p ≤ 0.05; ++ p ≤ 0.01).

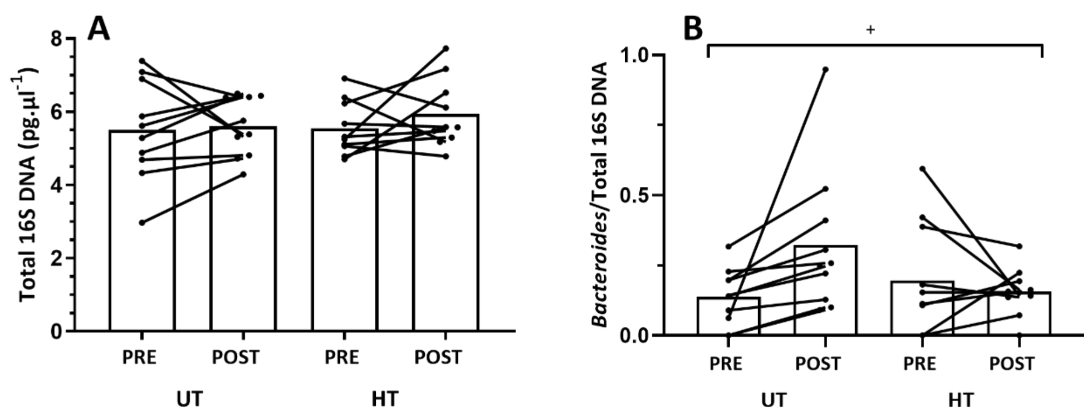

Figure 15. Gastrointestinal microbial translocation responses pre- and immediately post- the exertional-heat stress tests: (A) = total 16S DNA; (B) *Bacteroides*/total 16S DNA. UT = untrained group, HT = highly trained group. Significant group * time interaction (+ p ≤ 0.05; ++ p ≤ 0.01).

Associations

Associations between GI (DSAT, Δ I-FABP, Δ *Bacteroides*/total 16S DNA) and whole-body ($\dot{V}O_{2max}$, weekly training, age, body mass, body fat, peak T_{core}, peak T_{body}, mean HR, mean RPE) responses were conducted for the entire dataset (*n* = 20). Small positive associations were reported between the DSAT, with both absolute peak I-FABP concentrations ($r = 0.46$; $p = 0.04$) and *Bacteroides*/total 16S DNA ratio ($r = 0.43$; $p = 0.05$). No association was reported between the DSAT with Δ I-FABP or Δ *Bacteroides*/total 16S DNA ratio. Δ I-FABP displayed a small negative correlation with weekly training ($r = -0.55$; $p = 0.01$). Δ *Bacteroides*/total 16S displayed a small negative correlation with $\dot{V}O_{2max}$ ($r = -0.64$; $p < 0.01$), weekly training ($r = -0.55$; $p < 0.01$), body mass ($r = 0.48$; $p = 0.03$) and % body fat ($r = 0.54$; $p = 0.01$). Δ I-FABP correlated positively with mean RPE ($r = 0.57$; $p < 0.01$) and tended to correlate with peak T_{core} ($r = 0.42$; $p = 0.06$). Δ *Bacteroides*/total 16S DNA ratio displayed a small positive correlation with each: peak T_{core} ($r = 0.53$; $p = 0.02$), peak T_{body} ($r = 0.59$; $p < 0.01$), mean HR ($r = 0.60$; $p < 0.01$) and mean RPE ($r = 0.58$; $p < 0.01$). No further associations between Δ GI and whole-body responses were evident.

5.5 Discussion

The aim of this study was to determine the influence of aerobic fitness on GI barrier integrity (DSAT and I-FABP) and MT (*Bacteroides*/total 16S DNA) biomarkers following a fixed-intensity ecologically valid military EHST. The main findings were that GI permeability (serum DSAT) was comparable between the UT and HT groups following the EHST, however, small intestinal epithelial injury (I-FABP) increased to a greater extent (119% *versus* 16%) in the UT group following this protocol. In line with small intestinal epithelial injury, MT (*Bacteroides*/total 16S DNA) only increased following the EHST in the UT group. Weak-to-moderate associations ($r = 0.4$-0.7) were evident between certain whole-body thermoregulatory (e.g. mean RPE, peak T_{core}) and GI barrier integrity (I-FABP, *Bacteroides*/total 16S DNA) responses when combining data from the entire cohort. This would suggest that some of the benefits afforded by high aerobic fitness are likely attributable to a reduction in whole-body physiological strain. Given GI barrier integrity loss has been proposed as a key event within the pathophysiology of EHS (Lim, 2018), relevant doctrine should consider providing supplementary guidance for UT individuals to directly support GI barrier integrity (e.g. nutritional supplementation) and/or attenuate thermal strain (e.g. reduced load carriage, cooling) during group-paced occupational activities.

The DSAT is the gold-standard *in vivo* technique to assess GI permeability (Bischoff et al., 2014). In chapter 4, the serum DSAT increased ~2-fold above resting levels ([rest] = 0.014 ± 0.006, [post-EHST] = 0.028 ± 0.005; $p = 0.02$) in a cohort of participants with mixed aerobic fitness ($\dot{V}O_{2max}$ = 40-55 ml·kg·min^{-1}) when applying an identical EHST as herein. Contrary, to the *a priori* hypothesis, GI permeability was comparable between the UT and HT individuals following exertional-heat stress, whereby both groups presented absolute responses that were in line with chapter 4. This is the first study to assess the influence of aerobic fitness on GI permeability using the DSAT in response to either exercise or exertional-heat stress. Given the difficulty of obtaining intestinal biopsies in healthy humans, and the absence of available evidence from animal models, it is difficult to speculate whether aerobic fitness influences localised pathways (e.g. I-HSP concentration, GI tight junction structure) proposed to strengthen GI barrier integrity (Dokladny et al., 2016). Whole-body physiological mechanisms believed to disrupt GI barrier integrity, including pro-inflammatory cytokines (Landers-Ramos et al., 2014; Selkirk et al., 2008) and stress hormones (Wright et al., 2010; Reihmane et al., 2012), do not appear to be influenced by aerobic fitness in response to exertional-heat stress. In comparison, the expression and activity of I-HSP's in blood leukocytes both at rest (Fehrenbach et al., 2001) and in response to exertional-heat stress (Selkirk et al., 2008) are more pronounced in HT individuals, though whether a comparable response is initiated in GI tissue and the influence of this response on GI permeability is poorly understood.

I-FABP is the prominent biomarker of small intestinal epithelial injury and is tightly associated with localised splanchnic perfusion (van Wijck et al., 2011a; Bischoff et al., 2014). In the present study, the increase (Δ) in I-FABP following exertional-heat stress was comparable to previous research utilising similar intensity (60-70% $\dot{V}O_{2max}$) and duration (60-90 minute) EHSTs (e.g. Szymanski et al., 2017 [87%, Δ 0.800 ng·ml^{-1}]), including data reported in chapter 4 (56%, Δ 0.834 ng·ml^{-1}). In comparison, larger Δ I-FABP responses are reported in response to longer duration EHSTs ($\geq$ 120 minutes) of similar intensity (e.g. Snipe et al., 2018 [432%, Δ 1.230 ng·ml^{-1}]; Gaskell et al., 2019b [710%; Δ 1.805 ng·ml^{-1}]). In comparison to GI permeability responses, in the present study Δ I-FABP concentrations were more pronounced in the UT group than in the HT group following the EHST. This finding opposes previous evidence by Morrison et al. (2014) who reported HT individuals had more pronounced small intestinal injury than UT individuals during a 90-minute relative-intensity EHST. Whilst Morrison et al. (2014) proposed HT individuals might

redistribute a greater proportion of cardiac output away from the splanchnic organs than UT individuals during exertional-heat stress to support thermoregulation, they also acknowledge a limitation of their research was that their UT group had a lower overall thermal impulse given that 5/8 participants were unable to complete the EHST. The present finding that small intestinal epithelial injury is reduced in HT individuals in response to exertional-heat stress is supported by research in livestock showing aerobically trained animals better sustain splanchnic perfusion during passive heat stress than untrained animals (Sakurada and Hales, 1998). In humans, splanchnic cardiovascular stability has not been directly examined in response exertional-heat stress, however, given aerobic training characteristically increases blood plasma volume (Sawka et al., 2011) and superior-mesenteric arterial luminal area (Gabriel and Kindermann, 1996), comparable responses might be anticipated.

Bacterial DNA is an emerging biomarker of GI MT through high-sensitivity conserved 16S gene sequencing (Paisse et al., 2016). In comparison to traditional GI MT biomarkers (e.g. endotoxin), assessment of bacterial DNA is less susceptible to issues surrounding exogenous contamination given the ability to target microbial phyla/species (e.g. *Bacteroides*) with high GI specificity (March et al., 2019). The assessment of total 16S DNA is to control for co-variates that influence *Bacteroides* DNA concentration independent of GI MT, such as the efficiency of DNA extraction, immune function, and DNase concentrations (Velders et al., 2014; Paisse et al., 2016). In the present study, stable and comparable total 16S DNA concentrations were evident across both groups, however, only the UT group experienced a significant increase in the *Bacteroides*/total 16S DNA ratio following the EHST. In both chapter 4 and in previous research by March et al. (2019), similar basal *Bacteroides*/total 16S DNA ratios were reported as herein (~0 – 1.0). In both previous studies, participants' aerobic fitness was a potential co-variate, with $\dot{V}O_{2max}$ ranging between 40-60 ml·kg·min^{-1}.

In support of the present GI MT data, UT individuals experienced increased systemic endotoxin and LBP responses when T_{core} increased above 38.5 °C during exhaustive walking in the heat, however, this response was almost entirely absent in HT individuals despite exhibiting an improved exercise capacity (Selkirk et al., 2008). The findings of Selkirk et al. (2008), suggest that the protective benefits of aerobic fitness largely occur independent of absolute heat strain, given that blood was collected at fixed 0.5 °C T_{core} increments.

Correspondingly, given a lack of strong association between GI barrier integrity and MT biomarkers in the present study, it might be hypothesised that HT individuals also acquire an improved capacity for systemic microbial neutralisation. Though speculative, indirect evidence has previously shown improved aerobic fitness (e.g. $\dot{V}O_{2max}$) to increase certain anti-microbial defences, including: immunoglobulin G and M concentrations (Bosenberg et al., 1988; Camus et al., 1997); high density lipoprotein concentrations (Lippi et al., 2006); $CD14^{+}CD14$ monocyte profile (Selkirk et al., 2009); and hepatic reticuloendothelial (Kupffer cell) endotoxin phagocytosis (Komine et al., 2017).

Whole-body physiological responses – including thermoregulatory, cardiovascular, and perceptual strain – were all more pronounced in the UT *versus* HT individuals throughout the EHST. These results were anticipated, given that aerobic training is well-characterised to induce a plethora of physiological adaptations that both support thermoregulation (e.g. increased evaporative heat loss) and lower the relative metabolic cost (e.g. increased cardiac output) of fixed-absolute intensity exercise (Havenith et al., 1995; Cheung and McLellan, 1998). This reduction in whole-body physiological strain might have contributed towards blunting the Δ I-FABP and *Bacteroides*/total 16S DNA response in the HT participants in the present study. Relevantly, a recent systematic review outlined an exercise-induced T_{core} threshold of 38.6 °C for GI barrier integrity loss (DSAT, I-FABP and endotoxin) to be commonplace (>50% incidence) and of 39.0 °C for GI barrier integrity loss to be universal (100% incidence; Pires et al., 2017). In the present study, 9/10 UT participants had a peak T_{core} that exceeded 38.6 °C, including 4 participants whose T_{core} exceeded 39.0 °C. In comparison, only 2/10 HT participants surpassed 38.6 °C T_{core} and none 39.0 °C. Likewise, small associations were evident between peak T_{core} with both Δ I-FABP and *Bacteroides*/total 16S DNA when data for the entire participant cohort (n = 20) was accumulated. Mechanistically these responses would appear logical, given that hyperthermia disrupts GI barrier integrity in a broadly dose dependant manner (Dokladny et al., 2016). In the present study, small positive associations were also found between: (1) I-FABP and mean RPE; and (2) *Bacteroides*/total 16S DNA with peak T_{body}, mean HR, and mean RPE. The independent effect of these whole-body physiological responses on GI barrier integrity/MT have never been directly assessed and warrant future investigation utilising more valid methodologies (e.g. clamped T_{core}).

5.6 Limitations

Despite the execution of a tightly controlled methodological design, the present results were not without some limitations. First, the EHST only evoked moderate disturbance of GI barrier integrity and MT, potentially limiting the practical application of these findings in more severe thermal challenges indicative of EHS. However, the present EHST was selected as it had strong ecological validity in representing group-based military field activities (Spitz et al., 2012). Second, *Bacteroides* DNA analysis had poor analytical reliability (CV = 18.8%). This is largely attributable to a proportion of samples being close or below the assays minimum level of detection (1 copy·μl^{-1}). Despite this limitation, chapter 4 previously characterised the absolute test-retest reliability of *Bacteroides*/total 16S using the present EHST, whereby it is noted that the statistically significant Δ response between the UT and HT group reported herein exceeds the typical error of measurement (ratio = 0.077) and 95% limits of agreement (ratio = 0.213) previously reported. To further improve analytical reliability, future analysis might consider the assessment of whole-blood samples, given that bacterial 16S DNA concentrations in the buffy coat and red blood cells far exceed that of plasma (Paisse et al., 2016). Third, a basal DSAT was not performed to minimise the burden placed on participants with the aim of improving overall adherence. This lack of basal DSAT correction likely contributes to the lack of association between this biomarker with both Δ I-FABP and *Bacteroides*/total 16S DNA responses. Fourth, females were excluded from participation due to unavailability of menstruation hormone testing. Previous evidence has shown no influence of sex on GI barrier integrity responses to exertional-heat stress when females are in the follicular phase of the menstural cycle (Snipe and Costa, 2018a). Finally, the HT group were older and had a lower body fat percentage than the UT group. The difference in chronological age between the 2 groups is unlikely to have clinical relevance, given basal GI permeability appears relatively stable across the lifespan (Saweirs et al., 1985; Saltzman et al., 1995). In comparison, a small positive association was found between % body fat and the Δ *Bacteroides*/total 16S DNA ratio (but not DSAT or I-FABP responses). It is unknown whether deviations in body fat within the healthy physiological range influence GI permeability.

5.7 Conclusions

This is the first study to extensively assess the influence of aerobic fitness on GI barrier integrity and MT biomarkers in response to exertional-heat stress. There was no difference in GI permeability (serum DSAT) between the 2 fitness groups, but there was

more pronounced small intestinal epithelial injury (I-FABP) following the EHST in the UT group. These findings suggest that the GI barrier is more resistant to perturbation in HT individuals, though not to the extent where GI permeability is measurably altered. Likewise, GI MT (*Bacteroides*/total 16S DNA) only increased following the EHST in the UT group. Given that GI permeability was not different between the two groups, this suggests that GI MT neuralisation might also be increased in response to aerobic fitness training. These data broadly support conclusions drawn from studies assessing the impact of exertional heat stress on either GI barrier integrity or MT in isolation. It should be noted that peak thermoregulatory responses (e.g. T_{core} = 38-39.5°C) were sub-clinical when compared with situations were exertional-heat stroke predominately arise (e.g. T_{core} > 40.0°C). These findings help inform occupational EHS doctrine, in relation to the allocation of resources towards UT individuals during group-paced physical activity in the heat (e.g. reduced load carriage, increased cooling).

Chapter 6 - Gastrointestinal Tolerance of Low, Medium and High Dose Acute Oral $_{L}$-Glutamine Supplementation in Healthy Adults

6.1 Abstract

Purpose: $_{L}$-Glutamine (GLN) is a conditionally essential amino acid which supports gastrointestinal (GI) and immune function prior to catabolic stress (e.g. strenuous exercise). Despite potential dose-dependent benefits, GI tolerance of acute high dose oral GLN supplementation is poorly characterised.

Methods: 14 healthy males ingested 0.30 (LOW), 0.60 (MED) or 0.90 (HIGH) $g \cdot kg \cdot FFM^{-1}$ GLN beverages, in a randomised, double-blind, counter-balanced, cross-over trial. Individual and accumulated GI symptoms were recorded using a visual analogue scale at regular intervals up to 24-hours post ingestion. GLN beverages were characterised by tonicity measurement and microscopic observations.

Results: 24-hour accumulated upper-, lower- and total-GI symptoms were all greater in the HIGH, compared to LOW and MED trials ($p < 0.05$). Specific GI symptoms (discomfort, nausea, belching, upper GI pain) were all more pronounced on the HIGH *versus* LOW GLN trial ($p < 0.05$). Nevertheless, most symptoms were still rated as mild. In comparison, the remaining GI symptoms were either comparable (flatulence, urge to regurgitate, bloating, lower GI pain) or absent (heart burn, vomiting, urge to defecate, abnormal stools, stitch, dizziness) between trials ($p > 0.05$). All beverages were isotonic and contained a dose-dependent number of GLN crystals.

Conclusion: Acute oral GLN ingestion in dosages up to 0.90 $g \cdot kg \cdot FFM^{-1}$ are generally well-tolerated. However, the severity of mild GI symptoms appeared dose-dependent during the first 2 hours post prandial and may be due to high-concentrations of GLN crystals.

6.2 Introduction

L-glutamine (GLN) is the most abundant amino acid in the human body, with concentrations regulated between 600 to 800 μmol·l^{-1} in plasma, and 5 to 20 mmol·l^{-1} in tissue (Smith and Wilmore, 1990). Although traditionally classified as a non-essential amino acid, GLN was since reclassified as conditionally essential after discovery that intracellular concentrations are depleted during severe catabolism (Lacey and Wilmore, 1990). GLN has widespread physiological functions, including: nitrogen transportation; gluconeogenesis; acid-base regulation; gastrointestinal (GI) tight junction regulation, glutathione biosynthesis and increased heat shock protein expression (Wischmeyer, 2006). Furthermore, GLN is the preferred substrate for various types of rapidly proliferating cells including leukocytes, fibroblasts and GI epithelial cells (Newsholme et al., 2003). During severe catabolic events (e.g. trauma, surgery, sepsis), depletion of plasma GLN (≤ 420 μmol·l^{-1}) is a key predictor of both morbidity and mortality (Rodas et al., 2012). In comparison, normalisation of plasma GLN status through early exogenous supplementation (0.2-0.5 g·kg^{-1} body mass) improves clinical outcome (McRae, 2017; Wischmeyer, 2019).

The optimal dosage of GLN to support health during catabolic stress remains elusive. An umbrella meta-analysis evaluating the therapeutic benefits of GLN supplementation for intensive care patients concluded GLN supplementation reduced mortality in a dose-dependent manner (McRae, 2017). Despite this evidence, the European Society for Parenteral and Enteral Nutrition (ESPEN) only advise GLN supplementation in dosages *circa* 0.2-0.5 g·kg^{-1} body mass for up to 14 days (Singer et al., 2019), citing concerns that excessive supplementation may worsen clinical outcome in patients at risk of multiple organ failure (Heyland et al., 2013). In healthy individuals, habitual GLN supplementation is not generally recommended (Holecek, 2013). However, acute high dose oral GLN supplementation is suggested to support GI, immune and skeletal muscle function when ingested prior to an anticipated catabolic episode, such as prolonged-intense exercise (Gleeson, 2008) or an elective surgical operation (Gillis and Wischmeyer, 2019). For example, in young trained individuals, oral ingestion of 0.90 g·kg·FFM^{-1} of GLN improved GI permeability and systemic inflammatory responses when consumed 2 hours before 1-hour running at 70% of maximal oxygen uptake in the heat (Zuhl et al., 2015). These effects tended to be dose-dependent (Pugh et al., 2017b), but were not enhanced with continued

supplementation for 1 week (Zuhl et al., 2014). In pre-operative patients, 1 week of low-dose oral GLN supplementation (10 g·day^{-1}) reduced post-operative systemic inflammation and infection incidence (Martinez et al., 2019). Overall, acute high-dose oral GLN supplementation appears to be a potentially effected nutritional intervention to support GI and/or immune health prior to catabolic stress.

To date, the practicality of high-dose oral GLN supplementation on subjective GI tolerance has received little direct assessment. The observed safe dose recommended for chronic GLN ingestion is presently 14 g·day^{-1}, despite evidence of no observed adverse effects at doses *circa* ~45 g·day^{-1} (Shao and Hathcock, 2008). Understanding the incidence of severe GI symptoms following GLN supplementation has practical relevance for optimising nutritional programmes for target populations (e.g. athletes). In healthy adults, a phase-I clinical trial reported no measurable effects on routine clinical biochemistry, GLN metabolites or GI symptoms with acute oral GLN supplementation at 0.10 or 0.30 g·kg^{-1} body mass when assessed up to 4 hours post prandial (Ziegler et al., 1990). In paediatric oncology patients, acute GLN doses ranging from 0.35 to 0.65 g·kg^{-1} body mass were all well tolerated, but a 0.75 g·kg^{-1} body mass bolus induced vomiting in the first patient before the study was terminated (Ward et al., 2003). Previous laboratory studies supplementing GLN at 0.90 g·kg·FFM^{-1} prior to sub-clinical exertional-heat stress reported no difference in GI symptom severity compared with plain water when assessed 2-3 hours following ingestion (Pugh et al., 2017b; Osborne et al., 2019b).

Although several studies anecdotally report GI symptoms following acute oral GLN supplementation, the potential causes have not been investigated. Studies administering concentrated sugar solutions have attributed GI symptoms to high beverage osmolarity (Jeukendrup, 2017), whilst researchers administering creatine suggested GI symptoms might be due to undissolved creatine crystals in the GI tract (Ostojic and Ahmetovic, 2008). The aim of the present study was to determine the time-course of both upper- and lower-GI tract symptoms assessed up to 24 hours following acute ingestion of 0.30, 0.60 or 0.90 g·kg·FFM^{-1} of GLN in healthy adults. In addition, osmolality and light microscopy measures were performed to help determine if GI issues might be related to high osmolality and/or the presence of undissolved GLN crystals. It was hypothesised that GLN supplementation would induce GI symptoms in a dose-dependent manner.

6.2 Methods

Participants and Ethical Approval

14 healthy males volunteered to take part in this study (Table 29). All participants met the demographic (Section 3.3.1) and health (Section 3.3.3) criteria for inclusion. Informed consent was obtained for each participant following explanation of the experimental procedures (Section 3.1). The study protocol was approved by Plymouth MARJON University Research Ethics Committee (Approval Code: EP098) and conducted in accordance with the principles outlined in the *Declaration of Helsinki (2013)*.

