Effects of exercise and hyperthermia on gastrointestinal dysfunction and symptoms in recreational athletes

By

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University

for the degree of

DOCTOR OF PHILOSOPHY

May 2021

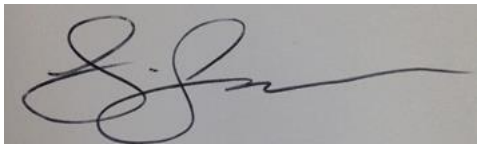
Author's Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of Liverpool John Moores University. Apart from the help and advice acknowledged, the work within was solely completed and carried out by the author.

Any views expressed in this thesis are those of the author and in no way represent those of Liverpool John Moores University and the School of Sport and Exercise Science.

This thesis has not been presented to any other University for examination either in the United Kingdom or overseas. No portion of the work referred to in this research project has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Signed



Date 01/05/2021

Acknowledgments

Firstly, I would like to express my sincere gratitude to my advisor Dr. Dominic Doran for the continuous support of my PhD study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I am sincerely grateful for his support throughout the difficult times experienced during this period of study. Without him, this thesis would not have been possible, and I could not have imagined having a better advisor and mentor for my Ph.D. study.

I would also like to thank Prof. Graeme Close, who took a chance by allowing me to study for an MSc, before providing me with the opportunity to undertake a PhD. Without the opportunities and advice provided by Graeme, my development as a practitioner and researcher would not have been possible, again, thank you.

Thirdly, I would also like to offer thanks to Prof. James Morton. Your work ethic and continuous pursuit of personal development have helped me develop as a professional. Your advice is always received in the highest regard, and your lectures always remind me of why I chose Sports Science as a profession. Thank you.

I would like to offer thanks to Dr. Ben Edwards, for always being happy to discuss ideas, training and presenting me with opportunities to develop as an academic; your friendship over the years has been treasured. To Dr. Mark Scott, for the many hours of unscheduled statistics support and advice, your 'open door' policy was truly appreciated. To the lab technicians, Gemma, Dean, George and Ian, for always being available to amend my (many) mistakes and provide guidance without question. I would also like to extend thanks to my fellow PhD students, who were always willing to assist with data collection and volunteering as participants without expecting anything in return.

Dedication

For my family: my wife Katie, for being my rock throughout this period of study, for believing in me and doing an amazing job at raising our two children through the many hours of my absence. To my parents for supporting me throughout writing this thesis and my life in general.

Abstract

Exercise leads to disturbances in the gastrointestinal (GI) tract, which have been suggested to contribute towards the appearance of symptoms such as bloating, vomiting, and diarrhoea. The mechanisms underlying the appearance of GI symptoms aren't fully understood and there is large variations in the individual susceptibility to symptoms (Karhu et al., 2017). Understanding this relationship is somewhat constrained by the methodological approaches applied in the field. The methods used to quantify the response in intestinal permeability and injury are subject to wide variations in response to a similar exertional and environmental stresses which would appear to be population independent. Whilst splanchnic hypoperfusion and hyperthermia have both been identified as key mechanism(s) contributing towards an increase in intestinal permeability, it is less clear which of these factors is most dominant. Furthermore, it is not yet clear whether these factors (hyperthermia and hypoperfusion) exist on a continuum, where one factor may dominate under certain conditions, and whether other factors such as psychological stress influence this relationship (van Wijck et al., 2011a; Pires et al., 2017). The aim of this thesis is to investigate the relationship between GI dysfunction (permeability and injury), subjective GI symptoms, hyperthermia and exercise. However, given the broad range of methods used in previous literature, a secondary aim of this thesis is to address methodological clarity about the timing of L/R test solution ingestion. The original contribution to knowledge from this work suggests, methodologically, the timing of dual-sugar probe test solution ingestion in relation to exercise does not significantly affect the subsequent serum Lactulose/L-Rhamnose ratio expressed (Chapter 4). Furthermore, GI permeability displays a dose-response relationship with exercise intensity, but this does not correlate with the expression of GI symptoms. The appearance of GI symptoms is likely due to a multitude of factors including physiological strain and exercise intensity, yet is highly individualistic (Chapter 5). Further, in Chapter 6 it is observed for the first time that hyperthermia implemented during resting conditions, in absence of exercise-induced mesenteric hypoperfusion, but does not augment GI permeability or markers of GI distress. Moreover, hyperthermia in absence of exercise-induced hypoperfusion results in the appearance of symptoms of heat illness, and core temperature is strongly correlated to nausea. Research has previously demonstrated that supplementation with glutamine can ameliorate intestinal permeability and core temperature. However, in Chapter 7, acute glutamine supplementation returned no protective effects on GI damage, permeability, core temperature or heat-illness symptoms in response to exposure of passive hyperthermia. Collectively, exercise causes an increase in GI permeability, a response not observed when similar levels of core temperature are induced by passive hyperthermia. In summary, the increase

in GI permeability observed during exercise is likely to be caused by intestinal ischemia, rather than an increase in core temperature. Furthermore, no association between intestinal permeability and GI symptoms appears to exist. Passive heat stress results in a rise in core temperature, GI damage and heat-illness symptoms, with glutamine supplementation demonstrating no protective effect on these outcomes.

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List of Abbreviations

Å	Angstrom
ATP	Adenosine triphosphate
Bpm	Beats per minute
BMI	Body mass index
BW	Body weight
cm	Centimetre
Da	Dalton
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
g	Gram
GI	Gastrointestinal
HCI	Hydrochloric acid
HIE	High intensity exercise
HPLC	High-performance liquid chromatography
hr	Hour

HR	Heart rate
HWI	Hot water immersion
I-FABP	Intestinal fatty acid-binding protein
FABP	Fatty acid-binding protein
IL	Interleukin
kDa	Kilo Dalton
kg	kilogram
LPS	Lipopolysaccharide
m	Meter
MDCK	Madin darby canine kidney cell
mL	Millilitre
MW	Molecular weight
NSAID	non-steroidal anti-inflammatory drugs
PPAR	peroxisome proliferator activated receptors
PHS	Passive heat stress
RER	Respiratory exchange ratio
SBF	Splanchnic blood flow
SKF	Skinfold thickness
SGLT-1	Sodium-dependent glucose transporter - 1
SST	Serum separating tube
TC	Thermal comfort
Tcore	Core temperature
TER	Transepithelial resistance
TNF	Tumour necrosis factor
VAS	Visual analogue scale
$\dot{V}O_{2\ max}$	Maximal oxygen uptake
$\dot{V}O_{2\ peak}$	Peak oxygen uptake
ZO	Zona occulden

Chapter 1: Introduction

1.1 Background

The gastrointestinal (GI) system, alternatively referred to as the gut, GI tract or alimentary canal, is responsible for the transportation, digestion and absorption of nutrients and fluids. It composes one of the two organ groups of the digestive system, the other group being the accessory digestive organs such as the salivary glands, liver, gallbladder and pancreas (Tortora and Derrickson, 2017). Exercise results in a plethora of physiological events, particularly involving the musco-skeletal and cardiorespiratory systems (Farrell et al., 2012). However, exercise also leads to disturbances in the gastrointestinal (GI) tract, which can ultimately lead to the appearance of symptoms such as vomiting, diarrhoea or bloating (Costa et al., 2017b). The appearance of these symptoms can cause impairments in performance for athletes, as they have to adjust their pace to reduce the magnitude of symptoms, or stop completely (de Oliveira and Burini, 2009). However, the mechanisms underlying the appearance of GI symptoms aren't fully understood and there is a large variation in the individual susceptibility to symptoms (Karhu et al., 2017). It is estimated that between thirty to ninety percent of athletes experience exercise-related GI symptoms (de Oliveira, Burini and Jeukendrup, 2014) such as nausea, abdominal pain, vomiting as well as diarrhoea (van Nieuwenhoven, Brouns and Brummer, 2004; ter Steege, Van der Palen and Kolkman, 2008; ter Steege et al., 2012; Costa et al., 2017b). There has been a considerable amount of research investigating the underlying mechanisms behind exercise-related GI symptoms (Costa et al., 2017b) with reduced splanchnic blood flow and an increase in core temperature being considered the main contributing factors (de Oliveira, Burini and Jeukendrup, 2014; Pires et al., 2017).

1.1.1: The Intestinal Epithelial Barrier

The small intestine displays structural characteristics to increase its absorptive surface area and capacity, including being very long (approximately three metres) and internally lined with finger-like folds known as villi and microvilli to increase absorptive surface area. In addition to being primarily responsible for the absorption of nutrients (water, organic molecules and ions) into the portal circulation, the gut also acts as a barrier to protect the internal environment against pathogenic micro-organisms (Grootjans et al., 2010b; Ward, Keely and Keely, 2014). Collectively, this protective barrier primarily consists of a mucosal layer comprised of secretions produced by specialist goblet cells found in the intestinal epithelium. Indeed, the intestinal epithelium itself acts as a secondary barrier and consists of a monolayer of columnar enterocyte cells and intercellular tight-junctions. Together the intestinal mucosal layer and enterocytes serve as both a physical and chemical barrier that prevents the adherence of micro-organisms to the epithelium and the translocation of pathogens into the internal environment (Grootjans et al., 2016).

1.1.2: Intestinal injury and permeability

Under resting conditions in healthy individuals, the regulation of nutrient absorption and inhibition of antigens transiting through the intestinal barrier is well controlled. However, mesenteric ischemia or hyperthermia can cause local intestinal injury, disruptions to the mucosal layer and reduced integrity of the enterocyte tight junctions (Anderson and Van Itallie, 2009; van Wijck et al., 2011a; Zuhl et al., 2014b; Dokladny, Zuhl and Moseley, 2016; Costa et al., 2017b; Pires et al., 2017; Snipe et al., 2017). Damage occurs through erosion of the enterocyte apical region and disruption to tight-junction integrity, leading to a loss of physical barrier functionality and an increase in intestinal permeability (Moses, 1990; Lambert, 2009; ter Steege and Kolkman, 2012; Dokladny, Zuhl and Moseley, 2016; Grootjans et al., 2016; Costa et al., 2017b).

Whilst short periods of intestinal ischemia are experienced daily through normal physiological function and do not normally disrupt the intestinal barrier (van Wijck et al., 2011a), prolonged exposure to ischemia causes intestinal injury and stimulates increased mRNA expression of pro-inflammatory cytokines such as interleukin (IL)- 1 beta, IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α) (Rhind et al., 2004; Ng et al., 2008; Selkirk et al., 2008; Gill et al., 2015; Snipe et al., 2017).

The increase in permeability facilitates augmented transport of larger macromolecules and endotoxin (lipopolysaccharide [LPS]), that is found on the surface of gram-negative bacteria within the gut, through the intestinal barrier into the portal circulation (Bosenberg et al., 1988; Brock-Utne et al., 1988; Hall et al., 2001a; Lambert et al., 2002; Selkirk et al., 2008). LPS is normally well contained within the intestinal lumen and small amounts within the blood are quickly cleared by anti-endotoxin mechanisms (Selkirk et al., 2008). However, a higher flux of LPS into the circulation can result in endotoxemia (Bosenberg et al., 1988; Brock-Utne et al., 1988; Ng et al., 2008; Selkirk et al., 2008; Gill et al., 2015; Costa et al., 2017b), a potentially life-threatening inflammatory response associated with pathological conditions such as exhaustion after ultra-distance events (Brock-Utne et al., 1988) and heat stroke (Selkirk et al., 2008).