Table 29. Participant demographic characteristics.

Measure	Mean ± SD
Age (years)	25 ± 5
Height (m)	1.79 ± 0.07
Body Mass (kg)	77.7 ± 9.8
Body Fat (%)	14.8 ± 4.6

Experimental Overview

This study utilised a randomised, double-blind, counterbalanced, cross-over design (Section 3.14.2). Participants visited the laboratory on 4 occasions. During the first visit, baseline eligibility and anthropometrics were assessed. The second, third and fourth visits consisted of GLN ingestion, followed by regular GI symptom monitoring over the subsequent 24 hours. Participants were supervised in the laboratory for the first 4 hours post-GLN ingestion. After this point, participants left the laboratory to reside within a free-living environment. GI symptom questionnaires were returned the following day. A $\geq$ 5 day wash-out period interspersed main experimental visits (Galera et al., 2010). A schematic illustration of the experimental protocol is shown in Figure 16.

Dietary and Lifestyle Controls

Trials were conducted following dietary (Section 3.5.2) standardisation. Conformation was self-attested using a pre-trial control questionnaire. Participants were permitted to drink a maximum of 200 $ml \cdot h^{-1}$ of plain water during the first 4 hours of all main experimental trials. Following this, participants were instructed to follow their standardised diet within a free-living environment and to match this as closely as possible

between repeat trials.

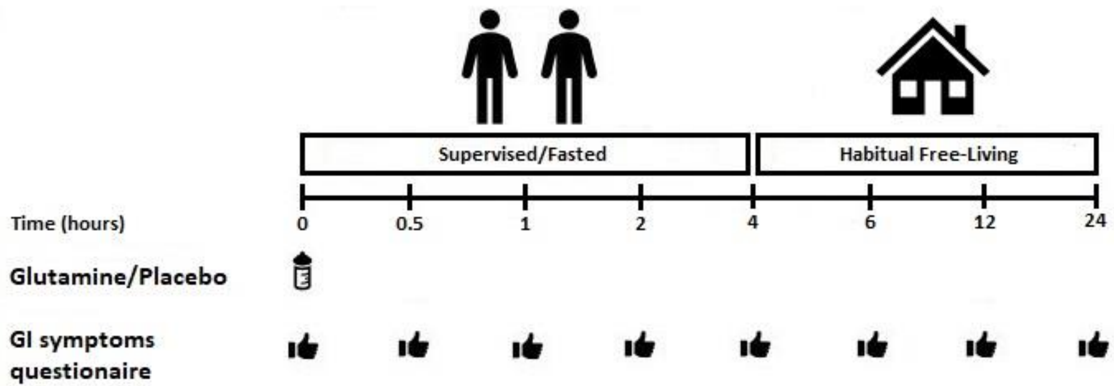

Figure 16. Schematic illustration of the experimental timings.

Anthropometric Measurements

Height (Section 3.4.1), mass (Section 3.4.2) and body fat (Section 2.4.3) were measured following the International Society for the Advancement of Kinanthropometry (ISAK) guidelines (Marfell-Jones et al. 2006). The duplicate coefficient of variation (CV) for skinfold thicknesses at 4-sites was 1.6%.

$_L$-Glutamine Supplementation

GLN supplementation consisted of 0.30 (LOW), 0.60 (MED) and 0.90 (HIGH) $g{\cdot}kg{\cdot}FFM^{-1}$ of unflavoured 100% GLN crystalline powder, which was freshly suspended in 500 ml of water/lemon flavour cordial in a 4:1 ratio (Section 3.6). Both drinks were consumed 1-hour before the EHST. The Bang Blinding Index (BI) was used to estimate the successfulness of trial blinding (Section 3.14.9).

Gastrointestinal Symptom Questionnaire

Perceived GI symptoms were measured using a modified visual analogue scale before, 0.5-, 1-, 2- 4-, 6-, 12- and 24-hours following GLN ingestion (Section 3.12.3). The mVAS is a 20-item questionnaire of common GI symptoms that range from 0 (absent), through 1-4 (mild GI symptoms that did not interfere with current activity), then 5-9 (severe GI symptoms that disrupted activity) to 10 (extremely severe GI symptoms) along a 10-point scale. For analysis, accumulated symptom scores were grouped as: upper- (belching, heartburn, bloating, upper abdominal pain, urge to regurgitate, regurgitation, vomiting), lower- (flatulence, bloating, urge to defecate, left/right lower abdominal pain, abnormal stools) and total- GI symptoms. Miscellaneous symptoms (nausea, dizziness, stitch and GI

discomfort) were only analysed individually. This grouping technique follows current recommendations (Gaskell et al., 2019a). Symptom incidence was indicative of scores rated ≥ 1 and severity indicative of the mean accumulated score of all reported symptoms ≥ 1.

Characterisation of GLN Beverages

The beverages were prepared with fruit squash and mixed as described above, using enough GLN to make each beverage representative of the mean dose consumed by the study participants in the LOW, MED and HIGH trials. To evaluate osmolality each LOW, MED and HIGH beverage was first agitated for 30 seconds just prior to removing a 0.5 ml sample from the middle of the container for analysis via freeze-point depression (Section 3.12.7; CV = 0.5%). For light microscope evaluation each LOW, MED and HIGH beverage were first agitated for 30 seconds just prior to removing a 20 µl sample onto a microscope slide from the middle of the container. Images were all collected using a 40x magnification (Swift SS110_25B-2P0, Germany). The length and diameter of the 10 largest crystals was determined using imaging software (Swift Easy View, V1.19.10.26, Germany).

Statistics

All statistical analyses were performed using Prism Graphpad software (Section 3.14). Comparisons were made after determining normal distribution and sphericity (Section 3.14.5). A 2-way analysis of variance (ANOVA) with repeated measures (time x trial) was used to identify differences in GI symptoms over time (Section 3.14.7.3, 3.14.7.4). Accumulated 24-hour GI symptoms were compared for trial differences using a non-parametric Freidman's test (Section 3.14.7.3, 3.14.7.4). Symptom severity are presented as mean ± range of accumulated scores (Section 3.12.3).

Power Analysis

Given the novelty of the dependent variables being evaluated, it was determined infeasible to perform an *a priori* sample size calculation (Section 3.14.1). Instead, general guidance on appropriate sample sizes (n = 12) for pilot studies were followed, whilst accounting for a ~20% anticipated participant drop-out rate (Julious, 2005).

6.4 Results

GLN Supplementation

The average GLN dose across the 3 trials were 19.8 ± 1.8 g (LOW), 39.5 ± 3.5 g (MED) and 59.3 ± 5.3 g (HIGH). Based on standard conversions (1 gram = 4 kcal), the energy density of the GLN dosages were: 79.2 ± 7.2 kcal (LOW), 158.0 ± 14 kcal (MED) and 237.2 ± 21.2 kcal (HIGH). Participant blinding was considered successful where 1 arm of the 95% confidence interval always covered 0 (Table 30).

Table 30. Assessment of trial blinding.

Assignment	Response				
	LOW	MED	HIGH	DK	BI (95% CI)
LOW	3	2	0	9	0.07 (-0.27, 0.34)
MED	1	2	2	9	-0.07 (-0.49, 0.35)
HIGH	0	2	4	8	0.14 (-0.18, 0.32)

Accumulated Gastrointestinal Symptoms (24-hour Total)

Accumulated (24-hour) upper-GI symptoms showed an overall trial effect ($p < 0.01$). Post hoc analysis revealed symptoms to be greater in the HIGH trial, compared with both the LOW ($p < 0.01$) and MED ($p < 0.01$) trials (Figure 17B). There was no difference between the LOW and MED trial ($p = 0.10$; Figure 17B). Accumulated (24-hour) lower-GI symptoms showed an overall trial effect ($p < 0.01$). Post hoc analysis revealed symptoms to be graded between each GLN dosage (LOW vs. HIGH $p < 0.01$; MED vs. HIGH $p = 0.05$; LOW vs. MED $p = 0.03$; Figure 17D). Accumulated (24-hour) total-GI symptoms showed an overall effect of trial ($p < 0.01$). Post hoc analysis revealed symptoms to be greater in the HIGH trial, compared with both the LOW ($p < 0.01$) and MED ($p < 0.01$) trials (Figure 17F). There was no difference between the LOW and MED trial ($p = 0.10$; Figure 17F). Specific GI symptoms responses are given in Table 31. Overall GI discomfort was greater in the HIGH compared with both the LOW ($p < 0.01$) and MED trials ($p < 0.01$). Nausea showed a graded response between each GLN dosage ($p < 0.05$; Table 31). Individual upper GI symptoms (bloating, belching, pain) were all greater in the HIGH *versus* LOW trial ($p < 0.05$; Table 31). However, individual lower GI symptoms (flatulence, urge to regurgitate, bloating, pain) were not different between trials ($p > 0.05$; Table 31). The remaining upper- (heart burn, projectile

vomiting), lower- (urge to defecate, abnormal stools) and miscellaneous (stitch, dizziness) GI symptoms were not experienced by any participant across any trial.

Gastrointestinal Symptoms (Time Course)

Upper-, lower- and total- GI symptoms all displayed time, trial and interaction effects ($p < 0.01$). Post hoc analysis revealed upper GI symptoms to be greater in the HIGH *versus* LOW trial at 1- ($p = 0.04$) and 2- ($p = 0.05$) hours post-ingestion, and greater in the HIGH *versus* MED trial at 0.5- ($p = 0.03$) and 1- ($p = 0.03$) hour post-ingestion (Figure 17A). There were no differences between the LOW and MED trials at any time point (Figure 17A). Lower GI symptoms were only greater in the HIGH *versus* LOW trial at 0.5- ($p < 0.01$) and 1- ($p = 0.03$) hour (Figure 17C). No other significant differences were observed for lower-GI symptoms. Total GI symptoms were greater at 0.5- ($p = 0.02$), 1- ($p < 0.01$) and 2- ($p < 0.01$) hours between both the HIGH *versus* LOW and HIGH *versus* MED trials, but not between the LOW *versus* MED trials (Figure 17E). There were no differences between trials at 4-hours onwards for any GI symptoms. Specific GI symptoms responses are given in Table 31. Only gut discomfort (1 and 2 hour), belching (1 hour) and nausea (1 and 2 hours) reached significance ($p < 0.05$) between the LOW and HIGH trials. Between the MED and HIGH trials, only belching (1 hour) reached significance ($p < 0.05$). There were no significant differences for any measure between the LOW and MED trials. 1 participant experienced severe symptoms (rating ≥ 5 one-hour post ingestion for belching, regurgitation) on the HIGH trial, with symptoms from the rest of the sample group consistently classified as mild (rating ≤ 4).

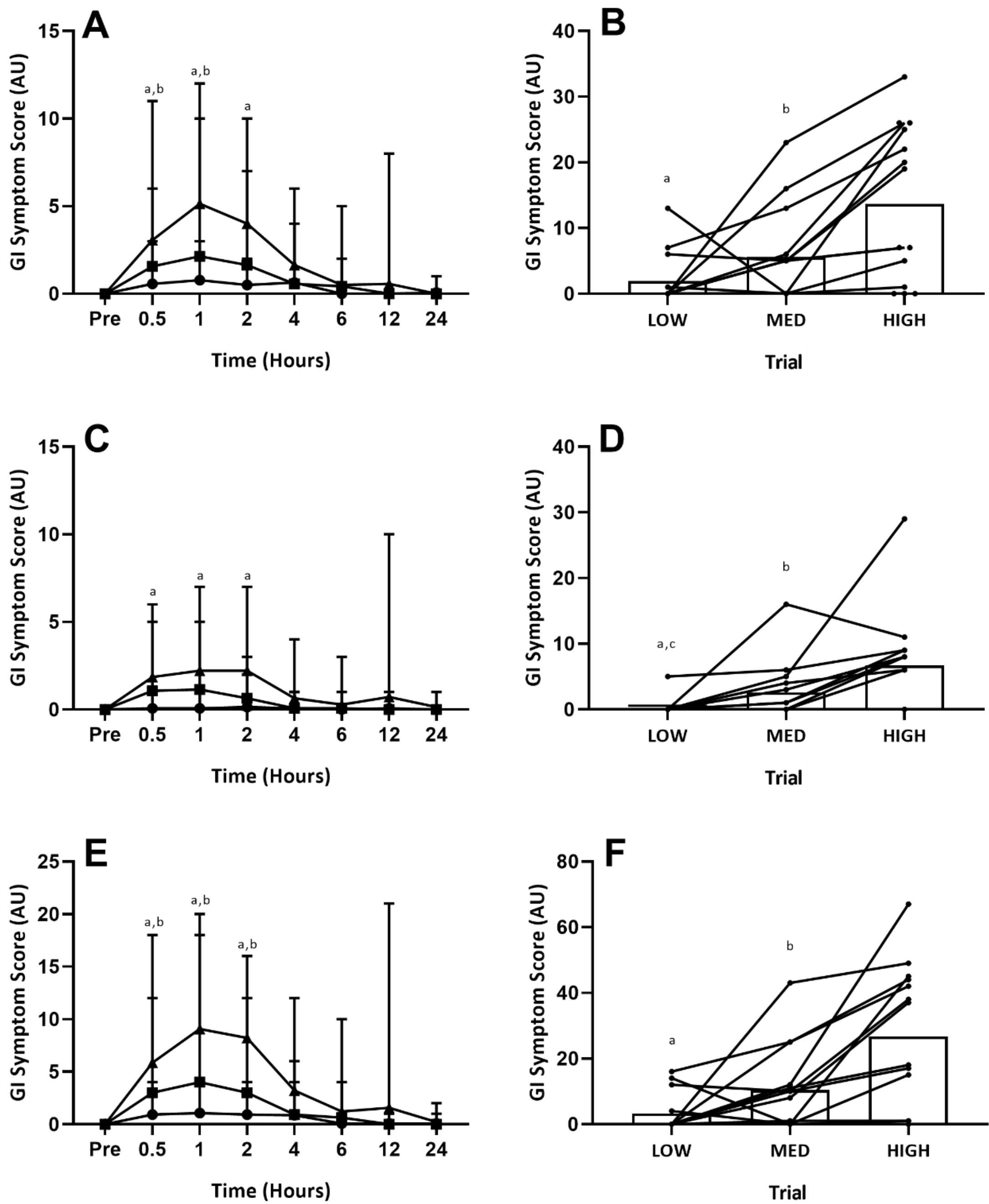

Figure 17. GI symptom scores: (A) = upper-GI symptoms over time; (B) = 24-hour accumulated upper-GI symptoms; (C) = lower-GI symptoms over time; (D) = 24-hour accumulated lower-GI symptoms; (E) = total-GI symptoms over time; (F) = 24-hour accumulated total-GI symptoms. Circles = LOW, squares = MID, Triangles = LOW. On (B), (D), (F) circles = individual participant data. Significant difference between LOW *versus* HIGH ([a] $p \leq 0.05$), MED *versus* HIGH ([b] $p \leq 0.05$) and LOW vs. MED ([c] $p \leq 0.05$) trials.

Table 31. Individual GI symptoms over time, incidence (%) and as a total 24-hour accumulated score following LOW, MED or HIGH GLN ingestion.

	Pre	0.5 h	1 h	2 h	4 h	6 h	12 h	24 h	Incidence (%)	Total
Gut Discomfort										
LOW	0 (0-0)	1 (1-2)	2 (1-2)[a]	1 (1-2)[a]	1 (1-1)	1 (1-1)	0 (0-0)	0 (0-0)	28	4 (1-6)[a]
MED	0 (0-0)	2 (1-2)	1 (1-3)	1 (1-2)	1 (1-1)	1 (1-1)	0 (0-0)	0 (0-0)	64	3 (1-8)[b]
HIGH	0 (0-0)	2 (1-3)	2 (1-4)	3 (1-5)	2 (1-4)	1 (1-2)	2 (1-3)	1 (1-1)	79	8 (1-12)
Belching										
LOW	0 (0-0)	1 (1-2)	2 (1-2)[a]	1 (1-2)	1 (1-1)	1 (1-1)	0 (0-0)	0 (0-0)	21	7 (4-12)[a]
MED	0 (0-0)	2 (1-2)	1 (1-3)[b]	1 (1-2)	3 (2-4)	1 (1-1)	0 (0-0)	0 (0-0)	50	4 (1-8)[b]
HIGH	0 (0-0)	2 (1-5)	3 (1-5)	2 (1-3)	3 (1-5)	2 (1-4)	1 (1-2)	3 (3-3)	64	8 (1-14)
Bloating (Upper)										
LOW	0 (0-0)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1)	0 (0-0)	0 (0-0)	1 (1-1)	43	2 (1-2)[a,c]
MED	0 (0-0)	2 (1-2)	1 (1-3)	1 (1-2)	1 (1-1)	1 (1-1)	0 (0-0)	0 (0-0)	71	3 (1-7)[b]
HIGH	0 (0-0)	2 (1-5)	3 (1-4)	2 (1-4)	2 (1-4)	2 (2-2)	4 (4-4)	0 (0-0)	64	8 (3-13)
Pain (Upper)										
LOW	0 (0-0)	1 (1-1)	2 (2-2)	2 (2-2)	2 (2-2)	0 (0-0)	0 (0-0)	0 (0-0)	14	4 (1-6)[a]
MED	0 (0-0)	1 (1-1)	1 (1-2)	2 (1-2)	1 (1-1)	1 (1-1)	0 (0-0)	0 (0-0)	50	2 (1-5)
HIGH	0 (0-0)	1 (1-2)	2 (1-3)	3 (1-4)	1 (1-1)	1 (1-1)	1 (1-1)	0 (0-0)	50	4 (1-8)
Urge to Regurgitate										
LOW	0 (0-0)	1 (1-1)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	7	1 (1-1)[a]
MED	0 (0-0)	1 (1-2)	3 (1-5)	2 (1-3)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	28	2 (2-3)[b]
HIGH	0 (0-0)	2 (1-4)	3 (1-5)	2 (1-4)	1 (1-1)	0 (0-0)	0 (0-0)	0 (0-0)	64	5 (1-13)

Continued.										
Flatulence										
LOW	0 (0-0)	0 (0-0)	0 (0-0)	1 (1-1)	0 (0-0)	0 (0-0)	1 (1-1)	0 (0-0)	14	1 (1-1)
MED	0 (0-0)	0 (0-0)	1 (1-1)	1 (1-2)	0 (0-0)	1 (1-1)	0 (0-0)	0 (0-0)	28	2 (2-2)
HIGH	0 (0-0)	2 (1-2)	2 (1-2)	2 (1-3)	2 (1-2)	1 (1-1)	4 (4-4)	1 (1-1)	28	6 (1-11)
Bloating (Lower)										
LOW	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0	0 (0-0)
MED	0 (0-0)	1 (1-1)	1 (1-1)	1 (1-1)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	21	2 (2-3)
HIGH	0 (0-0)	2 (1-3)	2 (1-2)	2 (2-2)	2 (1-2)	3 (3-3)	1 (1-1)	0 (0-0)	21	7 (4-11)
Pain (Lower)										
LOW	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0	0 (0-0)
MED	0 (0-0)	3 (3-3)	1 (1-1)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	7	4 (4-4)
HIGH	0 (0-0)	0 (0-0)	0 (0-0)	2 (2-2)	0 (0-0)	1 (1-1)	0 (0-0)	0 (0-0)	7	3 (3-3)
Nausea										
LOW	0 (0-0)	1 (1-1) [a]	1 (1-1) [a]	1 (1-1) [a]	1 (1-1)	0 (0-0)	0 (0-0)	0 (0-0)	7	4 (4-4)[a,c]
MED	0 (0-0)	2 (1-3)	2 (1-3)	1 (1-2)	1 (1-1)	0 (0-0)	0 (0-0)	0 (0-0)	57	3 (1-8)[b]
HIGH	0 (0-0)	2 (1-4)	2 (1-4)	2 (1-5)	1 (1-1)	0 (0-0)	2 (2-2)	0 (0-0)	71	6 (4-8)

Total incidence (%) of participants with reported symptoms ≥ 1 on the mVAS and summative accumulated severity of symptoms where reported (excluding data of no reported symptoms). Significant difference between LOW versus HIGH ([a] $p \leq 0.05$), MED versus HIGH ([b] $p \leq 0.05$) and LOW versus MED ([c] $p \leq 0.05$) trials. Note: symptoms that remained absent across all participants (e.g. heartburn) are not reported.

Characterisation of GLN Beverages

When mixed with fruit squash the LOW, MED and HIGH beverages were visually different, being progressively more turbid as the GLN content increased. The osmolality of the mean GLN dose for the LOW, MED and HIGH beverages were all isotonic at 269 mOsm·kg^{-1}, 281 mOsm·kg^{-1} and 278 mOsm·kg^{-1}, respectively. Microscopic evaluation of the LOW, MED and HIGH GLN beverages are shown in Figure 18. The LOW GLN beverage typically revealed less than 20 crystals in any field of view, which had a mean diameter of 0.054 ± 0.010 mm and a mean length of 0.121 ± 0.044 mm, giving them a rounded appearance (Figure 18A). In contrast, the MED beverage had ~180 crystals in the field of view, which had a more needle like appearance, with a mean diameter of 0.050 ± 0.10 mm and length of 0.201 ± 0.074 mm with some crystals being up to 0.24 mm in length (Figure 18B). The HIGH GLN beverage showed the highest density of crystals with around 290 per field of view, again with a needle like appearance with a mean diameter of 0.046 ± 0.014 mm and lengths of 0.239 ± 0.036 mm with crystals up to 0.27 mm in length (Figure 18C).

Figure 18. Microscopic illustration (40x) of the LOW (A), MED (B) and HIGH (C) GLN beverages

6.5 Discussion

A key aim of this study was to determine the time-course of potential total-, upper- and lower- GI symptoms up to 24 hours following acute ingestion of either 0.30 (LOW), 0.60 (MED) or 0.90 (HIGH) g·kg·FFM^{-1} of GLN in healthy adults. A second objective was to evaluate the characteristics of the GLN beverages, to give insights into potential mechanisms that might contribute to GI symptoms. The main findings were that accumulated 24-hour total-, upper- and lower- GI symptoms were all greater in the HIGH *versus* LOW trial. These responses appeared to be graded, although the effect size of the LOW *versus* MED trial generally did not reach statistical significance. When assessed over time, most participants experienced mild GI symptoms over the first 2 hours post-prandial,

which generally subsided by 4 hours. The GLN beverages were all found to be within the isotonic range of 270-330 $mOsm \cdot kg^{-1}$, so this is unlikely to be a cause of presented GI symptoms. In contrast, crystal structures were less frequent and more rounded in appearance in the LOW GLN beverage relative to either the MED or HIGH beverages, where the crystals had a more needle like structure. In addition, the HIGH beverage had a greater number of crystal structures in the field of view than the MED beverage. These findings have practical relevance to individuals looking to maximise the dose of GLN supplementation for clinical benefits relative to the risk of GI symptoms.