Gastrointestinal dysfunction presents a serious health hazard to athletes, with between thirty to ninety % of athletes experiencing GI symptoms during training or competition (de Oliveira, Burini and Jeukendrup, 2014; Pugh et al., 2017a). A decline in splanchnic blood flow during exercise, as blood is redirected to the periphery and working muscle, is a primary cause of GI dysfunction (van Wijck et al., 2011a). This results in a transient state of hypoperfusion in the mesenteric organs and subsequent damage accruing at the intestinal villus tips, as well as breakdown in enterocyte tight-junction integrity (Grootjans et al., 2016). As a result of damage to the epithelial layer, molecules translocate from within the intestinal tract into the portal blood, potentially leading to an inflammatory response and endotoxemia. Pires et al. (2017) recently explored the exercise-related rise in core temperature as a leading mechanism underpinning GI dysfunction. These authors identified two key thresholds of core temperature: 38.5°C where an increase in GI permeability is observed in some participants; and 39°C,

whereby augmented intestinal permeability is universal in participants. However, the relative contribution of each of these mechanisms towards the cascade of events leading to GI dysfunction and, possibly, the appearance of GI symptoms remains unclear. Whilst the underlying aetiology has yet to be fully delineated, practical approaches towards rescuing some of the reduced splanchnic blood flow and attenuating the damage caused by heat-stress to the intestinal mucosa have been investigated. The provision of carbohydrate during exercise (Rehrer et al., 2005) and acute glutamine supplementation (Mondello et al., 2010) have been shown to increase portal vein flow during exercise and attenuate ischaemia/reperfusion injury, respectively. Whilst glutamine supplementation has also been shown to attenuate, both, the increase in core temperature in response to acute heat stress (Soares et al., 2014) and exercise-induced GI permeability (Zuhl et al., 2015).

Understanding the mechanisms underlying exercise-induced GI dysfunction, with a view towards developing nutritional strategies to offset these effects, will inform the structure of this thesis. The aim is to investigate the relationship between GI dysfunction, subjective GI symptoms, and hyperthermia. The effects of exercise intensity and passive hyperthermia will be examined, along with potential nutritional strategies that could offset any disturbances to the intestinal barrier. Specifically, the potential of glutamine to reduce GI permeability and injury will also be examined. To achieve these aims, the following objectives will be addressed:

1. A comparison between the magnitudes of intestinal permeability elicited when the timing of the dual-sugar probe solution ingestion is altered relative to exercise performance (Chapter 4).
2. Investigate the effects of exercise intensity on GI permeability, damage and symptoms (Chapter 5).
3. Explore the responses in GI permeability, damage and symptoms in response to passive hyperthermia (Chapter 6)
4. Investigate the efficacy of glutamine supplementation to reduce GI permeability, damage and symptoms in response to passive hyperthermia (Chapter 7).

Chapter 2: Literature Review

2.0: The Gastrointestinal System

2.1: The Small intestine

Essentially a continuous long tube, the GI tract extends from the mouth to the anus. Foods and fluids enter the digestive system through the mouth and subsequently facilitated, via muscular contractions, down the oesophagus and into the stomach for further digestion (Farrell et al., 2012). These muscular contractions contribute to the physical breakdown and digestion of food through churning and mixing with secretions extracted within the GI tract, respectively. The stomach connects the oesophagus to the start of the small intestine (the duodenum) and essentially acts as a reservoir and mixing vessel for ingested food before it enters the small intestine (Tortora and Derrickson, 2017). The stomach also aids in the digestion of food and killing of bacteria by secreting a number of substances including gastric juices and hydrochloric acid. Together, these secretions combined with food and saliva form chyme, a thick liquid that leaves the stomach and enters the small intestine, where the major actions of digestion and absorption occur (Tortora and Derrickson, 2017). Following the small intestine, chyme enters the large intestine where, briefly, bacteria acts to further digest and absorb nutrients as well as water, ions and vitamins (Tortora and Derrickson, 2017).

2.1.1: Splanchnic blood flow

Splanchnic circulation is delivered via three branches of the aorta: the coeliac trunk, and the superior and inferior arteries (Harper and Chandler, 2015). Together these arteries perfuse the stomach, spleen, pancreas, liver and, both, the small and large intestine. Branches of the coeliac trunk supply the stomach, first 25-30 cm of the small intestine (upper duodenum) and the spleen and pancreas, while the superior mesenteric artery supplies blood to the rest of the small intestine (duodenum, jejunum and the ileum) as well as significant part of the large intestine. The inferior mesenteric artery supplies blood to the descending colon, sigmoid colon and upper rectum (Farrell et al., 2012). Of these three arteries, the superior mesenteric artery is the largest, delivering over ten % of the cardiac output to the splanchnic region (Harper and Chandler, 2015). These three arteries branch out into a further three vascular plexuses, namely the serosal, submucosal and mucosal plexuses. A capillary network from the mucosal plexus further supplies blood to the intestinal mucosa (Mensink et al., 2006). All blood outflow from the gastrointestinal tract is collectively delivered to the liver via the hepatic portal vein (Rehrer et al., 2001; Harper and Chandler, 2015).

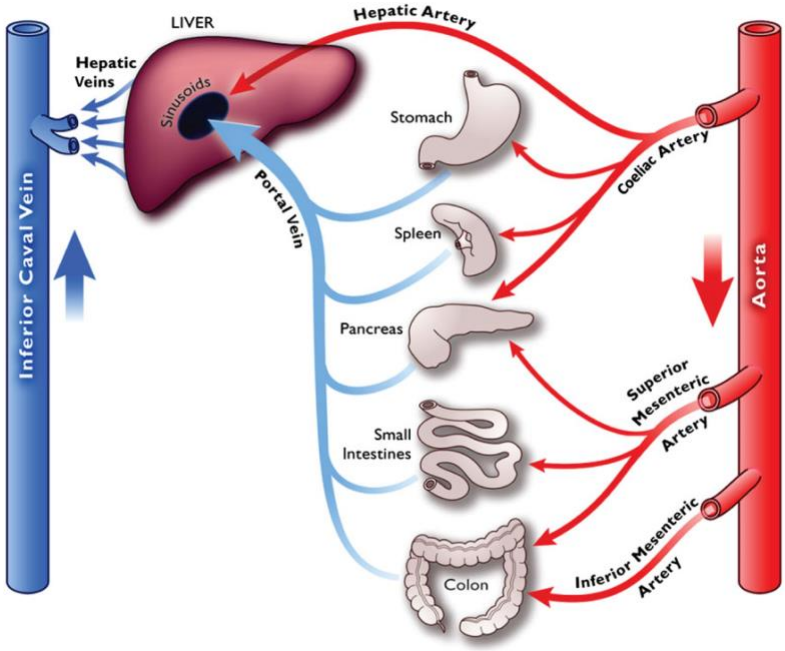


Figure 1: Schematic representation of the splanchnic circulation (Harper and Chandler, 2015)

Splanchnic blood flow (SBF) is highly adaptive, receiving approximately twenty to twenty five % of whole-body cardiac output at rest (Matheson, Wilson and Garrison, 2000), with perfusion values of approximately 30 mL^{-1} $100g^{-1}$ of tissue which can decrease to below 10 mL^{-1} $100g^{-1}$ under conditions of low cardiac output, or *increase* to approximately 250 mL^{-1} $100g^{-1}$ following a meal (Harper and Chandler, 2015). In response to feeding, SBF rises rapidly within five to fifteen minutes of ingestion and is sequentially increased during nutrient absorption to supply the relevant area of the GI tract with blood as the chyme moves over the mucosal surface, before returning to baseline once absorption is completed several hours later (Matheson, Wilson and Garrison, 2000). In contrast, SBF can decline under physiological, psychological or environmental conditions such as exercise, dehydration, stress, hyperthermia, medication or a combination (Rowell, 1974; Qamar and Read, 1987; Moses, 1990; Ryan, Chang and Gisolfi, 1996; van Nieuwenhoven et al., 2000; Rehrer et al., 2001; Lambert et al., 2002; Lambert et al., 2007; Lambert, 2009; Van Wijck et al., 2012b; Vargas and Marino, 2016; Pires et al., 2017; Wilson, 2017).

During vigorous exercise splanchnic vascular resistance is increased as a result of splanchnic vasoconstriction, due to increased sympathetic nervous system activity as norepinephrine, released from nerve endings during vigorous exercise, binds to α-adrenoreceptors on the sympathetic nervous system (Rowell, 1974; Pires et al., 2017). In contrast, the vascular resistance of organs under increased physiological stress (muscles, cardiovascular system, cerebral region and skin) will simultaneously decrease (Qamar and Read, 1987; Otte et al., 2005; Lambert, 2009; ter Steege and Kolkman, 2012), with the net result being a reduction in splanchnic blood flow. Specifically, intestinal blood flow has been shown to reduce by twenty % within ten minutes of the onset of steady-state exercise, increasing

to up to eighty % after one hour (Rehrer et al., 2001). This reduction in blood supply reduces the amount of oxygen available to the tissue, leading to splanchnic ischemia and hypoperfusion of the mesenteric organs (Ward, Keely and Keely, 2014), the magnitude of which is linearly associated with exercise intensity (ter Steege and Kolkman, 2012).

2.2: Gastrointestinal symptoms in athletes

2.2.1: Prevalence

The appearance of gastrointestinal symptoms is common amongst athletes, particularly endurance and ultra-athletes, where the sustained nature of the activities may cause a consistent reduction in splanchnic blood flow (Rehrer et al., 2001; Gaskell, Snipe and Costa, 2019). However, recent research suggests that team sport and combat sport athletes may also experience GI symptoms during a typical week (Pugh et al., 2017a).

Typically quantified using a subjective scale, an accurate quantification of prevalence rates of GI symptoms amongst athletes is difficult to obtain. Previous research has utilised various visual analogue scales, which have also included differing lists of symptoms, retrospective or prospective assessment, and online surveys (ter Steege et al., 2012; Guillochon and Rowlands, 2017; Snipe et al., 2017). GI symptoms range in their magnitude and severity, from feelings of nausea and bloating, to ischemic colitis and bloody stools. Nevertheless, de Oliveira, Burini and Jeukendrup (2014) estimate that between 30 – 90% of distance runners experience exercise-related GI symptoms; furthermore a more recent survey by Pugh et al. (2017a) suggests that 86% of athletes experience at least one GI symptom.

The accepted and convenient method of assessing GI symptoms has typically been by the 10 cm visual analogue scale (Bengtsson et al., 2013), which was recently modified by Gaskell, Snipe and Costa (2019) for specifically assessing GI symptoms during exercise. The results of previous studies are also confounded by debate as to what constitutes a symptom, as well as separating exercise-related GI symptoms and what an athlete may experience in normal daily life (Wilson, 2017). For example, in the case of an Ironman, where competition lasts over eight hours, an athlete may experience the urge to defecate, but whether this is related specifically to the exercise is debatable. Furthermore, the large range of symptoms employed on visual scales (nausea, bloating, urge to defecate, side stitch etc.) in addition to the wide 0 – 10 scale, representing “no symptom”, to “mild” and “severe”, makes the reporting of research difficult. Nonetheless, previous research indicates that the appearance of gastrointestinal symptoms in athletes is common, particularly those engaged in endurance sports, however further research is required investigating the contribution from underlying mechanisms.

2.2.2 Mechanisms of GI symptoms in athletes

Whilst we understand the prevalence of GI symptoms to be high amongst athletes, the aetiology is less well understood. A multifactorial, complex interplay is thought to exist between physiological mechanisms (ischemia), mechanical stress (due to the jarring nature of some sports such as running), (Coleman, 2019) and psychological factors as some athletes experience symptoms in competition but not during training or laboratory conditions (Wilson, 2019). An increase in gastrointestinal permeability was considered a primary factor, but correlations between GI permeability and symptoms have so far lacked association. Furthermore, the use of anti-inflammatory drugs, as well as the intake of food or fluids during exercise appear to exacerbate symptoms, both of which are common amongst athletes (Costa et al., 2017b; de Oliveira, 2017).