Accumulated upper- and lower- GI symptoms were more pronounced across the first 2 hours post prandial in the HIGH *versus* LOW trial. This finding supports previous evidence showing GLN doses ≤ 0.5 $g \cdot kg^{-1}$ body mass to be largely well tolerated without self-reported adverse responses in a range of participant demographics (Ziegler et al., 1990; Dechelotte et al., 1991; Valencia et al., 2002; Harris et al., 2012; Irimia et al., 2013). In comparison, the impact of acute oral GLN supplementation in doses *circa* 0.60-0.90 $g \cdot kg^{-1}$ body mass on GI tolerance has received far less research attention. This may be due to clinical (Savy, 1997) and sports (Gleeson, 2008) nutrition guidelines reporting such doses as being unpalatable. Despite this, several studies have shown the severity of symptoms with higher GLN doses to be below the limit where participants had to be removed from study protocols (Tjader et al., 2000; Darmaun et al., 2004). Likewise, supplementation with 0.90 $g \cdot kg \cdot FFM^{-1}$ of oral GLN dissolved with 500 ml of water did not exacerbate GI symptoms – compared with water alone – following assessment 2-3 hours post ingestion in healthy adults immediately after moderate intensity cycling in the heat (Pugh et al., 2017b; Osborne et al., 2019b). In contrast, 1 study reported 0.75 $g \cdot kg^{-1}$ body mass of oral GLN caused vomiting 2 hours post prandial (Ward et al., 2003), whilst another research group make anecdotal claims (Nava et al., 2019) that a single 0.90 $g \cdot kg \cdot FFM^{-1}$ GLN bolus was poorly tolerated when ingested 2 hours before exercise in previous research reported from their laboratory (Zuhl et al., 2014, 2015).

Specific GI symptoms (i.e. gut discomfort, belching and nausea), were all more pronounced over the first 2 hours post prandial in the HIGH *versus* LOW GLN trial. Nevertheless, the severity of symptoms over time were rated as either absent or-mild (mVAS ≤ 4) in 13/14 participants. The remaining participant had severe symptoms (mVAS = 5) for belching and regurgitation when assessed 1-hour post-prandial in the HIGH trial.

There are several potential physiological mechanisms that might explain the dose-dependent mild symptoms reported in response to GLN supplementation. First, increased GLN hydrolysis could elevate plasma ammonia concentrations, which in turn could cause symptoms of nausea (Savy, 1997). In previous studies supplementing GLN at doses < 0.65 $g{\cdot}kg^{-1}$ body mass, plasma ammonia concentrations remained relatively stable (<50 $\mu mol{\cdot}l^{-1}$) and/or had returned to basal levels (<30 $\mu mol{\cdot}l^{-1}$) by 2 hours post prandial (Ziegler et al., 1990; Ward et al., 2003). Unfortunately, investigations supplementing 0.90 $g{\cdot}kg{\cdot}FFM^{-1}$ of GLN (Zuhl et al., 2014, 2015; Pugh et al., 2017b; Osborne et al., 2019b) did not assess plasma ammonia concentrations, which prevents verification of this hypothesis. Given the LOW, MED and HIGH GLN beverages were all isotonic, differences in GI symptoms between trials cannot be attributed to osmolality. The solubility of pure GLN crystalline powder is ~3.3g per 100 ml of plain water (Furst et al., 1997). This means that each GLN beverage in the present study was a supersaturated solution. This gives GLN suspensions a chalky texture, which can be perceived as unpleasant; therefore might be argued as a direct cause of nausea.

In the present investigation, provision of supersaturated LOW, MED and HIGH GLN solutions was confirmed by light microscopy, which showed increasing numbers of crystal structures as GLN dose rose. As identical crystals were also present in the absence of fruit squash and their numbers increased with increasing GLN concentration, it is reasonable to assume that the crystal structures observed were undissolved GLN. GI tolerance to undissolved GLN is unknown, however, ingestion of supersaturated creatine and $_L$-arginine solutions has previously been suggested to trigger gastric distension and aggravate the intestinal mucosal lining (Ostojic and Ahmetovic, 2008; Evans et al., 2004). Previous studies reported that $_L$-arginine could result in high concentrations of nitric oxide, resulting in secretagogue functions for water and electrolytes (Grimble, 2007). As GLN may serve as a potential nitric oxide source, 1 potential mechanism to explain the GI symptoms in the present investigation is localised dysregulation of nitric oxide synthesis and aberrant cell signalling. In the present investigation the shape and number of crystals in GLN beverages highlights another possible mechanism of mucosal irritation. The needle like shape of GLN crystals may have irritated the gut mucosa by scratching the tissue surface; a proposal consistent with both the shape and increasing number of GLN crystals in the LOW, MED and HIGH beverages.

The clinical significance of findings from the present study that acute oral GLN supplementation cause mild GI symptoms are likely to be context dependant. For example, in sports settings, even minor GI symptoms have negative implications on performance (Hoffman and Fogard, 2011) and can result in withdrawal from competition (Jeukendraup et al., 2000). Likewise, in occupational settings where personnel may be required to work in the heat (e.g. military, firefighters), acute high-dose GLN supplementation could be used to protect against exertional-heat stroke. However, poor GI tolerance might increase the relative-risk of exertional-heat stroke if re-hydrated practises were compromised. Therefore, LOW dose oral GLN supplementation might be most appropriate during strenuous physical activity though this recommendation is made on the provision that the ingested dose still achieves the desired benefits (i.e. reduced GI permeability). Alternatively, athletes might consider HIGH dose GLN supplementation when ingested ≥ 4 hours prior to exercise, and/or trialling the GI tolerance of larger GLN doses outside of competition or perceived to be important occupational events. The opportunity may also exist to improve tolerance to each GLN dose by increasing the fluid volume of the beverages, thereby reducing the number of GLN crystals present. In comparison to athletes, the relevance of mild GI symptoms in pre-operative clinical care patients would only be considered a minor side-effect relative to the trauma induced by treatment (Gillis and Wischmeyer, 2019). Therefore, in these patients it is suggested that HIGH dose oral GLN supplementation offers acceptable GI tolerance. Furthermore, it might even be argued based on dose-dependent clinical benefits, GLN supplementation in dosages > 0.90 $g \cdot kg \cdot FFM^{-1}$ warrant future research consideration (McRae, 2017).

6.6 Limitations

Despite execution of a tightly controlled methodological design, the present results are not without some limitations. First, all trials were conducted in the fasted state, which may prevent the extrapolation of findings to scenarios where additional feeding is required. For example, general sports nutrition guidelines recommended athletes consume a meal containing 1-4 $g \cdot kg^{-1}$ of CHO between 1-4 hours before commencing prolonged aerobic exercise (Burke et al., 2019). Thus, it cannot be excluded whether participants reported certain GI symptoms (e.g. gut discomfort) as a result of low satiety. Second, though participants self-attested to closely matching their habitual diet around trials, strict dietary control was not enforced either prior to or during laboratory visits. Despite this limitation,

in instances were GI symptoms were reported (score >1), trials were rescheduled to mitigate any potential deleterious influence of habitual diet.

6.7 Conclusion

This study assessed the influence of acute oral GLN supplementation in dosages of 0.30 (LOW), 0.60 (MED) and 0.90 (HIGH) $g{\cdot}kg{\cdot}FFM^{-1}$ on subjective symptoms of GI intolerance. Accumulated upper-, lower- and total- GI symptoms were all greater in the HIGH trial over the entire 24-hour period, compared with both the LOW and MED trials. These responses appeared to be graded, although the effect size of the LOW *versus* MED trial did not reach statistical significance. When assessed over time, most participants experienced some mild GI symptoms, notably gut discomfort, belching and nausea, especially during the first 2 hours following GLN ingestion. The osmolality of each GLN beverage was isotonic and unlikely to be a cause of presented GI symptoms. In contrast, the needle like shape and greater presence of glutamine crystals with increasing GLN dose may be a direct contributor to GI symptoms. These results suggest that acute oral GLN supplementation in doses up to 0.90 $g{\cdot}kg{\cdot}FFM^{-1}$ are well tolerated, with only mild GI symptoms experienced by a proportion of healthy individuals.

Chapter 7 - Influence of Low Dose Acute L-Glutamine Supplementation on Gastrointestinal Barrier Integrity and Microbial Translocation in Response to a Military Exertional-Heat Stress Test

7.1 Abstract

Purpose: Exertional-heat stress adversely disrupts gastrointestinal (GI) barrier integrity and, through subsequent microbial translocation (MT), can have negative health consequences for physically active populations. Acute glutamine (GLN) supplementation is a potential nutritional countermeasure, although doses previously validated for this purpose are not universally well-tolerated.

Method: 10 moderately trained males completed two 80-minute exertional-heat stress tests (EHST) separated by 7-14 days with a double-blind, randomised, counterbalanced, cross-over design. Low dose oral GLN (0.30 $g \cdot kg^{-1}$ fat free mass) or placebo (PLA) beverages were ingested 1 hour before commencing the EHST. Venous blood was drawn immediately pre- and post- EHST. GI barrier integrity was assessed using the serum dual-sugar absorption test (DSAT) and plasma Intestinal Fatty-Acid Binding Protein (I-FABP). MT was assessed using plasma total 16S bacterial DNA and *Bacteroides*/total 16S DNA.

Results: Whole-body physiological and perceptual strain were comparable between the GLN and PLA trials during the EHST ($p > 0.05$). The GLN bolus was well tolerated, with no evidence of GI intolerance. Serum DSAT responses tended to be greater ($p = 0.06$) in the GLN (0.030 ± 0.012) than the PLA trial (0.023 ± 0.006), whilst the post-EHST elevation in I-FABP was more pronounced with GLN ($\Delta = 2.542 \pm 1.205$ $ng \cdot ml^{-1}$) than PLA ($\Delta = 1.374 \pm 1.101$ $ng \cdot ml^{-1}$) supplementation ($p = 0.01$). *Bacteroides*/total 16S DNA responses increased ($p = 0.04$) following the EHST, but there was no difference between the GLN ($\Delta = 0.06 \pm 0.09$) and PLA ($\Delta = 0.15 \pm 0.33$) trials ($p = 0.44$).

Conclusion: Acute low-dose (0.30 $g \cdot kg^{-1}$ fat free mass) oral GLN supplementation 1 hour before exertional-heat stress enhances I-FABP concentrations, but this does not translate to augmented GI MT.

7.1 Introduction

The gastrointestinal (GI) microbiota is a complex ecosystem formed of *circa* 100 trillion microbes (Cani, 2018). Whilst these microbes perform various functions symbiotic to human health, the GI barrier must effectively contain the microbiota to the non-sterile luminal space (Wells et al., 2017). However, in circumstances where GI barrier integrity is severely disturbed, highly pathogenic microbes can enter the systemic blood, triggering cytokine production by white blood cells (Armstrong et al., 2018). Exertional-heat stress is a well-characterised stimulus that disrupts GI barrier integrity, and in a manner broadly associated with the severity of thermal strain (Pires et al., 2017). Relevantly, the pro-inflammatory cascade that occurs following GI microbial translocation (MT) has been associated with adverse health consequences in physically active populations (e.g. military, athletes), including exertional heatstroke (Lim, 2018).

L-glutamine (GLN) is the most abundant free amino acid in the human body (Newsholme et al., 2003). It is classified as a conditionally essential nutrient, given it is the preferential energy source of rapidly proliferating cells (e.g. leukocytes), but becomes depleted during severe catabolic stress (Lacey and Wilmore, 1990). In addition to being an important energy substrate, GLN performs various other essential physiological roles, including: nitrogen transportation; gluconeogenesis; acid-base regulation; and the biosynthesis of glutathione, nitric oxide and heat shock proteins (Wischmeyer, 2006). Based on these functions, GLN supplementation has previously been recommended to strengthen GI barrier integrity in clinical care patients (McRae, 2017).

At present, sports nutrition guidelines do not endorse GLN supplementation to physically active individuals, given equivocal evidence to either improve immune function (Bermon et al., 2017) or exercise performance (Ahmadi et al., 2019). However, accumulating research has shown acute high-dose GLN supplementation supports GI barrier integrity during exertional-heat stress. Pioneering research by Zuhl et al. (2014) found 1 week of daily GLN supplementation (0.90 g·kg·fat free mass^{-1} [FFM]) attenuated the ~3-fold rise in GI permeability following 1-hour of running at 70% $\dot{V}O_{2max}$ in the heat (30°C). Promisingly, these findings were subsequently replicated by this research group (Zuhl et al., 2015) and others (Pugh et al., 2017b; Osborne et al., 2019b) but having provided a more practical single acute GLN bolus (0.90 g·kg·FFM^{-1}) 1-2 hours prior to exertional-heat stress.

Unfortunately, GLN supplementation has potential to induce acute dose-dependent GI symptoms (e.g. bloating, nausea), as proven in chapter 6. Two practical solutions to help combat this issue are to reduce the GLN dose to *circa* 0.30 $g \cdot kg^{-1}$ and/or to extend the post prandial time-period for absorption to > 2 hours before exertional-heat stress to allow any adverse symptoms time to reside. The former option is likely to be preferable in occupational settings (e.g. military, firefighters) if considering the financial and logistical constraints of population-level nutritional supplementation. Consequently, further research is warranted to determine whether the benefits of 0.90 $g \cdot kg \cdot FFM^{-1}$ GLN supplementation on GI barrier integrity is still achievable if providing a lower GLN dose (0.30 $g \cdot kg \cdot FFM^{-1}$) with the aim of achieving improved GI-tolerance (Chapter 6; Ziegler et al., 1990). A further limitation of previous research has been the lack of GI MT assessment, which is the key downstream pathophysiological event in the development of adverse health consequences.

The aim of the present study was to assess the influence of acute low-dose (0.30 $g \cdot kg \cdot FFM^{-1}$) oral GLN supplementation on GI barrier integrity and MT in response to exertional-heat stress. The primary hypothesis is that GLN supplementation would protect GI barrier integrity and reduce MT following exertional-heat stress. A secondary hypothesis was that GLN supplementation would be well tolerated without inducing subjective GI symptoms.

7.2 Methods

Participants and Ethical Approval

10 healthy males volunteered to take part in this study (Table 32). All participants met the demographic (Section 3.3.1) and health (Section 3.3.3) criteria for inclusion. Informed consent was obtained for each participant following explanation of the experimental procedures (Section 3.1). The study protocol was approved by Plymouth MARJON University Research Ethics Committee (Approval Code: EP097) and conducted in accordance with the principles outlined in the *Declaration of Helsinki (2013)*.

Experimental Overview

Participants visited the laboratory on 3 occasions. During the first visit, baseline anthropometrics and maximal oxygen uptake ($\dot{V}O_{2max}$) were assessed. The 2 subsequent visits were main experimental trials, where participants were supplemented with either

glutamine (GLN) or placebo (PLA) in a randomised, counterbalanced, double-blind, cross-over design (Section 3.14.2). These trials were separated by 7-14 days (Section 3.5.1). During both main experimental trials, participants completed a 100-minute fixed-intensity ecological military exertional-heat stress test (EHST) (Section 3.8.5). In comparison with chapters 4 and 5, when applying the present EHST, forearm cold water immersion (Section 3.6.7) and fluid replacement (Section 3.8.2) interventions were removed, with the aim to enhance GI barrier integrity loss (Costa et al., 2019). Data collection coincided cooler annual periods in Plymouth, United Kingdom (Section 3.5.1). A schematic illustration of the protocol is shown in Figure 19.

Table 32. Participant demographic characteristics.

Measure	Mean ± SD
Age (years)	32 ± 6
Height (m)	1.80 ± 0.07
Body Mass (kg)	83.6 ± 11.6
Physical Activity ($h \cdot week^{-1}$)	6 ± 2
Body Fat (%)	17.0 ± 4.0
$\dot{V}O_{2max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	48 ± 5

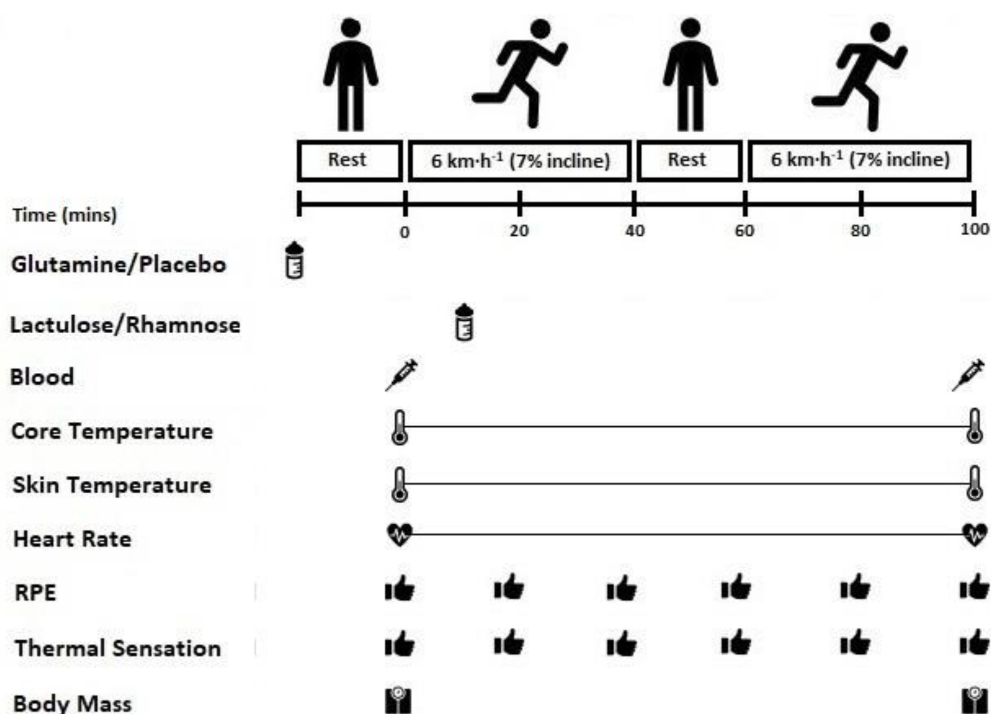

Figure 19. Schematic illustration of the experimental measurement timings.

Dietary and Lifestyle Controls

Trials were conducted following lifestyle (Section 3.5.1), dietary (Section 3.5.2) and hydration (Section 3.5.3) standardisation. Conformation was self-attested using a pre-trial control questionnaire. Participants remained fasted throughout all main experimental trials.

$_L$-Glutamine Supplementation

GLN supplementation consisted of 0.30 g·kg $^{-1}$ fat free body mass of GLN crystalline powder, which was freshly suspended in 500 ml of water/lemon flavour cordial in a 4:1 ratio (Section 3.6). The PLA was taste and consistency matched, comprised of the water/lemon flavour sugar-free cordial alone. Both drinks were consumed 1-hour before the EHST.

Anthropometrics

Height (Section 3.4.1), mass (Section 3.4.2) and body fat (Section 2.4.3) were measured following the International Society for the Advancement of Kinanthropometry (ISAK) guidelines (Marfell-Jones et al. 2006). The duplicate coefficient of variation (CV) for skinfold thicknesses at 4-sites was 2.2%.

Maximal Oxygen Uptake

Maximal oxygen uptake ($\dot{V}O_{2max}$) was determined using an incremental treadmill test to volitional exhaustion (Section 3.8.4) in normothermic laboratory conditions (Section 3.7). Expired metabolic gases were measured continuously using a breath-by-breath metabolic cart (3.10.1). Heart rate (HR; Section 3.10.2) and rating of perceived exertion (RPE; Section 3.12.1) were measuring during the final 10 seconds of each stage.

Exertional-Heat Stress Test

The EHST commenced at 08:30 ± 1 hour (Section 3.5.1). Upon laboratory arrival, participants provided a mid-flow urine sample to assess hydration status via urine osmolality (Section 3.12.7; CV = 0.5%) and urine specific gravity (Section 3.12.8; CV = <0.1%). A capillary blood sample was also collected for (CV = < 0.1%) plasma osmolality assessment (Section 3.13.6). Participants then measured their own nude body mass (Section 3.4.2), inserted a single use rectal thermistor (T_{core}; Section 3.9.1) and positioned a HR monitor (Section 3.10.2). Hard-wired thermistors were affixed to assess mean skin

temperature (T_{skin}) (Section 3.9.2). Participants then dressed in standardised summer-military clothing (Section 3.8.1) and entered the environment chamber that was regulated at ~35 °C (GLN: 35.3 ± 0.3 °C; PLA: 35.3 ± 0.2 °C; $p = 0.15$) and ~30% RH (GLN: 31 ± 1%; PLA: 30 ± 1%; $p = 0.44$) (Section 3.7).

Following 20 minutes seated rest, participants undertook a 100-minute fixed-intensity military EHST (Section 3.8.5). T_{core} (Section 3.9.1), T_{skin} (Section 3.9.2), mean body temperature (T_{body}; Section 3.9.3) and HR (Section 3.10.2) were continuously recorded throughout the EHST. RPE (Section 3.12.1), thermal sensation (TS; Section 3.12.2) and GI symptoms (Section 3.12.3) were reported at 20-minute intervals. Post-EHST nude mass was recorded for estimation of whole-body sweat rate (Section 3.11.1).

Blood Collection and Analysis

Venous blood samples (12 ml) were drawn immediately pre and post EHST (Section 3.13.1). Samples were centrifuged at 1300*g* for 15 minutes at 4 °C to separate serum and plasma. Aliquots were frozen at -80 °C until analyses. All blood handling was performed with sterile (pyrogen, DNA free) pipette tips and microtubes (Section 3.13.2).

Haematology

Haemoglobin (Section 3.13.3; CV = 0.4%) and haematocrit (Section 3.13.4; CV = 0.6%) were analysed in fresh whole blood for plasma volume estimation (Section 3.13.5). Post-exercise analyte concentrations were uncorrected for plasma volume change, given the similarity of between-trial response and low molecular weights of quantified analytes.