Reduced splanchnic blood flow and exercise-induced heat stress are thought to contribute to increased permeability (Lambert, 2008; Pires et al., 2017). Augmented permeability, whereby the tight-junctions bridging the enterocytes become dysfunctional, therefore allowing the translocation of otherwise impassable molecules, has often been considered a primary cause of exercise-induced GI symptoms (Karhu et al., 2017). With reduced blood flow, less oxygen is available to perfuse the intestinal organs, leading to ischemia and subsequent shedding of the villus tips. Whilst this shedding occurs as part of normal physiological function, sustained ischemia and villus shedding, such as that observed in response to endurance exercise, can lead to GI dysfunction (Grootjans et al., 2016). Indeed, GI hypoperfusion may result in physiological responses that contribute to GI barrier dysfunction, such as oxidative stress (Lambert et al., 2002) and an inflammatory cascade response (Grootjans et al., 2010a). Whilst low levels of oxidative stress may be beneficial for the GI tract (Lambert et al., 2002), excessive oxidative stress could be associated with the pathogenesis of GI diseases such as ulcers, cancer and inflammatory bowel disease (Bhattacharyya et al., 2014). Furthermore, as a result of blood flow returning to the gut, intestinal ischemia-reperfusion injury may occur through the production of cytokines, reactive oxygen species contributing to immune activation, inflammation and intestinal barrier compromise (Grootjans et al., 2010a).

The rise in core temperature associated with exercise results in hyperthermia within the gut wall, subjecting the tight-junctions of the epithelium to heat stress. As such, hyperthermia has consistently been shown to augment intestinal permeability in both animal and cell-culture models (Table 1) through tight-junction dysfunction, with evidence supporting a dose-response relationship (Pals et al., 1997; Dokladny, Zuhl and Moseley, 2016). Whilst the presence of such correlation is promising, this does not necessarily represent a causation effect. Indeed, the rise in core temperature and associated augmented intestinal permeability may be a proxy for other mechanistic, physiological events which research is yet to identify.

The translocation of potential antigens across the GI barrier, such as endotoxin (lipopolysaccharides), bacteriophages, large proteins (gliadin) or bacterial DNA, into the blood stream

can lead to an inflammatory response similar to that experienced by heat stroke patients (Ogden et al., 2020). Indeed, heat-stress appears to be a distinct contributor to GI dysfunction, as athletes have displayed endotoxemia in response to exercise in both laboratory and real-world situations such as competitive events subject to environmental conditions (Bosenberg et al., 1988; Brock-Utne et al., 1988; Yeh, Law and Lim, 2013). Participants have also displayed endotoxemia in response to exercise in hot (35°C), but not neutral (22°C), conditions (Yeh, Law and Lim, 2013; Snipe et al., 2018b). Together, these physiological events have seen athletes reporting the appearance of symptoms akin to heat illness, such as dizziness and nausea (Gill et al., 2015; Snipe et al., 2018b).

Outside of physiological consequences such as heat-stress and ischemia, the appearance of GI symptoms may be related to other factors such as nutritional intake before or during exercise, mechanical stresses, or the use of anti-inflammatory drugs (Gisolfi, 2000; Lambert et al., 2007; Pfeiffer et al., 2012). Gastric emptying is not likely affected at exercise intensities less than 70 % $\dot{V}O_{2\ max}$, however, intensities related to athletic competition, above 80% $\dot{V}O_{2\ max,}$ are likely to delay gastric emptying (Costill and Saltin, 1974). Further evidence suggests that gastric emptying is associated with exercise-induced GI symptoms such as nausea (van Nieuwenhoven et al., 2000). As such, athletes have been advised to practice 'gut training' to enhance gastric emptying during exercise and avoid meals high in nutrients such as fibre and protein prior to exercise, to prevent delayed gastric emptying (Costa et al., 2017a; Costa et al., 2017b). Collectively, it is apparent that the appearance of GI symptoms in response to exercise may not be related to one specific factor, but is instead governed by a mixture of factors, some of which are out of the athletes' control (such as environmental temperature).

Table 1: A summary of studies reporting exercise-induced GI symptoms.

Authors	Protocol	Model	Environmental Conditions	Outcome
(Ryan, Chang and Gisolfi, 1996)	60 min treadmill running at ~70% $\dot{V}O_{2\ max.}$ With or without aspirin ingestion	*n* = 7 healthy, active males. 100-mm visual analogue scale.	Thermoneutral conditions	Mild (<10%) symptoms. Neither running nor aspirin ingestion was associated with appearance of severe GI symptoms. No correlation with GI permeability.*
Pals et al. (1997)	60 min treadmill running at 40, 60 or 80% $\dot{V}O_{2\ max}$.	*n* = 6 healthy (*n* = male, *n* = 1 female) volunteers.	Thermoneutral conditions	One participant reported cramps (78 mm) and side stitch (82 mm).
(Lambert et al., 2007)	60 minutes running at 70% $\dot{V}O_{2\ max}$ 24ºC, 33% RH	*n* = 8 (*n* = 6 male; *n* = 2 female), healthy, recreational runners. 133 mm visual analogue scale.	24ºC, 33% RH	No appearance of symptoms
Lambert et al. (2008)	Treadmill running at 70% $\dot{V}O_{2\ max.}$ With or without fluid intake. Thermoneutral conditions	Twenty runners, 11 males and 9 females; 133 mm visual analogue scale.	Thermoneutral conditions	Appearance of some symptoms but all very low. Fluid intake resulted in higher occurrence of stomach fullness.
(ter Steege et al., 2012)	30-minute incremental exercise test on a cycling ergometer.	12 athletes (*n* = 7 male, *n* = 5 female). *n* = 5 runners, *n* = 6 cyclists,	Thermoneutral conditions	Athletes with GI symptoms reported increased susceptibility for the development of ischemia during exercise.

		n = 1 triathlete. No VAS. Exercise tonometry recorded		
Karhu et al. (2017)	Treadmill run at 80% of 10-km race speed. Thermoneutral conditions	17 runners, 9 asymptomatic and 8 symptomatic; 8-point VAS.	Thermoneutral conditions	No clear differences in symptoms between groups. Symptoms were low, with the highest reported symptom being flatulence (4/8)
Pugh et al. (2017b)	18 x 400m treadmill intervals at 120% $\dot{V}O_{2\ max}$. Thermoneutral conditions	11 male runners; 0-10 visual analogue scale	Thermoneutral conditions	HIIT increased the appearance of symptoms over rest. Symptoms were reported at the low end of the scale ('no problems at all", "very minor problems"). No correlation to intestinal permeability.
Snipe et al. (2017)	2h treadmill running at 60% $\dot{V}O_{2\ max}$ in hot (35°C) or temperate (22°C).	10 endurance trained runners (*n* = 6 male, *n* = 4 female). Visual analogue scale, range not reported	Hot (35°C) or temperate (22°C) conditions	Total upper and lower GI symptoms were significantly higher (all $p<0.05$) in HOT conditions compared to TEMP.
Snipe and Costa (2018b)	2h treadmill running at 60% $\dot{V}O_{2\ max}$ in hot (35°C) conditions. Participants received water at COLD (0.4 ±	12 endurance trained runners (*n* = 6 male, *n* = 6 female). Visual analogue scale, range not reported.	Hot (35°C) conditions.	75%, 92% and 92% incidence of GI symptoms in COLD, COOL and TEMP, respectively. No significant difference in the incidence and severity of GI symptoms between trials. Trends were

	0.4°C), COOL (7.3 ± 0.8°C), or TEMP (22.1 ± 1.2°C).			observed for increased upper-GI on TEMP compared to other conditions.
Snipe and Costa (2018a)	2h treadmill running at 60% $\dot{V}O_{2\ max}$ in hot (35°C) conditions.	24 endurance trained runners (*n* = 13 male, *n* = 11 female). Visual analogue scale, 10-point scale, 100 mm.	Hot (35°C) or temperate (22°C) conditions.	No difference in upper- and lower GI symptoms between sexes.
Snipe et al. (2018b)	2h treadmill running at 60% $\dot{V}O_{2\ max}$ in warm (30°C) or temperate (22°C).	10 endurance trained runners (*n = 6* male, *n* = 4 female). 10-point Likert-type rating scale	10 endurance trained runners (n = 6 male, n = 4 female). 10-point Likert-type rating scale	WARM conditions returned significantly higher total GI symptoms when compared to TEMP.
(Wilson, 2017)	Participants recorded GI symptoms during 30 days of habitual training.	150 endurance trained runners (*n* =74 male, *n* = 76 female). 10-point Likert-type rating scale. Stress and anxiety measures via Perceived Stress Scale.	Data not provided	Appearance of symptoms during 45.6% (16.6-67.3%) of training sessions. Age and running negatively correlated with occurrence of GI distress. Run RPE, probiotic food consumption and PSS scores positively correlated with GI distress
Pugh et al. (2019)	28 days of probiotic supplementation prior to	24 endurance trained runners (*n = 20* male, *n* = 4 female). 10-point	16 – 17 °C	Large range of scores for individual symptoms in days prior to the race.

a track-based marathon race.	Likert-type rating scale.	Probiotic supplementation reduced the incidence of moderate symptoms.*

*Data is shown from placebo arms where a study utilized multiple research interventions

2.3: The Gastrointestinal Barrier: Structure and Function

2.3.1: Intestinal Epithelial Tight-Junctions

Transportation of molecules through the intestinal epithelium occurs through paracellular or transcellular diffusion; where paracellular transport is mediated by the intercellular tight junctions (TJs) and transcellular transport is regulated by precise cellular membrane channels (Anderson and Van Itallie, 2009).

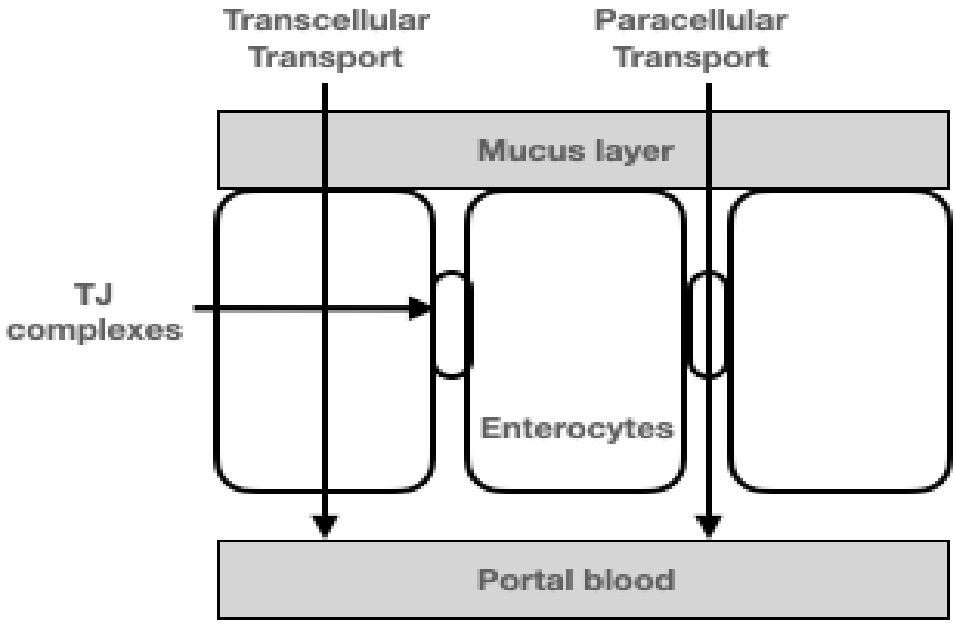


Figure 2: Schematic representation of transcellular and paracellular diffusion pathways.