Dual-Sugar Absorption Test

Participants orally ingested a standard dual-sugar probe solution (Section 3.13.9) 10-minutes into the EHST. Probe concentrations were determined from serum samples collected 90-minutes (i.e. post-EHST) post probe ingestion using high performance liquid chromatography (Section 3.13.9). The duplicate CV for lactulose/$_{L}$-rhamnose was 8.7%.

Intestinal Fatty-Acid Binding Protein

I-FABP was measured immediately pre- and post- the EHST using a solid-phase sandwich ELISA (Section 3.13.10). The intra-assay CV was 2.0%.

Bacterial DNA

Total 16S and *Bacteroides* DNA were measured immediately pre- and post- the EHST using quantitative real-time polymerase chain reaction assays (Section 3.13.13). The duplicate intra-assay CV were 6.4% (total 16S) and 24.5% (*Bacteroides*).

Statistics

All statistical analyses were performed using Prism Graphpad software (Section 3.14). Comparisons were made after determining normal distribution using a Shapiro-Wilk test (Section 3.14.5*).* A 2-way analysis of variance (ANOVA) with repeated measures (time x trial) was used to identify differences between the 2 trials for whole-body physiological, GI barrier integrity and MT data (Section 3.14.7.3, 3.14.7.4). When there was only a single comparison, a paired t-test or non-parametric Wilcoxon signed-ranks test was used to determine between-trial differences (Section 3.14.7.1, 3.14.7.2). Relationships were assessed using repeated-measures correlation (Section 3.14.8.1). Data are presented as mean ± standard deviation (SD).

Power Analysis

An *a priori* sample size estimation was calculated based on anticipated effect sizes (d = 0.9) derived from a previous study comparing the influence of acute glutamine supplementation (0.90 g·kg·FFM^{-1}) on DSAT responses (Zuhl et al., 2015) following exertional-heat stress (Section 3.14.1). In total, ≥ 4 participants per group were calculated necessary to detect a significant interaction effect for the DSAT using a 2-way dependant t-test with standard alpha (0.05) and beta (0.8) values.

7.4 Results

Thermoregulation

T_{core} increased to a similar extent (time; $p < 0.01$) throughout the EHST between the 2 conditions (Figure 20A; time x trial; $p = 0.98$). No significant difference in mean, peak and Δ T_{core} were evident between the GLN (37.76 ± 0.22 °C; 38.55 ± 0.39 °C; 1.85 ± 0.38 °C) and PLA (37.82 ± 0.35 °C; 38.63 ± 0.50 °C; 1.69 ± 0.48 °C) trials, respectively ($p > 0.05$). T_{skin} increased to a similar extent (time; $p < 0.01$) throughout the EHST between the 2 conditions (Figure 20B; time x trial; $p = 0.49$). No significant difference in mean, peak and Δ T_{skin} were evident between the GLN (35.01 ± 0.40 °C; 35.41 ± 0.66 °C; 1.30 ± 0.76 °C) and PLA (35.12 ± 0.32 °C; 35.37 ± 0.47 °C; 1.14 ± 0.68 °C) trials, respectively ($p > 0.05$). T_{body} was increased

to a similar extent (time; $p < 0.01$) throughout the EHST between the 2 conditions (Figure 20C; time x trial; $p = 0.93$). No significant difference in mean, peak and Δ T_{body} were evident between the GLN (37.61 ± 0.23 °C; 38.32 ± 0.37 °C; 1.63 ± 0.35 °C) and PLA (37.68 ± 0.31 °C; 38.38 ± 0.43 °C; 1.70 ± 0.44 °C) trials, respectively. Mean sweat rate (GLN: 1.84 ± 0.34 $l \cdot h^{-1}$; PLA: 1.80 ± 0.27 $l \cdot h^{-1}$; $p = 0.43$) and % body mass loss (GLN: 2.01 ± 0.32%; PLA: 1.95 ± 0.51%; $p = 0.48$) were similar between conditions.

Hydration and Cardiovascular

Basal urine osmolality (GLN: 383 ± 256 $mOsmol \cdot kg^{-1}$; PLA: 336 ± 217 $mOsmol \cdot kg^{-1}$; $p = 0.57$), urine specific gravity (GLN: 1.010 ± 0.008 AU; PLA: 1.009 ± 0.006 AU; $p = 0.53$) and plasma osmolality (GLN: 299 ± 3 $mOsmol \cdot kg^{-1}$; PLA: 298 ± 3 $mOsmol \cdot kg^{-1}$; $p = 0.15$) were similar between conditions. The Δ plasma volume following the EHST were similar (GLN: -3.26 ± 1.81%, PLA: -3.46 ± 1.59%; $p = 0.62$). HR increased to a similar extent (time; $p < 0.01$) throughout the EHST between the 2 conditions (Figure 20D; time x trial; $p = 0.96$). No significant difference in mean, peak and Δ HR were evident between the GLN (142 ± 17 $b \cdot min^{-1}$; 161 ± 13 $b \cdot min^{-1}$; 100 ± 17 $b \cdot min^{-1}$) and PLA (145 ± 21 $b \cdot min^{-1}$; 161 ± 19 $b \cdot min^{-1}$; 99 ± 21 $b \cdot min^{-1}$) trials, respectively ($p > 0.05$).

Perception

RPE increased to a similar extent (time; $p < 0.01$) throughout the EHST between the 2 conditions (Figure 20E; time x trial; $p = 0.59$). No significant difference in mean, peak and Δ RPE were evident between the GLN (12 ± 2 AU; 15 ± 3 AU; 5 ± 3 AU) and PLA (13 ± 2 AU; 14 ± 2 AU; 5 ± 2 AU) trials, respectively ($p > 0.05$). TS increased to a similar extent (time; $p < 0.01$) throughout the EHST between the 2 conditions (Figure 20F; time x trial; $p = 0.70$). No significant difference in mean, peak and Δ TS were evident between the GLN (6.0 ± 0.5 AU; 7.0 ± 1.0 AU; 2.0 ± 0.5 AU) and PLA (6.0 ± 0.5 AU; 7.0 ± 1.0 AU; 2.0 ± 0.5 AU) trials, respectively ($p > 0.05$). There were no reports of gut discomfort, nausea, total-, upper- or lower- GI symptoms in any participant throughout either the GLN or PLA trial.

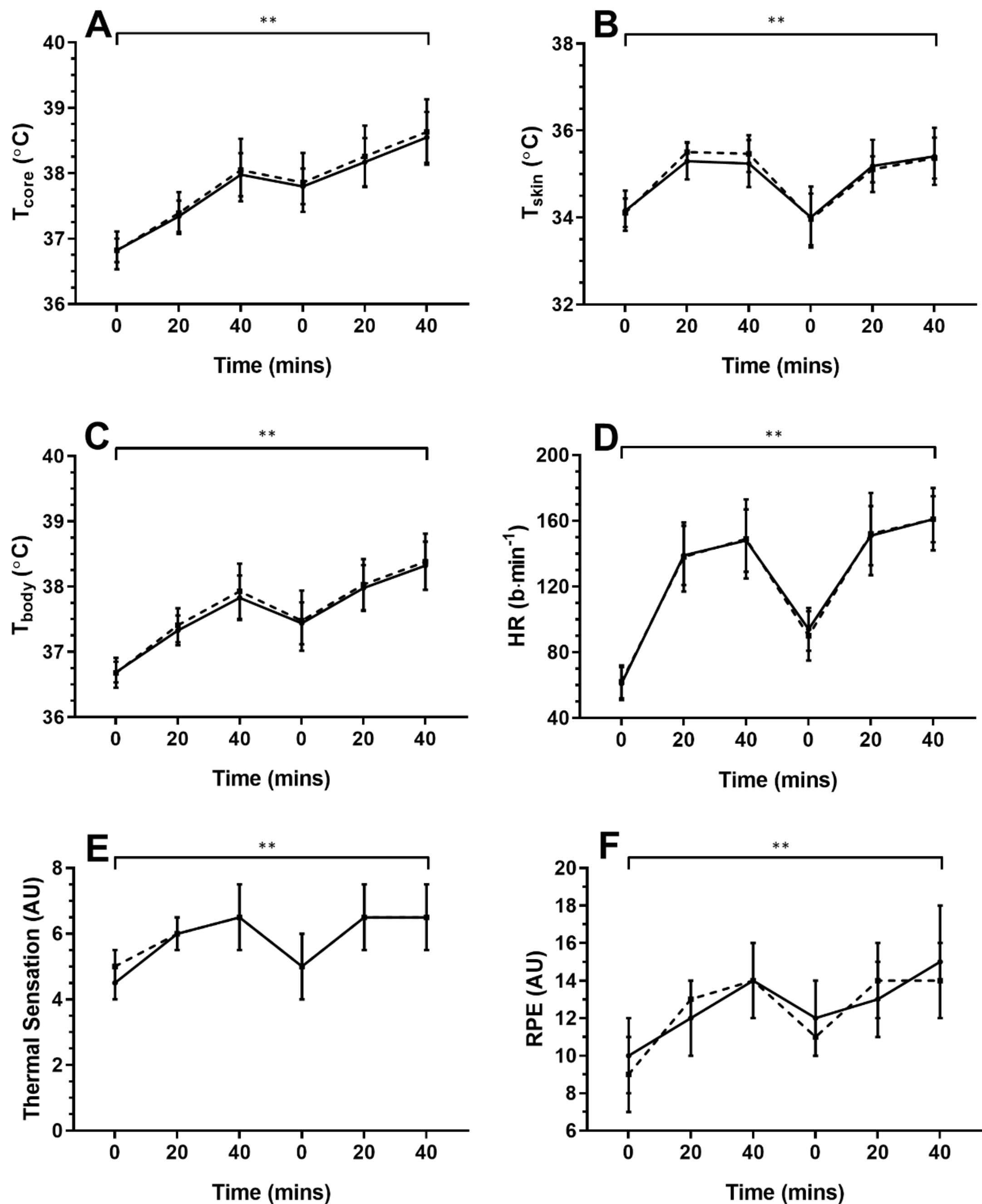

Figure 20. Whole-body physiological responses to EHSTs: (A) = core temperature; (B) = mean skin temperature; (C) = mean body temperature; (D) = heart rate; (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = GLN, broken line = PLA. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01).

Gastrointestinal Barrier Integrity

The DSAT (lactulose/$_{L}$-rhamnose ratio) tended to be greater (p = 0.06) in the GLN (0.030 ± 0.012), compared to the PLA (0.023 ± 0.006) trial (Figure 21A). I-FABP concentration increased from pre (GLN: 2.16 ± 0.98 ng·ml^{-1}; PLA: 2.60 ± 1.04 ng·ml^{-1}) to post-EHST (GLN: 4.70 ± 1.31 ng·ml^{-1}; PLA: 3.98 ± 1.70 ng·ml^{-1}) in both trials, but to a greater

extent in the GLN trial (Figure 21B; time x trial interaction; p = 0.03). Post hoc analysis revealed no significant difference between either time point (p > 0.05). The Δ I-FABP response was greater in the GLN (2.54 ± 1.21 $ng \cdot ml^{-1}$ [141 ± 85%]) *versus* PLA (1.37 ± 1.10 $ng \cdot ml^{-1}$ 1 [57 ± 49%]) trial (p = 0.01).

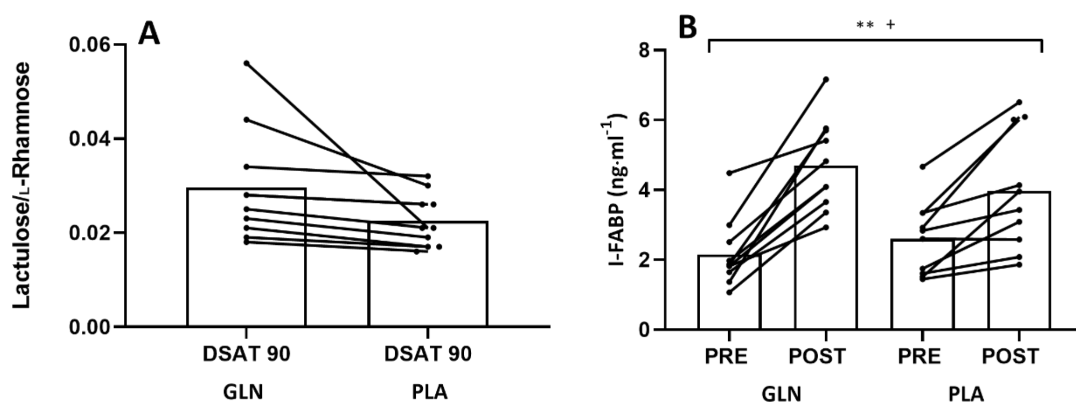

Figure 21. GI barrier integrity responses to EHSTs: (A) = L/R ratio (DSAT) at 90 minutes; (B) I-FABP. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). Significant trial * time interaction (+ p ≤ 0.05; ++ p ≤ 0.01).

Microbial Translocation

Total 16S DNA was unchanged (time; p = 0.73) from pre- (GLN = 6.08 ± 0.98 $\mu g \cdot ml^{-1}$; PLA = 6.55 ± 1.75 $pg \cdot \mu l^{-1}$) to post-EHST (GLN = 5.97 ± 0.98 $pg \cdot ml^{-1}$; PLA = 6.87 ± 0.91 $pg \cdot ml^{-1}$) in either trial (Figure 22A; time x trial interaction; p = 0.49). There was no difference in the Δ total 16S DNA response between the GLN (-0.10 ± 1.17 $\mu g \cdot ml^{-1}$) and PLA (0.29 ± 1.38 $\mu g \cdot ml^{-1}$) trials (p = 0.48). *Bacteroides*/total 16S DNA ratio increased (time; p = 0.04) from pre (GLN = 0.05 ± 0.05; PLA = 0.09 ± 0.08) to post-EHST (GLN = 0.12 ± 0.11; PLA = 0.25 ± 0.41) in both trials (Figure 22B; time x trial interaction; p = 0.21). The Δ *Bacteroides*/total 16S DNA ratio was comparable between the GLN (0.06 ± 0.09) and PLA (0.15 ± 0.33) trials (p = 0.44). Unfortunately, *Bacteroides* concentrations were below the limit of detection in 11/40 samples (ratio data presented as zero).

Associations

Associations between GI (DSAT, Δ I-FABP, Δ *Bacteroides*/total 16S DNA) and whole-body (peak T_{core}, mean HR, mean RPE) responses were conducted for the entire dataset (n = 20). The DSAT did not correlate with either I-FABP (Δ r = 0.52, p = 0.10; peak r = 0.27, p = 0.41) or *Bacteroides*/total 16S DNA responses (Δ r = -0.15, p = 0.66; peak r = 0.02, p = 0.96).

A small negative association was evident between Δ I-FABP and Δ *Bacteroides*/total 16S DNA (r = -0.61, p = 0.05). The DSAT was negatively associated with peak T_{core} (r = -0.67, p = 0.02) and mean HR (r = -0.66, p = 0.02), whilst Δ I-FABP was negatively associated with mean HR (r = -0.59, p = 0.05). No further associations were evident between Δ GI and whole-body responses.

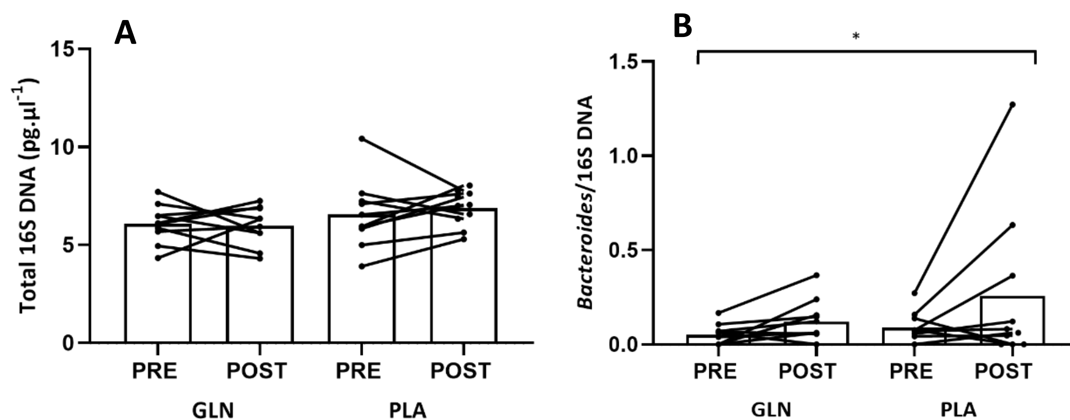

Figure 22. GI MT responses to EHSTs: (A) = total 16S DNA; (B) *Bacteroides*/total 16S DNA. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01).

7.5 Discussion

The aim of this study was to determine the influence of low-dose (0.30 g·kg·FFM^{-1}) acute oral GLN supplementation on GI barrier integrity and MT biomarkers in response to exertional-heat stress. The main findings were that low-dose acute oral GLN supplementation 1-hour before an EHST worsened GI permeability (serum DSAT) and small intestinal epithelial injury (I-FABP) in comparison to water-alone. Whilst GI MT (*Bacteroides*/total 16S DNA) increased following the EHST, this response was similar between the GLN and PLA trials. There was no evidence of subjective GI symptoms, whilst whole-body physiological (e.g. T_{core}, heart rate) responses were comparable between trials. Together, these data suggest no benefit, or even a harmful effect, of low-dose (0.30 g·kg·FFM^{-1}) acute oral GLN supplementation to support GI barrier integrity and GI MT following exertional-heat stress.

I-FABP is the prominent biomarker of small intestinal epithelial injury, whereas the DSAT assesses functional GI permeability (Bischoff et al., 2014). In the present study, overall mean Δ I-FABP (1.37 ± 1.101 ng·ml^{-1} [53%]) and absolute DSAT (0.023 ± 0.005) responses in the PLA trial where comparable to those previously reported (Δ I-FABP = 0.20-1.35 ng·ml^{-1}

[16-119%]; and DSAT = ~0.020-0.035) in chapters 4 and 5. This finding was unanticipated given in contrast to previous work, rehydration (12 g·kg·BM^{-1}) and forearm cold (15°C) water immersion (4 minutes) practises were intentionally curtailed in the present study, in attempt to increase GI barrier disturbance (Costa et al., 2019). However, given that post-EHST hypohydration (~2% vs ~1% body mass loss), thermoregulatory strain (e.g. peak T_{core} 38.6 vs. 38.5°C) and cardiovascular strain (mean HR = 145 vs. 150 bpm) were all similar to previous chapters, this finding is perhaps less surprising (Pires et al., 2017). For sake of comparison, in research by Costa et al. (2019) where permissive dehydration exaggerated small intestinal epithelial injury, here they were able to induce a greater divergence in hydration status between the hydrated (0.5% body mass loss) and hypohydrated conditions (3% body mass loss) than across experimental chapters of this thesis. Overall, the severity of small intestinal epithelial injury and GI permeability presently reported were comparable to many previous 60-to-90 minute moderate-intensity (60-70% $\dot{V}O_{2max}$) EHST protocols (e.g. Szymanski et al., 2017; Sheahen et al., 2018). It remains to be determined what intensity, duration and mode of exercise cause the greatest disturbance of GI barrier integrity, which should be established in future research.

Acute high-dose (0.90 g·kg·FFM^{-1}) oral GLN supplementation has been recommended to support GI barrier integrity during exertional-heat stress (Zuhl et al., 2015). The practicality of this supplementation regime is however questionable, given evidence of poor GI tolerance (e.g. nausea, bloating) in some individuals with this dosage (Ward et al., 2003; chapter 6). To overcome this issue, the present study instead supplemented participants with a reduced dose of GLN (0.30 g·kg·FFM^{-1}) 1-hour before exertional-heat stress, given that this dose was well-tolerated in chapter 6. However, in contrast to the *a priori* hypothesis, low-dose acute oral GLN supplementation in fact worsened small intestinal epithelial injury and GI permeability following exertional-heat stress in comparison to a non-calorific PLA by 85% and 30%, respectively. This result was surprising given previous literature on this topic showing acute high-dose (0.90 g·kg·FFM^{-1}) oral GLN supplementation to blunt Δ I-FABP responses by ~15-25%, and GI permeability responses by 40-50%, when ingested either 1 or 2 hours before exertional-heat stress (Zuhl et al., 2015; Pugh et al., 2017b; Osborne et al., 2019b). Whilst Pugh et al. (2017b) previously suggested that the benefits of acute oral GLN supplementation on GI barrier integrity might be dose-dependent in response to exertional-heat stress, the present finding is the first to report an adverse effect of GLN supplementation on GI barrier integrity in this setting.

In comparison to previous research reporting positive results with high-dose oral GLN supplementation on GI barrier integrity, there are several potential mechanisms that may explain the null results with low-dose GLN supplementation in the present study. First, the dose and/or timing of GLN ingestion might have been insufficient for meaningful induction of key protective mechanisms. These include: intracellular heat stroke protein (I-HSP) expression (Singleton and Wischmeyer, 2006); epithelial cell proliferation (Rhoads et al., 1997); glutathione biosynthesis (Harward et al., 1994); and GI TJ protein expression (Li et al., 2004). Whilst these mechanisms could not be examined in the present study due to the unavailability of intestinal tissue, previous *in vitro* studies on intestinal epithelial cells have shown dose-dependent benefits of GLN supplementation on these mechanisms inside 1-hour (Wischmeyer et al., 1997). Second, implementation of the serum DSAT at a single timepoint following probe ingestion is potentially confounded by the influence of prior GLN ingestion on alterations in probe transit throughout the GI tract (Du et al., 2018). Whilst this effect is theoretically controlled through paracellular/transcellular molecular probe ratio correction in 5-hour urine samples (Bjarnason et al., 1995), the time-courses of absorption of low/high molecular weight probes in the blood is not necessarily identical (Sequeira et al., 2014). Third, previous studies conducted trials following an overnight fast, where the PLA supplement was non-calorific (Zuhl et al., 2015, Pugh et al., 2017b). Given macronutrient ingestion improves intestinal vascular perfusion during exertional-heat stress (Snipe et al., 2017), favourable responses with GLN supplementation might simply relate to the dose-dependent effects of energy provision *per se*. Finally, the severity of GI barrier integrity loss would appear lower herein than in previous investigations (Zuhl et al., 2014, 2015; Pugh et al., 2017b), potentially concealing a true experimental effect.