TJs are multi-protein complexes located within the apical region of the enterocyte that regulate paracellular diffusion, epithelial permeability and, combined with mucous secretions and immune mediators, serve to bind the enterocytes together (Lambert, 2004; Anderson and Van Itallie, 2009; Shen et al., 2011). Transportation across TJs is regulated by two key pathways, namely the leak and pore pathways (Shen et al., 2011). The pore pathway is charge-selective, restricting the transport of charged molecules across the TJs, and controls paracellular flux of small solutes; in contrast, the leak pathway regulates transport of large (over four angstroms [Å]) molecules and is unrestrictive to charge (Shen et al., 2011).

The architecture of the tight-junctions is complex and a systematic description is beyond the scope of this review, however, the integrity of the TJs is governed by transmembrane barrier proteins (for example occludin and claudins), thus, greater accumulation of these proteins at the TJ site increases

the resistance of the barrier. TJs also consist of cytoplasmic scaffolding proteins such as the zona occludin (ZO) family, which link the (extracellular) occludin and claudins to intracellular actin cytoskeleton and regulatory proteins (Anderson and Van Itallie, 2009; Shen et al., 2011).

Permeability of the tight junction is controlled by the phosphorylation and de-phosphorylation of the epithelial actomyosin protein under the regulation of myosin light-chain kinase (MLCK) and myosin light-chain phosphatase (MLCP), respectively. Specifically, phosphorylation causes shortening and opening of the TJ, whereas de-phosphorylation elicits closure of the TJs (Rodgers and Fanning, 2011). Heat stress and exercise have both been shown to compromise TJ integrity and increase GI permeability, however, studies thus far have only utilised cell-culture and animal models (Dokladny, Zuhl and Moseley, 2016) (Table 2).

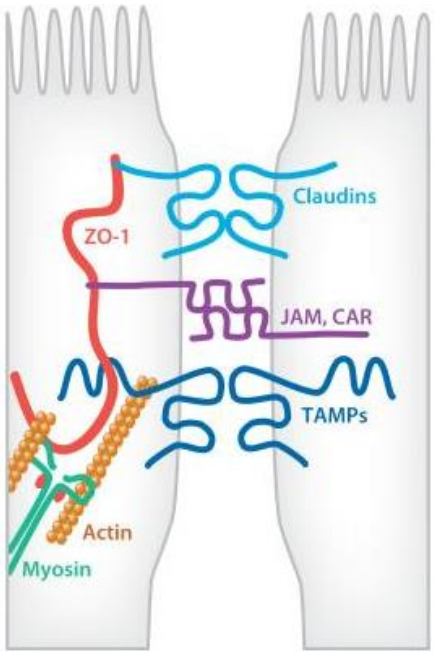


Figure 3: Schematic illustrating interactions between transmembrane proteins claudins; occludins (TAMPs); zona occludin (ZO); and actin and myosin light chains. Also showing junctional adhesion molecules (JAM) and the coxsackie adenovirus receptor (CAR) [not add

Table 2: A summary of studies showing the effects of exercise and heat on tight junction proteins.

Authors	Protocol	Model	Outcome
Rao, Baker and Baker (1999)	Oxidative stress induced by H_2O_2 exposure	In *vitro* Caco-2 intestinal epithelial cells	Epidermal growth factor (EGF) delayed TJ permeability
(Prosser et al., 2004)	Challenge with 1mM EGTA	MDCK cells	TER decreased by 60%, (effect was attenuated by colostrum exposure)
(Ikari et al., 2005)	Heat stress, 42°C for 3 h	Porcine renal epithelium LLC-PK_1 cells	Heat stress increased tight-junction permeability
Dokladny, Moseley and Ma (2006)	Heat Increase from 37°C to 41°C over 24 h.	In *vitro* Caco-2 intestinal epithelial cells	Increased TJ permeability
Yang, He and Zheng (2007)	Heat stress (37°C - 43°C) for 1 h	Human intestinal epithelial T84 cell monolayers	Heat stress increased tight-junction permeability is a dose-response manner
(Pearce et al., 2013)	Heat stress 35°C 20-35% RH, for 1,3 or 7 d	Crossbred gilts (pigs)	TER decreased by 30% indicating TJ dysfunction
Liu et al. (2012)	Heat stress, 40°C for 2 h daily over 3 days	Sprague Dawley rats	Heat stress damaged TJ structure
Zuhl et al. (2014b)	Glutamine supplementation on heat induced TJ protein expression	In *vitro* Caco-2 intestinal epithelial cells	Glutamine preserved the stability of occludin at the TJ

	Glutamine supplementation on exercise induced intestinal permeability	*n* = 8 male (*n* =5) and female (*n*=3*)* participants. 60 min treadmill running at ~ 70 % $\dot{V}O_{2\,max}$	Glutamine prevented exercise-induced intestinal permeability
Davison et al. (2016)	2°C rise (from 37°C to 39°C)	In *vitro* Caco-2 intestinal epithelial cells	Trans-epithelial resistance showed an inverse relationship with temperature.

2.4: Assessment of Gastrointestinal Permeability

The first human investigation into the absorption dynamics of different sugars through the intestine dates back to 1930 (McCance and Madders, 1930). These authors compared the rate of absorption of both orally and intravenously administered L-rhamnose, arabinose and xylose. However, it wasn't until the 1970s that the introduction of non-metabolisable oligosaccharides facilitated feasible means for measuring intestinal barrier function (Menzies, 1974). Since then, determination of human small-intestinal permeability has typically been conducted through the ingestion of a solution containing high-molecular weight dual-sugar probes followed by subsequent quantification of the ratio of the fractional excretion of the larger molecule to the smaller molecule in the urine (Menzies, 1974) or serum/plasma (Fleming et al., 1996). Two probes are used since quantifying the ratio of specific large and small molecules presents greater interpretive value than if only one probe was used (Menzies, 1974). For the assessment of intestinal permeability the most commonly utilized combination of molecules is lactulose and mannitol (Wang et al., 2015), however L-rhamnose is also used and more common in exercise-related studies (van Nieuwenhoven et al., 1999; Lambert, 2008; Lambert et al., 2012; van Wijck et al., 2013; Pugh et al., 2017b). The absorption rate of the sugar probes can be affected by certain physiological variations and diseases, so the need for participant screening and pre-experimental control is high. For example, in patients with coeliac disease, L-rhamnose absorption is decreased whilst lactulose absorption is increased. Furthermore, individual variations such as gastric emptying, intestinal transit and dilution by secretions can influence the absorption of the sugar probes (Fig. 2.3) (Travis and Menzies, 1992).