Bacterial DNA is an emerging biomarker to assess GI MT through high-sensitivity conserved 16S gene sequencing (Paisse et al., 2016). Compared with traditional GI MT biomarkers (e.g. endotoxin), bacterial DNA assessment is less susceptible to analytical issues surrounding exogenous contamination, given the ability to target phyla/species with high GI specificity (e.g. *Bacteroides*). To reduce the influence of potential covariates, such as the efficacy of DNA extraction, DNase concentrations and immune function, correction for total 16S DNA has previously been recommended (March et al., 2019). Absolute *Bacteroides*/total 16S DNA ratios (~0 – 1.0) reported in the present study were comparable to chapters 4 and 5. Likewise, the 89 ± 217% increase in *Bacteroides*/total 16S DNA following the EHST in the PLA trial was comparable to the 183 ± 336% increase in

participants with low aerobic fitness ($\dot{V}O_{2max}$ < 50 ml·kg·min^{-1}) in chapter 5. Thus, GI MT assessed using the *Bacteroides*/total 16s DNA ratio, is elevated following brief subclinical exertional-heat stress in most recreationally trained individuals. Nevertheless, given the large inter-individual variability and poor temporal reliability in participant responses, an increased sample size is likely required to detect the magnitude of effect observed in this study for GLN supplementation on small intestinal epithelial injury and GI permeability responses. Surprisingly, there was a moderate negative association between Δ I-FABP and *Bacteroides*/total 16S ratio. However, given a lack of mechanistic explanation and discordance with previous research, it is suggested that finding is most likely a false-positive result, arising from large heterogeneity in *Bacteroides* concentrations.

In contrast to the growing number of studies demonstrating oral GLN supplementation to support GI barrier integrity in response to exertional-heat stress, few studies have assessed whether these benefits translate into reduced downstream GI MT. In addressing this gap in the literature, the present study reported no difference in GI MT between the GLN and PLA trials following the EHST, which was inconsistent with the *a priori* hypothesis. This null effect of GLN supplementation on GI MT was surprising given that GLN simultaneously increased small intestinal epithelial injury and GI permeability. However, there is rationale to suggest that GLN supplementation might have improved microbial neutralisation capacity, independent of MT (Shu et al., 2016). For example, in experimental rodent sepsis models, GLN supplementation enhanced: lymphocyte function in gut-associate lymphoid tissue (Gianotti et al., 1995; Manhart et al., 2001); luminal immunoglobulin concentrations (Wu et al., 2016; Fan et al., 2018); and hepatic integrity upon pathological insult (Karatepe et al., 2010; Cruzat et al., 2014). Of the previous studies assessing the influence of GLN supplementation on GI barrier integrity around exertional-heat stress, only Zuhl et al. (2015) attempted to monitor GI MT through assessment of plasma endotoxin concentration. Unfortunately, endotoxin concentrations were unchanged following exertional-heat stress in this study, thus making it unfeasible to assess the efficacy of the GLN intervention (Zuhl et al., 2015). Therefore, the present study provides the first direct evidence that acute-oral GLN supplementation does not reduce systemic concentration of GI derived microbes in response to exertional-heat stress.

7.6 Limitations

Despite administering a tightly controlled methodological design, the present

results were not without limitations. First, the EHST only evoked moderate disturbance of GI barrier integrity and MT. A previous systematic review outlined an exercise induced hyperthermia threshold of 38.6 °C T_{core} for GI barrier integrity loss (DSAT, I-FABP and endotoxin) to be commonplace (>50% incidence) and of 39.0 °C for GI barrier integrity loss to be universal (100% incidence; Pires et al., 2017). In this study, in only 11/20 trials did participants exceed the 38.6 °C threshold, whilst only 3/20 exceeded the 39.0 °C threshold. Notwithstanding this limitation, the present EHST was chosen as it has robust ecological validity in representing group-based military field activities (Spitz et al., 2012) and previously was severe enough to induce GI MT (chapter 5). Second, *Bacteroides* DNA had poor analytical reliability (mean duplicate CV = 24.5%), which resulted from a large proportion of samples being close to the assays minimum level of detection. Future analysis should consider assessment of whole-blood samples, given that bacterial 16S DNA concentrations in the buffy coat far exceed that of plasma (Paisse et al., 2016). Third, a basal DSAT was not performed to minimise participants' time burden, with the overall aim on improving compliance. Consequently, this prevented direct assessment of the EHST on GI permeability. Fourth, females were excluded from participation due to unavailability of menstruation hormone testing. Previous evidence has shown no influence of sex on GI barrier integrity responses to exertional-heat stress (Snipe and Costa, 2018a). Finally, implementation of an isocaloric PLA or standardised pre-trial breakfast would have reduced concerns regarding the extraneous influence of macronutrient provision on GI barrier integrity. The decision to utilise a non-calorific PLA was selected to ensure consistency with previous research (Zuhl et al., 2014, 2015; Pugh et al., 2017b; Osborne et al., 2019b).

7.7 Conclusion

This study assessed the influence of low-dose (0.30 $g \cdot kg \cdot FFM^{-1}$) acute oral GLN supplementation on GI barrier integrity and MT biomarkers in response to exertional-heat stress. The GLN bolus was well-tolerated, with no adverse GI symptoms responses reported throughout this study. Unexpectedly, GI permeability (serum DSAT) and small intestinal epithelial injury (I-FABP) were worsened with GLN in comparison to PLA supplementation in response to exertional-heat stress. Therefore, unlike previous research on this topic supplementing with high-dose (0.90 $g \cdot kg \cdot FFM^{-1}$) acute oral GLN, these findings show that when supplemented in lower doses, GLN has the potential to enhance exertional-heat

stress associated GI barrier integrity loss. Downstream GI MT assessed via *Bacteroides*/total 16S DNA responses increased following the EHST, however, there was no significant impact of GLN supplementation. Given this response, it is speculatively recommended that the meaningfulness of heightened GI barrier integrity disturbance following GLN supplementation does not translate into an increased risk of clinical complications from exertional-heat stress (e.g. exertional-heat stroke). The peak thermoregulatory strain induced in the present study was sub-clinical (e.g. T_{core} = 38-39.5°C), which might warrant future investigation using a more arduous EHST. These findings do not support the use of acute low-dose oral GLN supplementation to protect GI barrier integrity and prevent MT in response to exertional-heat stress.

Chapter 8 - Low Dose Acute L-Glutamine Supplementation on Gastrointestinal Barrier Integrity and Microbial Translocation in response to High Intensity Exertional-Heat Stress

8.1 Abstract

Purpose: Exertional-heat stress adversely distrupts (GI) barrier integrity and, through subsequent microbial translocation (MT), can result in potentially fatal exertional-heat stroke. Acute glutamine (GLN) supplementation is a potential nutritional countermeasure, although the practical value of current supplementation regimens is questionable.

Method: 10 males completed 2 high-intensity exertional-heat stress tests (EHST) involving running in the heat (40 °C and 40% relative humidity) at lactate threshold to volitional exhaustion. Participants ingested GLN (0.30 g·kg·FFM^{-1}) or a non-calorific placebo (PLA) 1-hour prior to the EHST. Venous blood was drawn pre-, immediately post- and 1-hour post-EHST. GI barrier integrity was assessed using a serum dual-sugar absorption test (DSAT) and plasma Intestinal Fatty-Acid Binding Protein (I-FABP). MT was assessed using the plasma *Bacteroides*/total 16S DNA ratio.

Results: Volitional exhaustion occurred after 22:19 ± 2:22 (minutes: seconds), which was identical between conditions. Whole-body physiological responses and GI symptoms were not different between conditions ($p > 0.05$). GI permeability (serum DSAT) was greater following GLN (0.043 ± 0.020) than placebo supplementation (0.034 ± 0.019) ($p = 0.02$). Small intestine epithelial injury (I-FABP) similarly increased ($p = 0.22$) post EHST in both trials (GLN Δ = 1.25 ± 0.63 ng·ml^{-1}; PLA Δ= 0.92 ± 0.44 ng·ml^{-1}). GI MT (*Bacteroides*/total 16S DNA ratio) was unchanged following the EHST ($p = 0.43$).

Conclusion: Acute low-dose (0.30 g·kg^{-1} fat free mass) oral GLN supplementation 1 hour before high-intestity exertional-heat stress enhanced GI permeability, but did not influence either GI epithial injury or MT.

8.2 Introduction

Exertional Heat Stroke (EHS) is the most severe condition along a continuum of heat-related illnesses (Leon and Bouchama, 2011). The condition sporadically affects individuals engaged in arduous physical activity, including military personnel, firefighters, and athletes (Epstein and Yanovich, 2019). Whilst direct mortality from EHS is uncommon where rapid whole-body cooling is provided (Belval et al., 2018), many casualties still experience chronic morbidity due to residual organ damage (Wallace et al., 2007). The pathophysiology of EHS is widely considered to relate to a systemic inflammatory response, which arises following gastrointestinal (GI) microbial translocation (MT) into the systemic circulation (Lim, 2018). Consequently, contemporary research has focussed on evaluating the potential of nutritional (Ogden et al., 2020), pharmacological (Walter and Gibson, 2020a) and thermoregulatory (Costa et al., 2020b) countermeasures to support GI barrier integrity during exertional-heat stress.

$_{L}$-glutamine (GLN) is a conditionally essential nutrient that is often depleted during severe catabolism (Lacey and Wilmore, 1990). It is the preferential energy source of various rapidly proliferating cells (e.g. GI enterocytes), whilst performs important actions relating to the biosynthesis of glutathione, nitric oxide and heat shock proteins (Wischmeyer, 2006). Based on these functions, GLN supplementation has been recommended to strengthen GI barrier integrity in both clinical care patients (McRae, 2017) and in individuals performing exertional-heat stress (Zuhl et al., 2015).

In relation to exertional-heat stress, high-dose (0.90 $g \cdot kg^{-1}$ fat free body mass [FFM]) GLN supplementation has been repeatedly shown to protect GI barrier integrity when consumed 1-2 hours before ~60 minutes of moderate intensity (70% $\dot{V}O_{2max}$) exercise in the heat (Zuhl et al., 2015; Pugh et al., 2017b; Osborne et al., 2019b). However, despite this compelling evidence, from a practical standpoint this supplementation regime regularly causes adverse GI symptoms, which in chapter 6 were shown to persist for several hours in some individuals. In attempt to overcome this issue, a reduced dose (0.30 $g \cdot kg^{-1}$ FFM) GLN supplement was trialled 1-hour before an 80-minute fixed-intensity exertional-heat stress test in chapter 7, though unexpectedly exacerbated GI barrier integrity disturbance compared to a non-calorific placebo. Given these inconsistent findings, further research is still warranted before guidance for applied practise can be recommended.

Short-duration, high-intensity exercise (e.g. 5-10 km fitness tests) is a common risk factor for EHS in military settings (Shibolet et al., 1967; Abriat et al., 2014). For example, in Israeli soldiers, retrospective analysis of 150 EHS casualties, concluded 57% of incidents to occur within the first 5-km of exercise onset (Epstein et al., 1999). Whilst the influence of exercise intensity on GI barrier integrity has never been directly examined, short-duration (< 30 mins) high-intensity (>75% $\dot{V}O_{2max}$) exercise typically causes greater GI permeability (Marchbank et al., 2011; Davison et al., 2016; March et al., 2017) and MT (Lim et al., 2009; Shing et al., 2013; Barberio et al., 2015) in comparison to long-duration (1-3 hours) low-intensity (≤ 70% $\dot{V}O_{2max}$) exercise. Despite this evidence, virtually all previously recommended nutritional countermeasures to support GI barrier integrity in response to exertional-heat stress have only ever demonstrated been examined in response to prolonged duration moderate intensity exercise . Thus, it is important to consider whether acute low-dose GLN supplementation can protect GI barrier integrity and prevent downstream MT during more intense forms of exercise.

The aim of the present study was to assess the influence of low-dose (0.30 $g{\cdot}kg{\cdot}FFM^{-1}$) acute GLN supplementation on GI barrier integrity and MT in response to a high-intensity short-duration EHST to exhaustion. The primary hypothesis was that GLN supplementation would worsen GI barrier integrity and reduce GI MT in response to exertional-heat stress. A secondary hypothesis was that GLN supplementation would be well tolerated without inducing subjective GI symptoms.

8.3 Methods

Participants and Ethical Approval

10 healthy males volunteered to take part in this study (Table 33). All participants met the demographic (Section 3.3.1) and health (Section 3.3.3) criteria for inclusion. Informed consent was obtained for each participant following explanation of the experimental procedures (Section 3.1). The study protocol was approved by Plymouth MARJON University Research Ethics Committee (Approval Code: EP082) and conducted in accordance with the principles outlined in the *Declaration of Helsinki (2013)*.

Table 33. Participant demographic characteristics.

Measure	Mean ± SD
Age (years)	29 ± 7
Height (m)	1.78 ± 0.10
Body Mass (kg)	81.8 ± 9.3
Physical Activity (h·week^{-1})	8 ± 3
Body Fat (%)	16.5 ± 5.0
$\dot{V}O_{2max}$ (ml·kg^{-1}·min^{-1})	48 ± 6

Experimental Overview

Participants visited the laboratory on 3 occasions. During the first visit, baseline anthropometrics, maximal oxygen uptake ($\dot{V}O_{2max}$) and lactate threshold (LT) were assessed. The 2 subsequent visits were main experimental trials, where participants were supplemented with either glutamine (GLN) or placebo (PLA) in a randomised, counterbalanced, double-blind, cross-over design (Section 3.14.2). These trials were separated by 7-14 days (Section 3.5.1). During both main experimental trials, participants completed an exertional-heat stress test (EHST), consisting of a 30-minute fixed-intensity treadmill run at normothermic LT intensity in the heat (Section 3.8.3). Data collection coincided cooler annual periods in Plymouth, United Kingdom (Section 3.5.1). A schematic illustration of the protocol is shown in Figure 23.

Dietary and Lifestyle Controls

Trials were conducted following lifestyle (Section 3.5.1), dietary (Section 3.5.2) and hydration (Section 3.5.3) standardisation. Conformation was self-attested using a pre-trial control questionnaire. Participants remained fasted throughout all main experimental trials, but were permitted water (28-30°C) to drink following the EHST (Section 3.8.2).

$_{L}$-Glutamine Supplementation

GLN supplementation consisted of 0.30 g·kg^{-1} fat free body mass (FFM) of GLN crystalline powder, which was freshly suspended in 500 ml of water/lemon flavour cordial in a 4:1 ratio (Section 3.6). The PLA was taste and consistency matched, comprised of the water/lemon flavour sugar-free cordial alone. Both drinks were consumed 1-hour before the EHST.

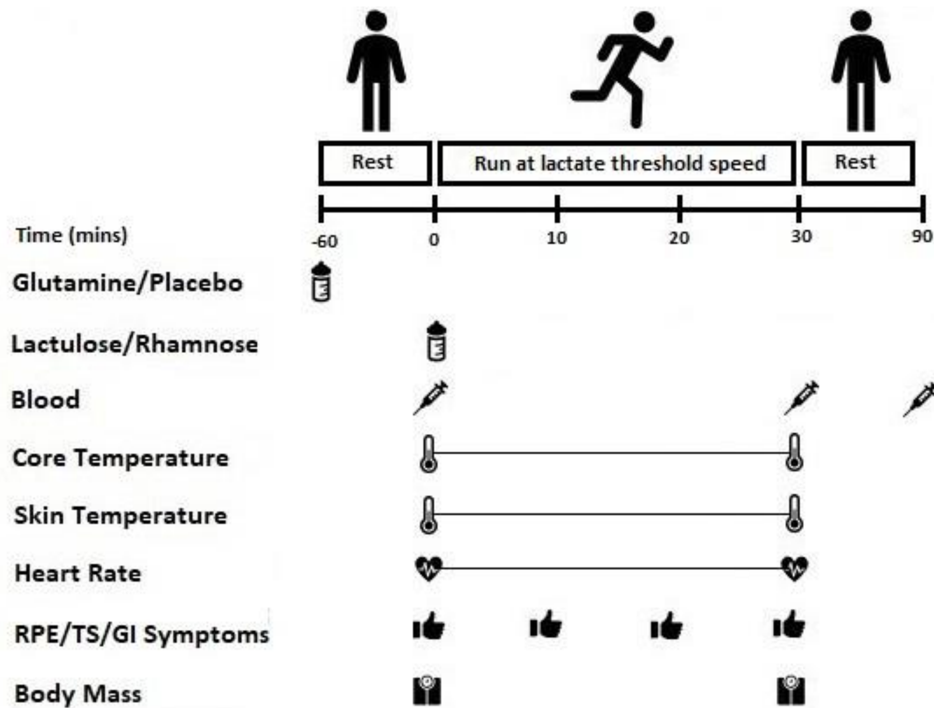

Figure 23. Schematic illustration of the experimental measurement timings.

Anthropometrics

Height (Section 3.4.1), mass (Section 3.4.2) and body fat (Section 2.4.3) were measured following the International Society for the Advancement of Kinanthropometry (ISAK) guidelines (Marfell-Jones et al. 2006). The duplicate coefficient of variation (CV) for skinfold thicknesses at 4-sites was 2.2%.

Maximal Oxygen Uptake

Maximal oxygen uptake ($\dot{V}O_{2max}$) was determined using an incremental treadmill test to volitional exhaustion (Section 3.8.4) in normothermic laboratory conditions (Section 3.7). Expired metabolic gases were measured continuously using a breath-by-breath metabolic cart (3.10.1). Heart rate (HR; Section 3.10.2) and rating of perceived exertion (RPE; Section 3.12.1) were measuring during the final 10 seconds of each stage.

Lactate Threshold

Lactate threshold (LT) was determined using a discontinuous incremental treadmill test (Section 3.8.3) in normothermic laboratory conditions. Participants dismounted the treadmill between stages for exactly 1 minute to collect a 20 µl fingertip capillary blood sample. Blood samples were gently inverted within a lysing stabilising agent and bulk analysed in duplicate (CV = 0.4%) for $_{L}$-lactate concentration (Section 3.8.3).

Exertional-Heat Stress Test

The EHST commenced at 08:30 ± 1 hour (Section 3.5.1). Upon laboratory arrival, participants provided a mid-flow urine sample to assess hydration status via urine osmolality (Section 3.12.7; CV = 1.3%) and urine specific gravity (Section 3.12.8; CV = <0.1%). A capillary blood sample was also collected for (CV = 0.1%) plasma osmolality assessment (Section 3.13.6). Participants then measured their own nude body mass (Section 3.4.2), inserted a single use rectal thermistor (T_{core}; Section 3.9.1) and positioned a HR monitor (Section 3.10.2). Hard-wired thermistors were affixed to assess mean skin temperature (T_{skin}) (Section 3.9.2). Participants then dressed in standardised summer-military clothing (Section 3.8.1) and entered the environment chamber that was regulated at ~40 °C (GLN: 40.5 ± 0.3 °C; PLA: 40.0 ± 0.5 °C; p = 0.44) and ~40% RH (GLN: 38 ± 1%; PLA: 38 ± 1%; p= 0.59) (Section 3.7).

Following ~30 minutes seated rest, participants undertook a high-intensity EHST (Section 3.8.6). Termination of the EHST was 30-minutes of running, volitional exhaustion, or physiological maximum end-points, whichever came first (Section 3.8.8). If the first trial was terminated early, EHST duration was successfully matched in the second trial. T_{core} (Section 3.9.1), T_{skin} (Section 3.9.2), mean body temperature (T_{body}; Section 3.9.3) and HR (Section 3.10.2) were continuously recorded throughout the EHST. RPE (Section 3.12.1), thermal sensation (TS; Section 3.12.2) and GI symptoms (Section 3.12.3) were reported at 10-minute intervals. Post-EHST nude mass was recorded for estimation of whole-body sweat rate (Section 3.11.1).

Blood Collection and Analysis

Venous blood samples (12 ml) were drawn immediately pre and post the EHST (Section 3.13.1). Samples were centrifuged at 1300*g* for 15 minutes at 4 °C to separate serum and plasma. Aliquots were frozen at -80 °C until analyses. All blood handling was performed with sterile (pyrogen, DNA free) pipette tips and microtubes (Section 3.13.2).

Haematology

Haemoglobin (Section 3.13.3; CV = 0.6%) and haematocrit (Section 3.13.4; CV = 0.3%) were analysed in fresh whole blood for plasma volume estimation (Section 3.13.5). Post-exercise analyte concentrations were uncorrected for plasma volume change, given the similarity of between-trial response and low molecular weights of quantified analytes.

Dual-Sugar Absorption Test

Participants orally ingested a standard dual-sugar probe solution (Section 3.13.9) at the start of the EHST. Probe concentrations were determined from serum samples collected 90-minutes (i.e. 1-hour post EHST) post probe ingestion using high performance liquid chromatography (Section 3.13.9). The duplicate CV for lactulose/$_L$-rhamnose was 6.5%.

Intestinal Fatty-Acid Binding Protein

I-FABP was measured immediately pre- and post- the EHST using a solid-phase sandwich ELISA (Section 3.13.10). The intra-assay CV was 5.5%.

Bacterial DNA

Total 16S and *Bacteroides* DNA were measured immediately pre- and post- the EHST using quantitative real-time polymerase chain reaction assays (Section 3.13.13). The duplicate intra-assay CV were 6.4% (total 16S) and 27.7% (*Bacteroides*).

Statistics

All statistical analyses were performed using Prism Graphpad software (Section 3.14). Comparisons were made after determining normal distribution using a Shapiro-Wilk test (Section 3.14.5*).* A 2-way analysis of variance (ANOVA) with repeated measures (time x trial) was used to identify differences between the 2 trials for whole-body physiological, GI barrier integrity and MT data (Section 3.14.7.3, 3.14.7.4). When there was only a single comparison, a paired t-test or non-parametric Wilcoxon signed-ranks test was used to determine between-trial differences (Section 3.14.7.1, 3.14.7.2). Relationships were assessed using a repeated-measures correlation (Section 3.14.8.1). Data are presented as mean ± standard deviation (SD).

Power Analysis

An *a priori* sample size estimation was calculated based on anticipated effect sizes (d = 0.9) derived from a previous study comparing the influence of acute glutamine supplementation (0.90 g·kg·FFM^{-1}) on DSAT responses (Zuhl et al., 2015) following exertional-heat stress (Section 3.14.1). In total, ≥ 4 participants per group were calculated necessary to detect a significant interaction effect for the DSAT using a 2-way dependant t-test with standard alpha (0.05) and beta (0.8) values.

8.4 Results

EHST Duration

Treadmill speed at lactate threshold was 11.7 ± 1.4 km·h^{-1}. All participants were able to replicate EHST duration (22:19 ± 2:22 minutes: seconds) across the 2 trials and exceeded the *a priori* minimum duration threshold of 20-minutes.

Thermoregulation

T_{core} increased to a similar extent throughout the EHST between the 2 trials (Figure 24A; time x trial; p = 0.92). No significant difference in mean, peak and Δ T_{core} were evident between the GLN (37.72 ± 0.30°C; 38.67 ± 0.40°C; 1.76 ± 0.39°C) and PLA (37.67 ± 0.33°C; 38.59 ± 0.37°C; 1.81 ± 0.44°C) trials, respectively ($p > 0.05$). T_{skin} increased to a similar extent throughout the EHST between the two trials (Figure 24B; time x trial; p = 0.99). No significant difference in mean, peak and Δ T_{skin} were evident between the GLN (36.59 ± 0.37°C; 37.45 ± 0.49°C; 2.07 ± 0.74°C) and PLA (36.65 ± 0.39°C; 37.51 ± 0.51°C; 2.11 ± 0.86°C) trials, respectively ($p > 0.05$). T_{body} was increased to a similar extent throughout the EHST between the 2 trials (Figure 24C; time x trial; p = 0.95). No significant difference in mean, peak and Δ T_{body} were evident between the GLN (37.89 ± 0.23°C; 38.83 ± 0.36°C; 1.82 ± 0.35°C) and PLA (37.82 ± 0.24°C; 38.78 ± 0.34°C; 1.87 ± 0.40°C) trials, respectively. Mean estimated sweat rate (GLN: 2.31 ± 0.89 l·h^{-1}; PLA: 2.33 ± 0.86 l·h^{-1}; p = 0.89) and % body mass loss (GLN: 1.07 ± 0.45%; PLA: 1.08 ± 0.44%; p = 0.73) were similar between trials.

Hydration and Cardiovascular

Basal urine osmolality (GLN: 322 ± 176 mOsmol·kg^{-1}; PLA: 227 ± 102 mOsmol·kg^{-1}; p = 0.12), urine specific gravity (GLN: 1.006 ± 0.005 AU; PLA: 1.004 ± 0.004 AU; p = 0.37) and plasma osmolality (GLN: 300 ± 5 mOsmol·kg^{-1}; PLA: 299 ± 3 mOsmol·kg^{-1}; p = 0.25) were similar between trials. The Δ plasma volume following the EHST was similar (GLN: -0.79 ± 1.53%; PLA: -0.52 ± 1.67%; p = 0.72) between trials. HR increased to a similar extent throughout the EHST between the 2 trials (Figure 24D; time x trial; p = 0.66). No significant difference in mean, peak and Δ HR were evident between the GLN (171 ± 13 b·min^{-1}; 186 ± 11 b·min^{-1}; 117 ± 9 b·min^{-1}) and PLA (170 ± 11 b·min^{-1}; 185 ± 10 b·min^{-1}; 119 ± 10 b·min^{-1}) trials, respectively ($p > 0.05$).

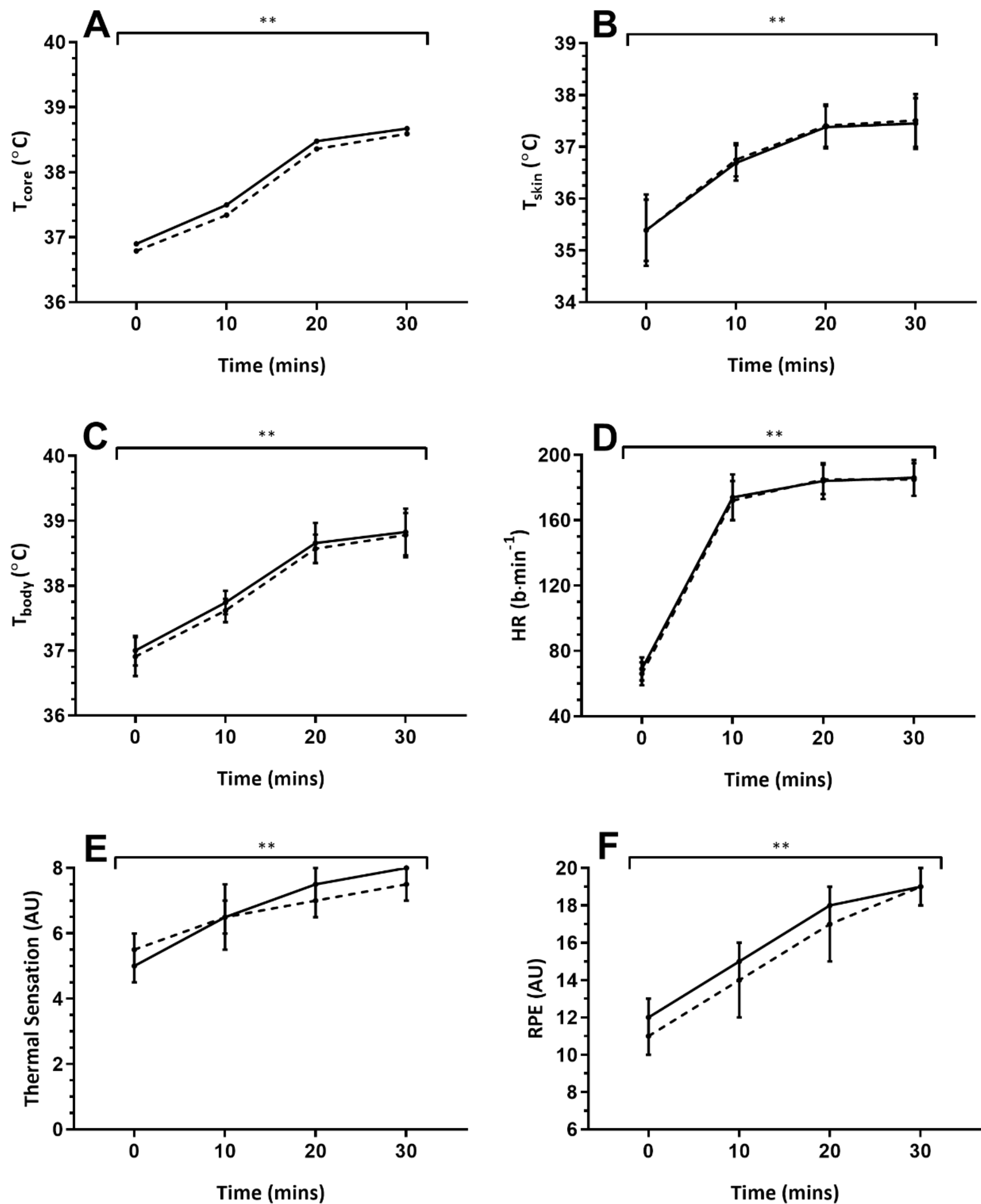

Figure 24. Whole-body physiological responses to EHSTs: (A) = core temperature; (B) = mean skin temperature; (C) = mean body temperature; (D) = heart rate; (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = GLN, broken line = PLA. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01).

Perception

RPE increased to a similar extent throughout the EHST between the 2 trials (Figure 24E; time x trial; p = 0.61). No significant difference in mean, peak and Δ RPE were evident between the GLN (16 ± 1 AU; 19 ± 1 AU; 7 ± 1 AU) and PLA (16 ± 1 AU; 19 ± 1 AU; 7 ± 1 AU) trials, respectively ($p > 0.05$). TS increased to a similar extent throughout the EHST between the 2 trials (Figure 24F; time x trial; p = 0.33). No significant difference in mean, peak and Δ TS were evident between the GLN (6.5 ± 0.5 AU; 8.0 ± 0.5 AU; 2.5 ± 0.5 AU) and PLA (7.0 ± 0.5 AU; 8.0 ± 0.5 AU; 2.0 ± 0.5 AU) trials, respectively ($p > 0.05$). The incidence and severity of gut discomfort, total-, upper- or lower- GI symptoms and nausea were comparable ($p > 0.05$) over time between the GLN and PLA trials (Table 34). No participant gave a rating indicative of severe GI symptoms (score ≥ 5) across either trial.

Gastrointestinal Barrier Integrity

The DSAT (lactulose/$_L$-rhamnose ratio) was 26% greater (p = 0.02) following the GLN (0.043 $\pm$ 0.020), in comparison to the PLA (0.034 $\pm$ 0.019) trial (Figure 25A). There was no difference in I-FABP responses between the 2 trials (Figure 25B; time x trial interaction; p = 0.20). In both conditions, I-FABP concentration increased from pre- (GLN: 1.21 $\pm$ 0.67 ng·ml^{-1}; PLA: 1.62 $\pm$ 0.82 ng·ml^{-1}) to post-EHST (GLN: 2.46 $\pm$ 1.17 ng·ml^{-1}; PLA: 2.54 $\pm$ 1.07 ng·ml^{-1}), There was no difference in the Δ I-FABP response between the GLN (1.25 $\pm$ 0.63 ng·ml^{-1} [117 $\pm$ 63%]) and PLA (0.92 $\pm$ 0.44 ng·ml^{-1} [63 $\pm$ 29%]) trials (p = 0.22).

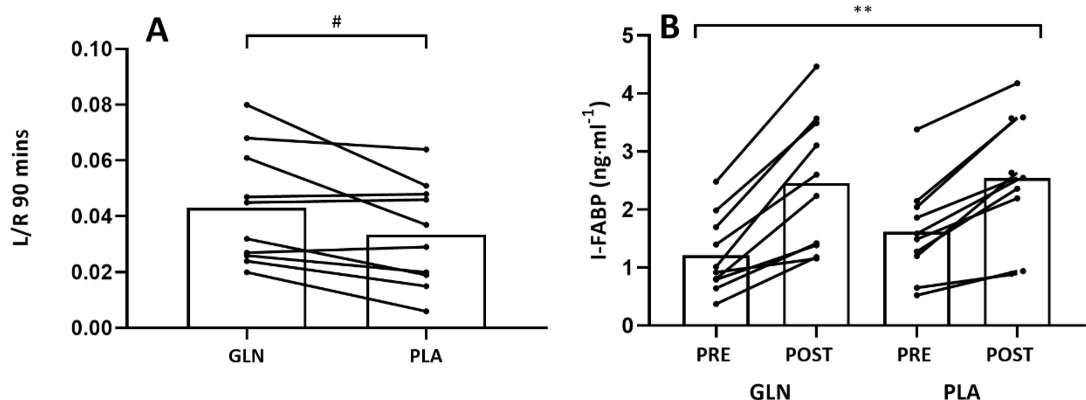

Figure 25. GI barrier integrity responses to EHSTs: (A) = L/R ratio (DSAT) at 90 minutes; (B) I-FABP. Significant overall effect of time (*p ≤ 0.05; ** p ≤ 0.01). Significant effect of trial (# p ≤ 0.05).

Microbial Translocation

Total 16S DNA concentrations responded comparably across both trials (Figure 26A; time x trial interaction; $p = 0.84$), albeit total 16S DNA concentrations were 13% lower throughout the GLN trial (trial; $p = 0.04$). The EHST had no influence on total 16S DNA concentration (pre: GLN = 3.56 ± 0.74 $\mu g \cdot ml^{-1}$; PLA = 4.00 ± 1.05 $pg \cdot \mu l^{-1}$; post: GLN = 3.38 ± 0.40 $pg \cdot ml^{-1}$; PLA = 3.86 ± 0.43 $pg \cdot ml^{-1}$). There was no difference in the Δ total 16S DNA response between the GLN (-0.18 ± 0.78 $\mu g \cdot ml^{-1}$) and PLA (-0.14 ± 1.33 $\mu g \cdot ml^{-1}$) trials ($p = 0.95$). *Bacteroides*/total 16S DNA ratio was unchanged ($p = 0.34$) from pre- (GLN = 0.07 ± 0.07; PLA = 0.02 ± 0.05) to post-EHST (GLN = 0.05 ± 0.09; PLA = 0.05 ± 0.05) in both trials (Figure 26B; time x trial interaction; $p = 0.37$). The Δ *Bacteroides*/total 16S DNA ratio was comparable between the GLN (-0.04 ± 0.11) and PLA (0.03 ± 0.08) trials ($p = 0.45$). 1 participant was removed from all *Bacteroides*/total 16S DNA analysis as an outlier. *Bacteroides* concentrations were below the limit of detection in 24/40 samples (ratio data presented as zero).

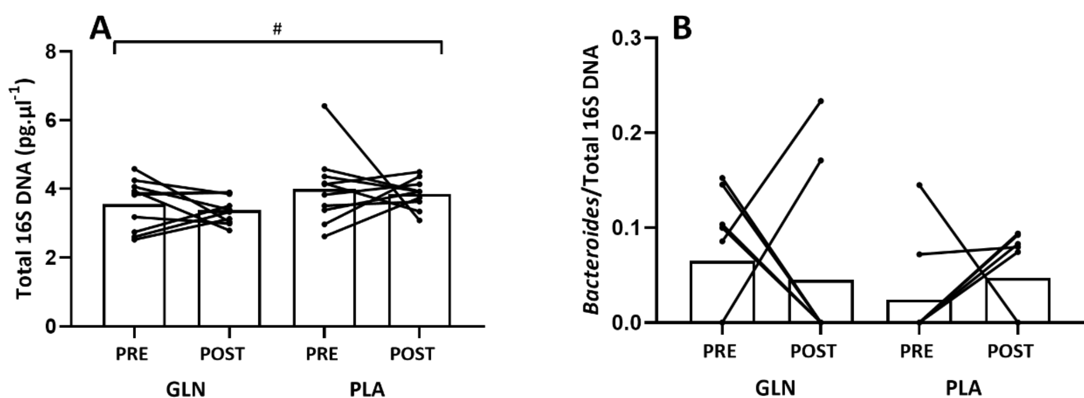

Figure 26. GI MT responses to EHSTs: (A) = total 16S DNA; (B) *Bacteroides*/total 16S DNA ($n = 9$). Significant overall effect of trial (# $p \leq 0.05$).

Associations

Associations between GI (DSAT, Δ I-FABP, Δ *Bacteroides*/total 16S DNA) and whole-body (peak T_{core}, mean HR, mean RPE) responses were conducted for the entire dataset ($n = 20$). The DSAT did not correlate with either I-FABP (Δ $r = 0.25$, $p = 0.46$; peak $r = 0.23$, $p = 0.50$) or *Bacteroides*/total 16S DNA responses (Δ $r = -0.19$, $p = 0.60$; peak $r = 0.03$, $p = 0.94$). A small positive association was evident between Δ I-FABP and Δ *Bacteroides*/total 16S DNA ($r = 0.61$, $p = 0.05$). No associations were evident between Δ GI and whole-body responses.

Table 34. Incidence and severity of gastrointestinal symptoms during the EHST.

	Pre		10 mins		20 mins		End	
Symptom	Incidence (%)	Severity	Incidence (%)	Severity	Incidence (%)	Severity	Incidence (%)	Severity
Gut Discomfort								
GLN	10	1 (1-1)	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)
PLA	0	0 (0-0)	10	1 (1-1)	0	0 (0-0)	0	0 (0-0)
Total GI symptoms								
GLN	30	1 (1-1)	10	3 (3-3)	50	6 (2-24)	40	9 (5-13)
PLA	10	1 (1-1)	40	3 (1-3)	40	4 (2-7)	40	10 (2-18)
Upper GI symptoms								
GLN	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)
PLA	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)
Lower GI symptoms								
GLN	10	1 (1-1)	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)
PLA	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)	0	0 (0-0)
Nausea								
GLN	10	1 (1-1)	0	0 (0-0)	40	5 (4-5)	40	5 (4-5)
PLA	0	0 (0-0)	0	0 (0-0)	20	4 (2-6)	30	7 (6-8)

Total incidence (%) of participants with reported symptoms ≥ 1 on the mVAS and summative accumulated severity of symptoms where reported (excluding data of no reported symptoms).

8.5 Discussion

The aim of this study was to determine the influence of low-dose (0.30 g·kg·FFM^{-1}) acute oral GLN supplementation on GI barrier integrity and MT biomarkers in response to a high-intensity short-duration EHST to exhaustion. This protocol has ecological relevance to situations where EHS arises in occupation settings, whilst the nutritional intervention has fewer practical limitations than previously recommended protocols (Zuhl et al., 2015). The main findings were that acute low-dose oral GLN ingestion worsened GI permeability (serum DSAT) compared to PLA, but small intestinal epithelial injury (I-FABP concentration) was similar between the 2 trials. There was no evidence of increased GI MT (*Bacteroides*/total 16S DNA ratio) following the EHST in either the GLN or PLA trial. The GLN bolus was well-tolerated, with few adverse subjective GI symptoms and comparable whole-body physiological (e.g. T_{core}, heart rate) responses reported across both trials. Thus, these data suggest no benefit of low-dose (0.30 g·kg·FFM^{-1}) acute oral GLN supplementation, or even detrimental effects, on GI barrier integrity and MT in response to exertional-heat stress.

I-FABP is a high-sensitivity biomarker of small intestinal epithelial injury, whereas the DSAT assesses functional GI permeability (Bischoff et al., 2014). In the present study, overall mean Δ I-FABP (0.88 ng·ml^{-1} [57%]) and absolute DSAT (L/R = 0.034 ± 0.019) responses were comparable in the PLA trial to previous research in this thesis that adopted a low-intensity (6 km·h^{-1}; 7% incline) 80-minute EHST in a 35 °C (25% RH) environment (e.g. chapter 4: I-FABP = Δ 0.83 ng·ml^{-1} [53%]; DSAT = 0.028 ± 0.012). The participant demographic (e.g. $\dot{V}O_{2max}$ = ~50 ml·kg·min^{-1}), analytical methodology, and severity of physiological strain (e.g. peak T_{core} = ~38.5-39.0°C) were all comparable between these studies. Taken together, these data suggest that the range of exercise intensities and durations used in these trials have little influence on GI barrier integrity, when confounding variables are tightly controlled. Whilst the small increase in I-FABP in the present study is comparable to other high-intensity, short-duration (20-40 minute) exercise protocols (e.g. Barberio et al., 2015 [Δ 0.30 ng·ml^{-1}; 46%]; March et al., 2017 [Δ 0.35 ng·ml^{-1}; 61%]), a greater increase in GI permeability *circa* 200% was anticipated with this form of exercise (e.g. Marchbank et al., 2011; Davison et al., 2016; March et al., 2017; [DSAT = ~0.09 - 0.11]). Despite widespread suggestions that prolonged duration (≥2 hours), moderate-intensity (~60% $\dot{V}O_{2max}$) exertional-heat stress initiates the greatest disruption of GI barrier integrity,

virtue of a 200-400% increase in plasma I-FABP concentration, typically these protocols have little influence on downstream GI permeability (e.g. Snipe et al., 2018a, 2018b; Pugh et al., 2019). Surprisingly, no individual randomised-control trial has examined the independent effects of exercise intensity and duration on GI barrier integrity, whilst controlling whole-body physiological strain (e.g. peak T_{core}), which is consequently worthy of further research.

Acute oral GLN supplementation at doses *circa* 0.90 $g{\cdot}kg{\cdot}FFM^{-1}$ is a proposed nutritional countermeasure to support GI barrier integrity during exertional-heat stress (Ogden et al., 2020). Despite previous favourable evidence on this topic, notably a ~40-50% reduction in GI permeability and ~25% reduction in epithelial injury following 1-hour of moderate intensity (70% $\dot{V}O_{2max}$) exertional-heat stress (Zuhl et al., 2015; Pugh et al., 2017b), this supplementation regimen has potential practical limitations, including poor GI tolerance (Ward et al., 2003; chapter 6). In comparison, GLN doses *circa* 0.30 $g{\cdot}kg{\cdot}FFM^{-1}$ are largely well tolerated (Ziegler et al., 1990; Ward et al., 2003) and still favourably improve GI barrier integrity in clinical care settings (Shu et al., 2016). However, contrary to the *a priori* hypothesis, the present study found an acute 0.30 $g{\cdot}kg{\cdot}FFM^{-1}$ bolus of GLN ingested 1-hour before running at LT pace to exhaustion in the heat (40 °C/40% RH), increased GI permeability (serum DSAT) by 26% and had no influence on small intestinal epithelial injury, when compared to a non-calorific PLA supplement. This finding supports the data presented in chapter 7, that low-dose (0.30 $g{\cdot}kg{\cdot}FFM^{-1}$) GLN supplementation negatively impacts GI barrier integrity, when consumed 1-hour before exertional-heat stress. The mechanism underpinning the negative influence of GLN supplementation on GI barrier integrity in the present thesis, in comparison to previous positive research in this field, has previously been discussed in detail in section 7.5. Briefly, low-dose acute-GLN supplementation was either insufficient to upregulate key physiological pathways (e.g. I-HSP biosynthesis) that protect GI barrier integrity during exertional-heat stress; or aspects of the experimental design (e.g. influence of non-isocaloric placebo supplement on probe absorption with the serum DSAT) attenuated true experimental effects.

Bacterial DNA is an emerging biomarker to assess GI MT (Paisse et al., 2016), which negates commonplace analytical issues relating to traditional assessment of plasma endotoxin (March et al., 2019). In the present study, *Bacteroides*/total 16s DNA concentrations were stable over time across both trials, although, many samples (24/40)

were below the assays sensitivity of analysis (hence the high duplicate CV). Previous research from this thesis (chapters 4, 5 and 7) and other laboratories (March et al., 2019) have previously reported comparable basal *Bacteroides*/total 16S DNA ratios (~0 – 1.0) as herein, though the proportion of samples with *Bacteroides* DNA concentrations below the assay sensitivity was significantly lower than present. In response to exertional-heat stress, March et al. (2019) found the *Bacteroides*/total 16S DNA ratio tended to increase though statistically insignificantly (p = 0.07) by ~65% (placebo arm) directly following 1-hour moderate intensity (70% $\dot{V}O_{2max}$) treadmill running in the heat (30 °C/60% RH). Similarly, in chapters 5 and 7, *Bacteroides*/total 16S DNA ratio increased following an 80-minute fixed-intensity EHST in recreationally trained individuals. Based on reported findings to date, future developments in bacterial DNA methodology are warranted to reduce inter- and intra-individual heterogeneity in biomarker concentrations. Considerations for such development includes: (1) assessment in whole blood samples, whereby GI microbial DNA concentrations are several magnitudes greater than plasma (Paisse et al., 2016); and (2) simultaneous assessment of other major GI microbial phyla (Thursby and Juge, 2017).

Though research has previously demonstrated acute GLN supplementation to maintain GI barrier integrity in response to exertional-heat stress, there is no evidence that these functional benefits translate into reduced downstream GI MT. A key aim of the present study was to address this gap in the literature, hence the selection of a high-intensity EHST previously proven to induce GI MT (Shing et al., 2013; Barberio et al., 2015). However, inconsistent with the *a priori* hypothesis, the *Bacteroides*/total 16S DNA ratio was not different between the GLN and PLA trials. This finding was perhaps unsurprising given the lack of influence of GLN on small intestine epithelial injury, which was positively associated with the *Bacteroides*/total 16S DNA ratio in both present study and in previous research (March et al., 2019). Concordant with the present findings, previous studies examining the influence of GLN supplementation on GI barrier responses following exertional-heat stress, were also unable to induce GI MT, measured by plasma endotoxin concentration, despite evoking a ~200% increase in GI permeability (Zuhl et al., 2015) and an ~83% increase in small intestinal injury (Osborne et al., 2019b). Likewise, in chapter 7, although *Bacteroides*/total 16S DNA concentrations increased by *circa* 150% following exertional-heat stress, GLN supplementation did not influence the magnitude of this response. In the present study, total 16S DNA concentrations exhibited a significant overall trial effect, where concentrations were 13% lower throughout the GLN trial. This response

was unexpected given that GLN simultaneously increased GI permeability and had no influence on the *Bacteroides*/total 16S DNA ratio. Whilst total 16S DNA concentrations have previously been suggested as a method to assess GI MT in clinical settings (Fukui, 2016), this analysis is potentially confounded by variables independent of GI MT (e.g. DNase concentrations) when applied in response to exercise (March et al., 2019). Therefore, despite previous evidence suggesting GLN supplementation to improve systemic microbial neutralisation capacity in clinical care patients (Shu et al., 2016), these benefits were not reproducible following high-intensity exertional heat stress.

8.6 Limitations

Despite execution of a tightly controlled methodological design, the present results were not without some limitations. First, the EHST only evoked moderate disturbance of GI barrier integrity and MT, likely attributable to only a moderate rise in T_{core} given all participants failed to complete the 30-minute EHST (Pires et al., 2017). This result was surprising given that critical internal temperature theory suggests humans reliably reach volitional exhaustion during fixed-intensity exercise at a T_{core} of ~40°C, independent of the rate of heat storage (Nybo and Gonzalez-Alonso, 2015). Conversely, rectal temperature might have underestimated T_{core} given the typical 5-10 minute lag in this measurement during high-intensity physical activities in comparison with central blood temperature (Teunissen et al., 2012). Second, a basal DSAT was not performed to minimise participants' time burden, with the overall aim of improving compliance. Consequently, this prevented direct assessment of the EHST on GI permeability. Third, females were excluded from participation due to unavailability of menstruation hormone testing. Previous evidence has shown no influence of sex on GI barrier integrity responses to exertional-heat stress (Snipe and Costa, 2018a). Finally, implementation of an isocaloric PLA or standardised pre-trial breakfast would have reduced concerns regarding the extraneous influence of macronutrient provision on GI barrier integrity. The decision to utilise a non-calorific PLA was selected to ensure consistency with previous studies in the field (Zuhl et al., 2014, 2015; Pugh et al., 2017b; Osborne et al., 2019b).

8.7 Conclusion

This study assessed the influence of low-dose (0.30 $g \cdot kg \cdot FFM^{-1}$) acute oral GLN supplementation on GI barrier integrity and MT biomarkers in response to a high intensity exertional heat stress test to volitional exhaustion. Unexpectedly, GI permeability assessed

via the serum DSAT was increased with GLN supplementation following the EHST, whereas I-FABP a biomarker of small intestinal epithelial injury was comparable between the 2 trials. These findings suggest that GI barrier integrity was potentially compromised with GLN supplementation. Downstream GI MT assessed via *Bacteroides*/total 16S DNA responses was unchanged in response to the EHST, and there was no significant impact of GLN supplementation. Total 16S DNA responses were 13% lower across the GLN trial, however, this finding should be treated with caution considering potential bias from other factors that may influence this biomarker independent of GI MT. The GLN bolus was well-tolerated, with few adverse subjective GI symptoms and comparable whole-body physiological responses reported across both trials. These findings do not support the use of acute low-dose oral GLN supplementation in occupational settings (e.g. military) to protect GI barrier integrity and prevent MT in response to high-intensity exertional-heat stress to volitional exhaustion.

Chapter 9 - General Discussion

9.1 Summary

Exertional-heat stroke (EHS) is a life-threatening medical emergency and most severe condition along a continuum of heat-related illnesses. The pathophysiology of EHS is incompletely understood, though growing research demonstrates gastrointestinal (GI) microbial translocation (MT) following structural barrier integrity disturbance to influence disease outcome. Despite this knowledge, there is minimal evidence to inform effective countermeasures against EHS that target this mechanistic pathway. Of the various countermeasures to have previously been examined, acute high-dose (0.90 $g \cdot kg \cdot FFM^{-1}$) oral $_L$-glutamine (GLN) supplementation appears one of the most promising. The aims of this thesis were to characterise the nature of GI barrier integrity responses to subclinical exertional-heat stress and confirm whether acute oral GLN supplementation is an effective nutritional countermeasure. This topic has applied relevance, including to the military, due to the high incidence of EHS in Defence personnel and limited options for mitigation strategies during field operations.

Various techniques are available to examine *in vivo* GI barrier integrity and MT. An important first step in informing experimental design was to determine technique reliability. Chapter 4 reported that the serum DSAT, I-FABP, CLDN-3, LBP and total 16S bacterial DNA had acceptable reliability when examined in response to exertional-heat stress. There are many intrinsic risk factors for EHS, including low aerobic fitness, but whether these risk factors relate to GI barrier integrity is poorly understood. Chapter 5 confirmed that individuals with low-aerobic fitness experience greater small intestinal epithelial injury and MT in response to exertional-heat stress than individuals with high-aerobic fitness. Acute oral GLN supplementation (0.90 $g \cdot kg \cdot FFM^{-1}$) is one potential nutritional countermeasure to protect GI barrier integrity, but the benefits of this dose has been questioned due to associated adverse GI symptoms. In chapter 6, acute oral GLN supplementation (0.30, 0.60, 0.90 $g \cdot kg \cdot FFM^{-1}$) caused mild dose-dependent GI symptoms that subsided by 4 hours post-prandial. Applying this knowledge, experimental studies presented in chapters 7 and 8 examined whether a lower dose of GLN (0.30 $g \cdot kg \cdot FFM^{-1}$) conferred similar benefits as previously validated doses on GI barrier integrity following exertional-heat stress. Acute low-dose oral GLN in fact worsened GI barrier integrity during both low- and high-intensity exertional heat stress.

This thesis has provided novel and important insights regarding: (1) the reliability of commonly applied markers used in the assessment of how GI barrier integrity; (2) intrinsic and extrinsic risk factors for GI barrier integrity loss in response to exertional heat stress; and (3) the practicality and effectiveness of acute oral GLN as a nutritional intervention to protect GI barrier integrity (Table 35). These findings have important implications for policy, practice and the direction of future research in this field.

Table 35. Primary study hypotheses and outcomes.

Chapter	Primary Hypothesis	*Accept/Reject*
4	Biomarkers of GI barrier integrity and microbial translocation would display acceptable test-retest reliability in response to exertional-heat stress.	*Accept*. The DSAT, I-FABP, CLDN-3, LBP and total 16S DNA all had acceptable test-retest reliability, although *Bacteroides*/total 16S DNA ratio had unacceptable test-retest reliability.
5	Highly trained individuals would experience elevated GI barrier integrity loss, but reduced GI microbial translocation in comparison to untrained individuals in response to exertional-heat stress.	*Reject*. Untrained individuals experienced elevated GI barrier integrity loss (I-FABP) and GI microbial translocation (*Bacteroides*/Total 16S DNA) compared to highly trained individuals.
6	L-glutamine supplementation would induce GI symptoms in a dose-dependent manner.	*Accept*. 0.9 $g \cdot kg \cdot FFM^{-1}$ L-glutamine caused greater 24-hour upper-, lower- and total-GI symptoms than either 0.3 or 0.6 $g \cdot kg \cdot FFM^{-1}$ L-glutamine. 0.6 $g \cdot kg \cdot FFM^{-1}$ L-glutamine induced greater 24-hour lower-GI symptoms than 0.3 $g \cdot kg \cdot FFM^{-1}$.
7	L-glutamine supplementation would protect GI barrier integrity and reduce microbial translocation following exertional-heat stress.	*Reject*. L-glutamine supplementation increased GI barrier integrity loss (DSAT and I-FABP) and had no influence on GI microbial translocation. L-glutamine supplementation was well-tolerated with adverse GI symptoms absent for all participants.
8	L-glutamine supplementation would protect GI barrier integrity and reduce microbial translocation following high-intensity exertional-heat stress.	*Reject*. L-glutamine supplementation increased GI barrier integrity loss (DSAT) and had no influence on GI microbial translocation. L-glutamine supplementation was well-tolerated with similar GI symptoms compared with placebo supplementation.

9.2 Gastrointestinal Barrier Integrity Assessment

Several techniques are available to assess *in vivo* GI barrier integrity. These can be broadly categorised as either: (1) *active* tests (i.e. GI permeability), based upon the extracellular recovery of orally ingested inert probes; or (2) *passive* tests (i.e. GI injury) based on monitoring extracellular GI specific biomarkers. In this thesis, multiple GI barrier integrity and MT techniques were examined, with the intention of providing data on the nature of GI barrier integrity (Grootjans et al., 2010). Previous work has typically demonstrated only weak associations between techniques when examined simultaneously in response to exertional-heat stress (van Wijck et al., 2011a; Yeh et al., 2013; Sessions et al., 2016; March et al., 2017, 2019). Similarly, throughout this thesis, a lack of positive association was consistently found between any two techniques. Plausibly, this finding is explained by inconsistencies in: biomarker kinetics; regional variability in GI barrier integrity loss; non-GI mediated cross-contamination (e.g. immune/hepatic function); analytical sensitivity; and analytical reliability. Likewise, none of the experimental chapters were primarily designed to characterise the association between biomarkers, therefore null findings might be attributed to a type 2 error virtue of a lack of statistical power. Many investigators in the field of exercise physiology are limited by logistical constraints that seldom permit multi-biomarker assessment. This thesis was able to highlight new perspectives on the strengths and weaknesses of several popular GI barrier integrity and MT assessment techniques, which should help to inform researchers when designing future research on this topic.

The dual-sugar absorption test (DSAT) is the gold-standard active GI functional test (Galipu and Verdu, 2016) and has received almost exclusive application in exercise physiology research (Costa et al., 2017). Although the test traditionally involves measurement in urine over a 5-hour period following sugar probe ingestion, recent advancements in analytical sensitivity have permitted assessment in blood plasma/serum over a reduced 1-2.5 hour time-course (Fleming et al., 1996; van Wijck et al., 2012b). In previous exercise studies, the serum DSAT offered improved sensitivity in detecting mild GI barrier integrity loss in comparison to the urine DSAT (van Wijck et al., 2011a; Janssen-Duijghuijsen et al., 2017a; Pugh et al., 2017a). However, given the transient kinetics of sugar probe appearance in blood and low absolute lactulose concentrations, questions remained pertaining to the reliability of the technique and optimal time-point for blood collection.

Chapter 4 assessed the reliability of the serum DSAT both 90- and 150- minutes following sugar-probe ingestion that occurred 10-minutes into a 100-minute EHST. From the results it was evident that the serum DSAT, had similar reliability at both time-points to that previously observed for the 5-hour urine DSAT test (van Elburg et al. 1995; Marchbank et al., 2011). The most valid time-point for blood collection with the serum DSAT was unresolved in chapter 4, given that a simultaneous urine-DSAT was not undertaken; this was due to logistical challenges regarding participant recruitment and adherence. However, it was highlighted that the test-rest reliability of both blood collection time-points were similar, and in future selection can be made based on logistical convenience.

Inconsistent with the primary hypothesis, chapters 7 and 8 reported GLN supplementation to aggravate GI permeability using the 90-minute serum DSAT. One unforeseen issue with the application of the serum DSAT in these studies was the potential extraneous influence of GLN on gastric emptying rate compared to a non-isocaloric placebo. This potential issue could be resolved by applying a 5-hour urine DSAT, where alterations in probe kinetics can be controlled by accumulative (e.g. area under the curve) biofluid collection (Bjarnason et al., 1995). To promote participant adherence, none of the studies in this thesis performed basal DSATs, and this prevented comparison of this technique against others for sensitivity in response to exertional-heat stress.

Intestinal-fatty acid binding protein (I-FABP) is the only tissue specific biomarker of small-intestinal epithelial injury (Wells et al., 2017). Its popularity has increased exponentially since initial application in exercise physiology research, which is largely due to reduced logistical constraints compared to the DSAT (van Wijck et al., 2011a). Throughout this thesis, absolute I-FABP responses were at the high-end of the healthy reference range (e.g. 0.1-2.0 ng·ml^{-1}) (Treskes et al., 2017). Within the literature, this variability in I-FABP concentrations is thought to be explained by discrepancies in ELISA assays produced by individual manufacturers (I-FABP antibody, wash procedure). On this basis, it is recommended for clarity of data reporting that researchers present both the absolute (Δ) and relative (%) change in I-FABP concentrations. In chapter 4, the reliability thresholds for I-FABP were defined relative to exertional-heat stress and considered to be acceptable. In chapters 5 and 7, I-FABP was sufficiently responsive to detect the impact of aerobic fitness and low-dose oral GLN supplementation on GI integrity in response to exertional-heat stress, which was not detected using the serum DSAT. However, the clinical

relevance of subtle, transient changes in I-FABP concentration – especially in situations where GI permeability is unchanged – is poorly understood. Assessment of the sensitivity and specificity of I-FABP in predicting morbidity and mortality from EHS is an important next step for understanding the results of sub-clinical exertional heat stress research.

Claudin-3 (CLDN-3) is a pore-sealing tight junction (TJ) protein located predominately in GI tissue (Marcov et al., 2010). Despite initial introduction as a valid biomarker of GI TJ integrity in 2009 (Thuijls et al., 2009), the healthy reference range for plasma CLDN-3 has not been consistently characterised (Osborne et al., 2019a). In chapter 4, basal CLDN-3 concentrations ranged between 0.9-14.3 $ng \cdot ml^{-1}$, which was comparable (0.8-12.6 $ng \cdot ml^{-1}$) to that previously reported by Yeh et al. (2013). In response to exertional-heat stress, CLDN-3 concentrations remained relatively stable, increasing by *circa* 10-15%, which is in agreement to Yeh et al. (2013) who reported a 24% increase in CLDN-3 following 1-hour of moderate-intensity (70% $\dot{V}O_{2max}$) running in the heat (33 °C/50% RH). This stability of CLDN-3 likely explains its strong reliability reported in chapter 4. Histological tissue expression of the TJ proteins occludin and zonula-occludin-1 have been shown to strongly predict GI paracellular permeability, therefore examination of systemic concentrations of these proteins using recently developed ELISA analysis warrants future investigation as an alternative to claudin-3 (Assimakopoulos et al., 2019).

MT defines the migration of live bacteria or their products from the GI lumen into the circulating blood (Fukui, 2016). Endotoxin is the hydrophobic domain of lipopolysaccharides located on the outer membrane of gram-negative bacteria (Gnauck et al., 2016). All previous evidence characterising the GI EHS paradigm has relied on endotoxin as a biomarker to examine GI MT. Despite widespread application, endotoxin assessment has well-publicised analytical limitations (Gnauck et al., 2016). These include: frequent exogenous contamination during sample collection/analysis; and rapid hepatic detoxification from portal blood before passage into the systemic circulation. Based on these factors, endotoxin has a wide universal healthy reference-range (0.15-61 $EU \cdot ml^{-1}$; Gnuack et al., 2016), whilst the biomarker's poor test-retest reproducibility (basal CV = 24%; Guy et al., 2016) likely explains its inconsistent response to exertional-heat stress (Table 9).

In chapter 4, endotoxin examination using the chromogenic LAL assay was curtailed given unphysiologically high values reported during analysis, which were indicative of exogenous contamination. As such, attention was instead given to the application of a novel bacterial 16S DNA methodology developed by March et al. (2019). This technique involves extraction of plasma cell free DNA and subsequent amplification of *Bacteroides* target genes through real-time quantitative PCR. Major advantages of this novel technique include: minimal hepatic bacterial DNA removal making assessment in systematic blood feasible (Mortensen et al., 2013); low basal plasma concentrations (Paisse et al., 2016); and improved specificity via correction for non-GI mediated factors (March et al., 2019). However, despite the proposed benefits of the method, plasma *Bacteroides* concentrations were low and sometimes undetectable during PCR analysis (sensitivity = 1 copy·μl^{-1}) in each chapter of this thesis. The low concentrations explain the poor test-retest reliability in chapter 4, and poor analytical reliability of duplicate samples across all chapters. From chapter 5 onwards, prior to DNA extraction, the elution buffer was heated to 65 °C to enhance extraction concentrations. Whilst this methodology increased absolute total 16S DNA concentrations approximately 2-3 fold, it did not consistently increase *Bacteroides* concentrations above the assays limit of detection. Based on these findings, future development of this methodology might improve reliability by: (1) DNA extraction from whole blood samples, where microbial DNA concentrations are several magnitudes greater than plasma; (2) simultaneous assessment of *Firmicutes* DNA, which are the second dominant GI microbial phyla ; and (3) biochemical assessment in triplicate (Paisse et al., 2016). In comparison to *Bacteroides* responses, total 16S DNA and LBP offered acceptable reliability in chapter 4. However, total 16s DNA and LPB appeared unresponsive to the exertional-heat stress tests employed throughout this thesis. Whether these biomarkers would have been more responsive to more severe exertional-heat stress remains to be determined.

9.3 Intrinsic and Extrinsic Risk Factors

Various intrinsic (e.g. age, sex) and extrinsic (e.g. clothing, WGBT) risk factors predispose military personnel to EHS (Westwood et al., 2020). GI barrier integrity loss potentially underpins some of these risk factors (Lim, 2018; Armstrong et al., 2018). Despite this, evidence to support these claims in humans is unavailable, due to ethical constraints of examining EHS in the laboratory. Instead, the evidence base is currently limited to characterising associations between EHS risk factors from epidemiological studies and biomarkers of GI barrier integrity during subclinical laboratory randomised-controlled trials. This thesis reports supplementary data on the impact of aerobic fitness, whole-body physiological strain, and the intensity/duration of exertional-heat stress on GI barrier integrity. Knowledge of these risk factors should help develop strategies to monitor (e.g. body composition) and mitigate (e.g. healthy weight management) this risk in the field (Moore et al., 2015).

Low aerobic fitness, typically classified by poor performance (e.g. failure) in a recent mandatory fitness test, is well-demonstrated to increase the odds-ratio of EHS in military personnel by 2-8 fold (Wallace et al., 2006; Moore et al., 2016; Nelson et al., 2018). The mean estimated aerobic fitness of serving British Armed Forces personnel in ground close combat roles is typically 50 ± 5 $ml \cdot kg \cdot min^{-1}$ (Lunt et al., 2013; Fallowfield et al., 2014, 2018). The UK Ministry of Defence (Military Headquarters of the Surgeon General, 2019) and international North Atlantic Treaty Organisation (Spitz et al., 2012) doctrine endorse an individual risk assessment for unfit personnel to determine ability for high risk activities. Nevertheless, the development of targeted interventions that support this demographic has clear ecological relevance. Prior to this thesis, equivocal evidence found low-aerobic fitness to either strengthen (Selkirk et al., 2008) or weaken (Morrison et al., 2014) GI barrier integrity during exertional-heat stress. In chapter 5, highly trained individuals ($\dot{V}O_{2max}$ > 60 $ml \cdot kg \cdot min^{-1}$; ≥ 3 $h \cdot week^{-1}$ aerobic training) did not experience GI barrier integrity disturbance to a fixed-intensity EHST, whilst untrained individuals ($\dot{V}O_{2max}$ < 50 $ml \cdot kg \cdot min^{-1}$; ≤ 3 $h \cdot week^{-1}$ aerobic training) displayed a *circa* 2-fold increase in I-FABP and *Bacteroides*/total 16S DNA concentrations. One limitation of this study was the implication of a fixed-intensity EHST that induced unequal physiological strain between the two fitness cohorts. However, this experimental design had clear ecological relevance, given that group-paced occupational activities are a frequent context for EHS in the military (Epstein

et al., 1999; Stacey et al., 2015). A comparably untrained participant cohort to chapter 5 was recruited for chapters 7 and 8, who again developed a similar magnitude of GI integrity loss in response to exertional-heat stress. Together, these findings support previous epidemiological EHS research, suggesting that low-aerobic fitness is a risk factor for GI barrier integrity loss during exertional-heat stress that requires careful management.

Since introduction of the GI EHS paradigm in 1993 (Gisolfi, 1993), the impact of thermal strain (e.g. elevated T_{core}) on GI barrier integrity has been a specific topic of investigation. Numerous randomised-controlled trials report matched-intensity exercise in a hot (T_{amb} ≥ 30 °C) *versus* temperate (T_{amb} ≤ 22 °C) environment, induces a greater magnitude of GI barrier damage, measured via either plasma I-FABP, CLDN-3 and endotoxin concentration (Yeh et al., 2013; Snipe et al., 2018a, 2018b; Osborne et al., 2019a). A systematic review conducted in 2016 on this topic found a tight positive association (r = 0.79) between T_{core} and GI barrier integrity (Pires et al., 2017). Furthermore, T_{core} thresholds of 38.6 °C and 39.0 °C were proposed for commonplace (50% incidence) and universal (100% incidence) GI barrier integrity damage, respectively. In comparison to Pires et al. (2017), inclusion of recent data on this topic up to February 2020 presented in Tables 7, 8 and 9 of chapter 2 (Ogden et al., 2020a), report T_{core} to be only weakly associated with I-FABP (Δ; r = 0.52; p = <0.001), and not associated with DSAT (5-h urine only; r = 0.30; p = 0.19) or endotoxin (Δ; r = 0.14; p = 0.56) responses. Though not a primary aim of this thesis, correlational analysis was performed between T_{core} and GI barrier integrity in chapters 5, 7 and 8. In chapter 5, T_{core} was weakly associated with I-FABP (r = 0.42; p = 0.06) and *Bacteroides*/total 16S DNA (r = 0.53; p = 0.02). However, no positive associations were evident between any T_{core} and any GI biomarker in later chapters. Given the small homogenous sample population in these chapters, these conclusions probably lacked statistical power. In comparison, the DSAT was insensitive to detect mild subclinical variations in T_{core} throughout this thesis, though as previously reported, no basal corrections were performed for this measure. Future research with appropriate statistical power should look to clarify the influence of separate whole-body physiological variables on GI barrier integrity.

The independent effects of exercise intensity and duration on GI barrier integrity have never been directly assessed. However, conclusions may be drawn by pooling data from individual studies (Tables 7, 8 and 9). I-FABP generally increases by ≤ 100% following

short-duration (≤ 1 hour) exertional-heat stress, irrespective of exercise intensity (40-80% $\dot{V}O_{2max}$; e.g. Barberio et al., 2015; Szymanski et al., 2017). In comparison, moderate-intensity (60% $\dot{V}O_{2max}$) exertional-heat stress of durations ≥ 1.5 hours trigger large increases in I-FABP *circa* 200-500% (e.g Snipe et al., 2018a, 2018b; Pugh et al., 2019). In contrast with these responses, downstream GI permeability (DSAT) is largely unchanged in response to 1-2 hours of moderate-intensity (60% maximal oxygen uptake) exercise (Pals et al., 1997; Lambert et al., 2008), but increases by 150-200% following higher intensity exercise (≥ 70% $\dot{V}O_{2max}$) of ≥ 20 minutes duration (e.g. Marchbank et al., 2011; Davison et al., 2016). In comparison to assessment of GI barrier integrity, GI MT responses to exertional-heat stress are less consistent, which is likely a reflection of the methodological constraints associated with endotoxin analysis.

Variability in biomarker responses to exertional-heat stress is a challenge for investigators to manage when designing experiments to balance the conflicting constraints of maximising GI barrier disturbance and ensuring participant adherence and safety. In chapters 4 and 5, an 80-minute EHST designed to have ecological validity in military settings was adopted. This protocol induced a peak T_{core} of 39.07 ± 0.29 °C during initial pilot work (*n* = 6), which subsequently caused a ~2-fold increase in GI permeability above rest (90-minute serum DSAT = 0.014 ± 0.006 to 0.028 ± 0.005). However, a limitation of applying this EHST in later chapters was the inability to consistently elevate T_{core} above 39.0°C, which likely contributed to the only mild disturbance of GI barrier integrity. Chapter 8 therefore adopted a high-intensity EHST utilised by Barberio et al. (2015), which had previously been associated with a peak T_{core} > 39 °C and induced significant GI MT. However, unlike Barberio et al. (2015), 80% of participants reached volitional exhaustion at a T_{core} < 39 °C in chapter 8. This finding is inconsistent with widely cited theory that humans reliably terminate fixed-intensity submaximal exercise at a T_{core} of ~40 °C irrespective of the rate of heat storage (Nybo and Gonzalez-Alonso, 2015). Explanations for this early termination of trials in chapter 8 might relate to the previous inexperience of participants in performing vigorous exertional-heat stress (Flouris and Schlader, 2015) and/or from metabolic fatigue caused by a suppression of lactate threshold with high T_{amb} (Lorenzo et al., 2011). Given that the magnitude of thermal strain observed in chapter 8 was comparable to all previous chapters, both low-intensity and high-intensity exercise would appear to damage the GI barrier comparably. A future randomised controlled trial is recommended to compare the

influence of exercise intensity/duration on GI barrier integrity whilst tightly controlling whole-body physiological strain (e.g. T_{core} peak, total power output).

9.4 Nutritional Intervention

Investigation of nutritional interventions to support GI barrier integrity during exertional-heat stress has been a rapidly expanding area of research. An early focus of this thesis was to synthesise the current body of evidence on this topic to identify the most promising strategies for potential future application in military personnel (Ogden et al., 2020). At least one randomised-control trial for each strategy was found to support the use of carbohydrates, bovine colostrum, nitric oxide precursors ($_{L}$-citrulline, inorganic nitrate), probiotics, polyphenols, or zinc carnosine to protect GI barrier integrity during sub-clinical exertional-heat stress. Of these strategies, a single acute dose of GLN (0.90 g·kg·FFM^{-1}) consumed 1-2 hours before exertional-heat stress offered clear protection of small intestinal epithelial injury (I-FABP) and GI permeability (DSAT) in three separate studies (Zuhl et al., 2015; Pugh et al., 2017b; Osborne et al., 2019b). Furthermore, this nutritional intervention appeared to present few challenges to wide-scale, real-world applications. For example, GLN is widely available, inexpensive, lightweight, non-perishable, vegan and potentially only requires acute supplementation to deliver meaningful physiological benefits.

Supplementation with 0.90 g·kg·FFM^{-1} of GLN, 1-2 hours before exertional-heat stress, had previously been shown to be effective in protecting GI barrier integrity. However, during pilot work for chapter 7, this dose was shown to cause vomiting in 2 of 3 participants. This finding was surprising, given that Pugh et al. (2017b) and Osborne et al. (2019b) had both previously reported this dosage to be well-tolerated in comparison to a non-calorific placebo, when examined immediately following exertional-heat stress. Unfortunately, these studies did not assess GI symptoms either before or during exertional-heat stress, whereby there is potential that symptoms had reduced by the point of assessment. Given that GI symptoms are widely documented to hinder exercise performance (Hoffman and Fogard, 2011), and might potentially increase EHS risk if vomiting/diarrhoea cause dehydration (Westwood et al., 2020), chapter 6 was designed to assess the time-course of potential GI symptoms up to 24-hours following ingestion of low (0.30 g·kg·FFM^{-1}), medium (0.6 g·kg·FFM^{-1}) and high (0.90 g·kg·FFM^{-1}) GLN doses. The main findings of chapter 6 were that GLN supplementation caused dose-dependent GI symptoms

(e.g. gut discomfort, belching, nausea) over the first 2-hours post-prandial. These symptoms were classified as mild in all except one participant, who returned a score of 5/10 for *belching* and *urge to regurgitate* during the first 2-hours postprandial on the high dose. Based on these data, it is recommended that GLN supplementation is administered either in a smaller dose (~0.30 g·kg·FFM^{-1}) or earlier (> 2 hours) before exertional-heat stress, to minimise the risk of adverse GI symptoms on occupational performance. In circumstances where a larger GLN dose might be considered preferential, GI tolerance should be thoroughly examined on an individual basis alongside habitual diet prior to implementation during high-risk activities. A limitation of chapter 6 was the inability to monitor routine clinical biochemistry and waste-products of GLN metabolism (e.g. ammonia). This analysis would have been useful to establish the safety of acute oral GLN supplementation, which has never been rigorously examined for doses exceeding 0.30 g·kg·body mass^{-1} (Ziegler et al., 1990).

In consideration of both the conclusions drawn from chapter 6 and the logistical constraints of supplying nutritional supplements across large military populations (Parsons et al., 2019), chapter 7 aimed to determine the influence of supplementing with 0.30 g·kg·FFM^{-1} of GLN on GI barrier integrity when ingested 1-hour before exertional-heat stress. To expand on previous evidence where 0.90 g·kg·FFM^{-1} of GLN was shown to protect GI permeability and small intestinal epithelial injury in response to a 1 hour of moderate intensity (70% $\dot{V}O_{2max}$) EHST (Zuhl et al., 2015; Pugh et al., 2017b), chapter 7 aimed to: (1) extend the portfolio of analysis to include biomarkers of downstream MT (*Bacteroides*/total 16S DNA); and (2) prevent re-hydration during exertional-heat stress with the aim to increase the severity of GI barrier integrity disturbance (Costa et al., 2019). However, in contrast to earlier research on this topic by Zuhl et al. (2015), Pugh et al. (2017b) and Osborne et al. (2019b), chapter 7 surprisingly found GLN supplementation to increase small intestinal epithelial injury and GI permeability by 85% and 30% respectively, in comparison to the placebo trial. An explanation for this null result is not forthcoming given that GLN supplementation has only previously been shown to upregulate pathways that strengthen GI barrier integrity. Likewise, due to ethical constraints of collecting intestinal biopsy samples in healthy humans, it was not possible to collect data to examine any potential mechanistic responses further. The clinical meaningfulness of these null results is unclear, though given MT was uninfluenced by GLN supplementation, the likelihood that GLN supplement in fact increases the risk of EHS appears low.

To further investigate the surprising finding that GLN supplementation increased GI barrier integrity loss in response to exertional-heat stress, chapter 8 was undertaken as a replication study, but instead applying a high-intensity exhaustive exertional-heat stress test previously shown to induced measurable MT (Barberio et al. 2015). In agreement with chapter 7, GI permeability was again increased by 24% in the GLN trial when compared to the placebo trial, but in this chapter did not influence small-intestinal epithelial injury. Together, chapters 7 and 8 do not support the use of acute low-dose oral GLN supplementation to protect GI barrier integrity and prevent MT in response to either low-intensity or high-intensity exertional-heat stress. One hypothetical limitation of this null conclusion, is the potential that GI permeability measured at a single-time point using the serum DSAT could have been a type 2 error attributable to the application of non-isocaloric nutritional interventions that influenced the kinetics (e.g. unbalanced gastric emptying) of lactulose appearance in the blood (Sequeira et al., 2014). To this point, it could be speculated that altering the time-point of blood serum collection around probe-ingestion might have achieved a different outcome. Notwithstanding the null results presented in chapters 7 and 8, future research into the efficacy of acute GLN supplementation on GI barrier integrity in response to exertional-heat stress is still warranted. This is particularly pertinent given the previous body of favourable literature on this topic. Highlighted areas for future investigation might focus to address the following research questions.

First, *what is the optimal time-point to administer GLN around exertional-heat stress?* Previous beneficial studies had participants ingest the GLN supplement 2-hours before exertional-heat stress (Zuhl et al., 2015; Pugh et al., 2017b), whereas in this thesis the timing of GLN pre-ingestion was reduced to 1-hour concordant with Osborne et al. (2019b). No *in vitro* or *in vivo* study has previously examined the influence of GLN timing on GI barrier integrity under any form of stress. Mechanistically, GLN supplementation (0.75 g·kg·BM^{-1}) increases basal colonic I-HSP70 expression in rodents between 1-72 hours after supplementation (Wischmeyer et al., 2001), whilst in GLN deprived Caco-2 monolayers recovered the loss of GI TJ integrity inside 1-hour (DeMarco et al., 2003). Therefore, whilst the timing of GLN supplementation to exertional-heat stress test is unlikely responsible for the null findings reported in this thesis, potentially stronger results might be achieved in future if GLN is ingested earlier (2-72 hours) prior to exertional-heat stress.

Second, *what is the optimal dose of GLN to administer around exertional-heat stress?* When including the data presented in this thesis, GLN in doses ≤ 0.30 g·kg·FFM^{-1} appear ineffective in protecting GI barrier integrity in response to exertional-heat stress (Lambert et al., 2001; Pugh et al., 2017b). However, at a larger dose of 0.90 g·kg·FFM^{-1} beneficial results are consistently reported (Zuhl et al., 2015; Pugh et al., 2017b; Osborne et al., 2019b). Mechanistically, *in vitro* studies show GLN to increase intracellular I-HSP concentrations (Wischmeyer et al., 2001) and GI TJ protein expression (Seth et al., 2004) in a dose-dependent manner. Consequently, future research could re-focus on larger GLN doses, but supplemented ≥ 4 hours before exertional-heat stress to minimise the influence of adverse GI symptoms.

Third, *are the benefits of acute-oral GLN supplementation solely attributable to increased post-prandial splanchnic perfusion?* A limitation of the present and previous research on this topic was the initiation of trials following an overnight fast. However, it has since been hypothesised that feeding can protect GI barrier integrity during exertional-heat stress independent of nutritional composition (Snipe et al., 2017). Consequently, future research should look to examine whether the benefits of GLN supplementation are maintained when trials are conducted in the post-prandial state.

Fourth, *are the benefits of GLN supplementation homogenous?* Aerobic exercise training (Selkirk et al., 2008) and heat acclimation (Barberio et al., 2015) are believed to confer protective adaptations to the GI barrier, which might negate the potential benefit of GLN supplementation in such populations. If an effective and practical GLN supplementation intervention can be developed, it must be validated across larger heterogenous populations.

Finally, if the efficacy of GLN supplementation can be demonstrated in accordance with the abovementioned considerations, future prospective research is warranted in humans to examine the influence of GLN supplementation on overall EHS incidence, morbidity (e.g. multi-organ injury, cytokinemia), and mortality.

9.5 Continuation of the Gastrointestinal Paradigm

Historically, the pathophysiology of EHS was solely attributed to attaining a critical level of hyperthermia (Bouchama and Knochel, 2002). However, this model lacked sophistication and does not explain many of the clinical manifestations of EHS (Lim, 2018). For example, almost all EHS cases occur at a T_{core} below the threshold for cellular denaturation (< 43°C), whilst the magnitude of reported thermal tolerance is diverse (e.g. 39-43°C) at incapacitation (Laitano et al., 2019). Because of the significant health, financial and operational burden of EHS to populations like the UK Armed Forces, there is an ongoing requirement to better understand the pathophysiology of the disease and to improve management guidance (Parsons et al, 2019). Over the last two decades, the GI EHS paradigm has gained momentum as a supplementary pathway to explain associations between immune function and EHS morbidity (Figure 27). Throughout this thesis, various risk factors and countermeasures were presented that have previously been demonstrated to influence GI barrier integrity in response to exertional-heat stress (Table 36). Future prospective research should look to clarify the importance of GI barrier integrity on EHS prognosis and determine the efficacy of management policy that looks to prevent EHS virtue of influencing this pathophysiological pathway (Figure 27).

Figure 27. The Gastrointestinal Exertional-Heat Stroke Paradigm: An updated Model to Incorporate Identifiable Risk Factors and Modifiable Interventions

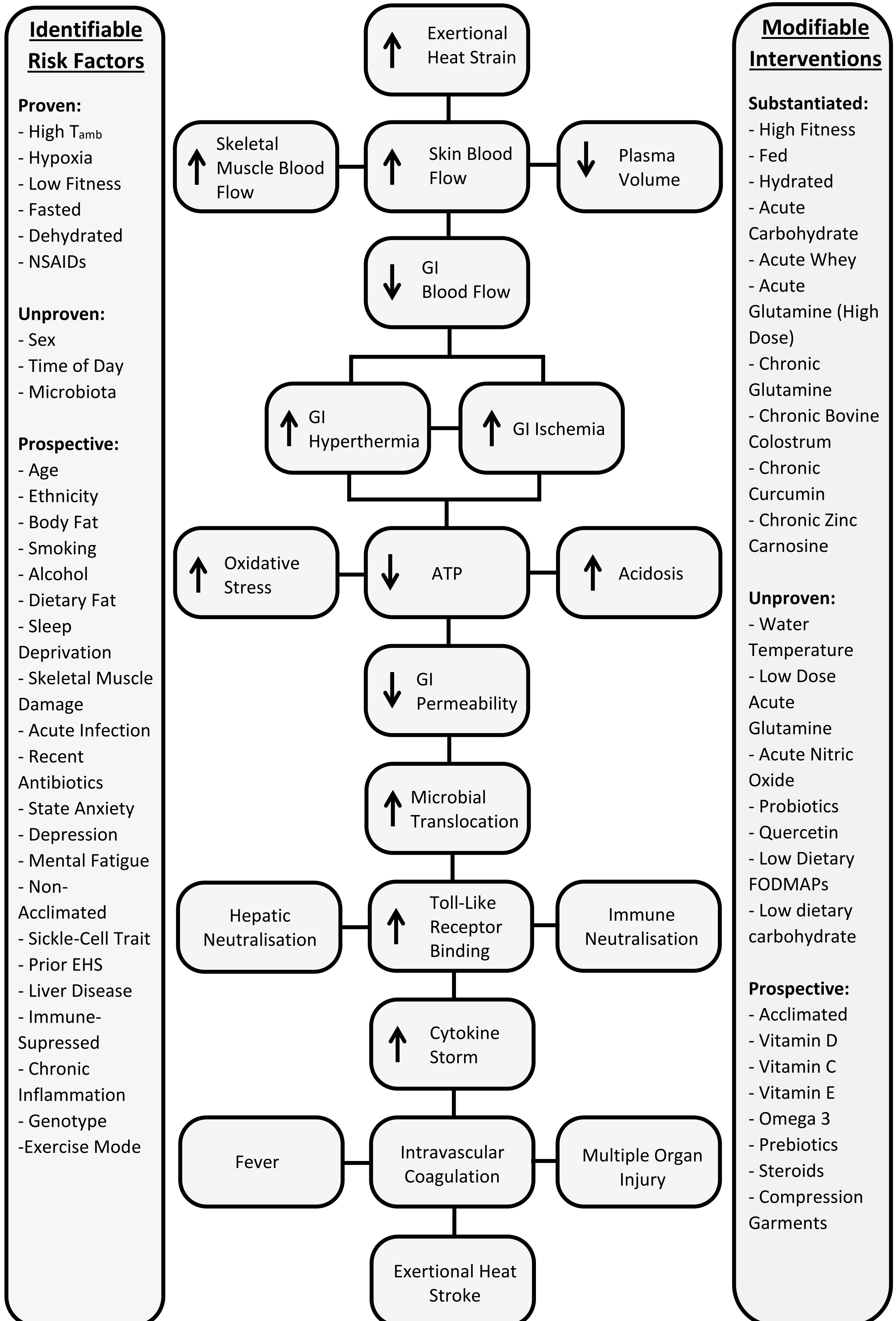

Table 36. Identifiable risk factors and modifiable interventions on GI barrier integrity in response to exercise or exertional-heat stress

Risk Factor	Reference
High T_{amb}	Yeh et al. (2013) [+]; Snipe et al. (2018a) [+]; Snipe et al. (2018b) [+]; Sheahen et al. (2018) [|]; Osborne et al. (2019a) [+]; Kartaram et al. (2019) [+]; Wallett et al. (2020) [+]
Hypoxia	Lee and Thake (2017) [+]; Hill et al. (2019) [+]
Low Fitness	Selkirk et al. (2008) [+]; Morrison et al. (2014) [-]; Chapter 5 [+]
Female Sex	Snipe et al. (2018d) [|]
Microbiota	Bennett et al. (2020) [|]
Fasted	Lambert et al. (2001) [|]; van Nieuwenhoven et al. (2004) [|]; Zuhl et al. (2014) [+]; Zuhl et al. (2015) [+]; Snipe et al. (2017) [+]; Trommelen et al. (2017) [+]; Edinburgh et al. (2017) [+]; Pugh et al. (2017b) [+]; Osborne et al. (2019) [+]; Salvador et al. (2019) [+]; Jonvik et al. (2019) [+]; Flood et al. (2020) [+]
Dehydration	Lambert et al. (2008) [+]; Costa et al. (2019) [+]; Kartaram et al. (2019) [+]
NSAIDs	Smetanka et al. (1999) [+]; Lambert et al. (2001) [+]; Lambert et al. (2008) [+]; van Wijck et al. (2012) [+]
Time of Day	Gaskell et al. (2020) [+]
Countermeasure	
High Fitness	Selkirk et al. (2008) [+]; Morrison et al. (2014) [-]; Chapter 5 [+]
Fed	Lambert et al. (2001) [|]; van Nieuwenhoven et al. (2004) [|]; Zuhl et al. (2014) [+]; Zuhl et al. (2015) [+]; Snipe et al. (2017) [+]; Trommelen et al. (2017) [+]; Edinburgh et al. (2017) [+]; Pugh et al. (2017b) [+]; Osborne et al. (2019) [+]; Salvador et al. (2019) [+]; Jonvik et al. (2019) [+]; Flood et al. (2020) [+]
Rehydration	Lambert et al. (2008) [+]; Costa et al. (2019) [+]; Kartaram et al. (2019) [+]
Water Temperature	Snipe et al. (2018c) [|]
Acute Carbohydrate	Lambert et al. (2001) [|]; van Nieuwenhoven et al. (2004) [|]; Snipe et al. (2017) [+]; Trommelen et al. (2017) [+]; Edinburgh et al. (2017) [+]; Salvador et al. (2019) [+]; Jonvik et al. (2019) [+]; Flood et al. (2020) [+]
Acute Whey	Snipe et al. (2017) [+]
Acute Glutamine	Zuhl et al. (2015) [+]; Pugh et al. (2017b) [+]; Osborne et al. (2019b) [+]; Chapter 7 [-]; Chapter 8 [-]
Acute Quercetin	Kuennen et al. (2011) [-]
Acute Nitric Oxide	van Wijck et al. (2014) [|]; Jonvik et al. (2019) [|]
Chronic Glutamine	Zuhl et al. (2014) [-]
Chronic Bovine Colostrum	Marchbank et al. (2011) [+]; Davison et al. (2015) [+]; Morrison et al. (2014) [|]; March et al. (2017) [+]; McKenna et al. (2017) [|]; March et al. (2019) [+]
Chronic Curcumin	Szymanski et al. (2017) [+]
Chronic ZnC	Davison et al. (2015) [+]
Chronic Probiotics	Shing et al. (2013) [+]; Gill et al. (2014) (-); Pugh et al. (2019) [|]; Axelrod et al. (2019) [|]; Mooren et al. (2020) [|]

[+] = positive effect; [-] negative effect; [|] = no effect

Chapter 10 - Conclusions

The main findings from this thesis include:

1. Gastrointestinal barrier integrity is reliably examined in blood samples taken both at rest and following exertional-heat stress using the dual-sugar absorption test, intestinal-fatty acid binding protein and claudin-3 (chapter 4).

2. Gastrointestinal microbial translocation is reliably examined in blood samples taken both at rest and following exertional-heat stress using lipopolysaccharide binding protein and total 16S bacterial DNA (chapter 4).

3. *Bacteroides* DNA has unacceptable biological and analytical reliability when examined in plasma samples, likely due to extremely low concentrations found in this biofluid. Future studies should look to validate this method in whole-blood, given bacterial DNA concentrations are several magnitudes greater than in plasma (chapters 4, 7, 8).

4. Individuals with high-aerobic fitness experience reduced small intestinal epithelial injury and microbial translocation compared with untrained individuals during a fixed load exertional-heat stress test (chapter 5).

5. Acute oral $_{L}$-glutamine supplementation (0.30, 0.60, 0.90 $g{\cdot}kg{\cdot}FFM^{-1}$) caused mild dose-dependent GI symptoms at rest and typically lasted less than 4 hours (chapter 6).

6. An acute 0.30 $g{\cdot}kg{\cdot}FFM^{-1}$ $_{L}$-glutamine supplement did not protect gastrointestinal permeability, small intestinal epithelial injury or microbial translocation when consumed 1-hour before either a low-intensity ecological (chapter 7) or a high-intensity exhaustive- exertional-heat stress test (chapter 8).

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