It is known that the probes transit through different areas of the crypt-villus axis of the small intestine dependent on their size. As the cells mature along the axis (from bottom to top) the channels decrease in size but become more abundant with the channels at the tip of the villus being approximately <6 A, compared to those at the crypt (50-60 A). At the villus base there are also intermediate-sized channels approximately 10 – 15 A (Fihn, Sjoqvist and Jodal, 2000). This data would suggest that larger molecules such as disaccharides and probes of this structure e.g. lactulose, would be confined to the lower channels, whereas L-rhamnose would readily transport across the villus tips (Arrieta, Bistritz and Meddings, 2006).

Lactulose is a disaccharide comprised of galactose and glucose with a large molecular weight (MW) (342Da) and (due to this high MW) is believed to only translocate across the intestinal wall through the paracellular pathway (Vojdani, 2013). Since lactulose is non-digestible in the small intestine and only begins to be degraded in the colon by colonic bacteria, its appearance in urine or plasma can be utilized as a marker of small intestinal permeability (Arrieta, Bistritz and Meddings, 2006). In contrast, L-rhamnose is a monosaccharide with a molecular weight of 164Da and transfers through the gut wall via the transcellular pathway (Vojdani, 2013). Monosaccharides are consumed as part of the

test to control for non-barrier-related factors between experimental conditions such as: tissue distribution (Bjarnason, MacPherson and Hollander, 1995); the surface area of the epithelium; and the available time for permeation (Arrieta, Bistritz and Meddings, 2006). Despite these differences, a common factor between the sugars is that they remain largely intact and unaltered throughout the digestion process and their rate of appearance in the urine or plasma is directly correlated to their absorbed quantity (Sequeira et al., 2014).

The majority of studies utilize a 'classical' dual-sugar probe assay consisting of lactulose and, either, mannitol or L-rhamnose. This assay provides insight into the permeability characteristics of the upper GI tract due to colonic degradation of the sugar probes (van Wijck et al., 2011b). However, researchers began adding sucrose to sugar-probe assays to provide an analysis of gastroduodenal permeability (Lambert et al., 2008) since sucrose is not completely broken down until reaching the jejunum (Sutherland et al., 1994; Meddings, Wallace and Sutherland, 1995). Furthermore, sugar-probe assays should contain additional probes, such as erythritol to enable a thorough analysis of permeability throughout the length of the GI tract. However, these more complex assays require analysis through highly sensitive liquid chromatography tandem mass spectrometry (van Wijck et al., 2011b).

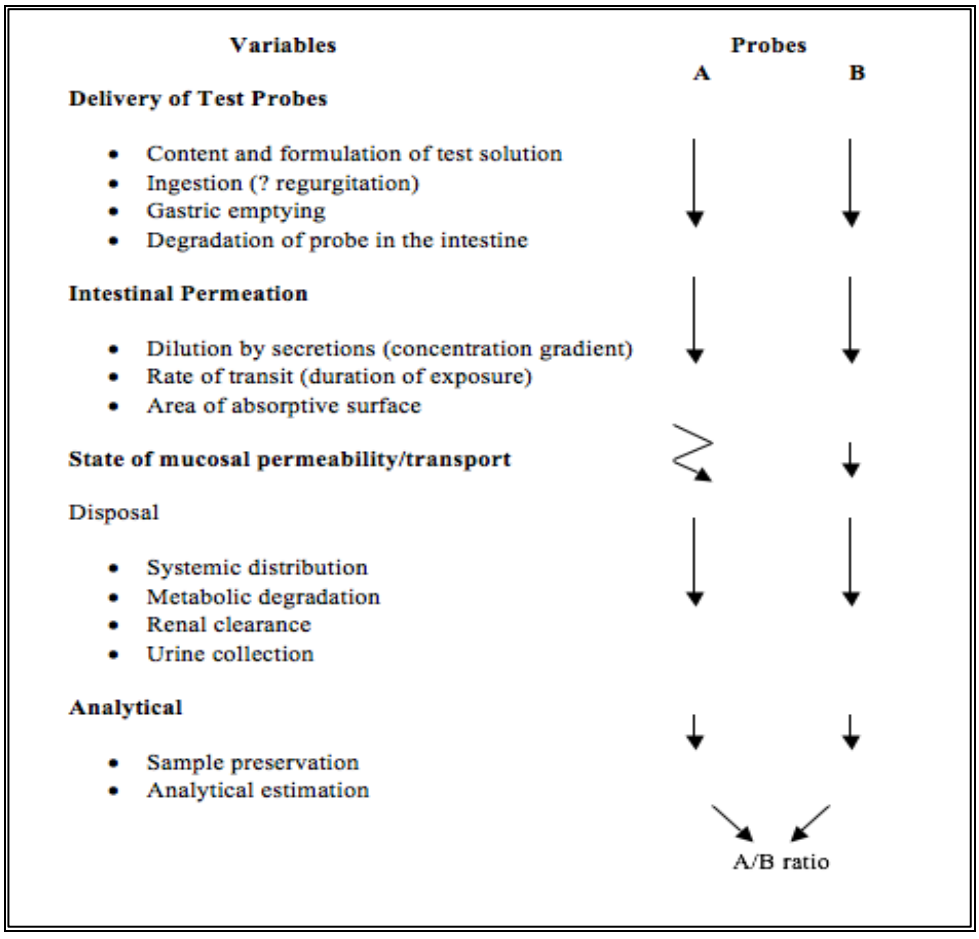


Figure 4: Non-Mucosal factors likely to influence the quantification of GI permeability (Travis and Menzies, 1992).

2.4.1: Urine and blood determination of dual sugar probes

Analysis of intestinal permeability can be performed through urine, plasma or serum sample analysis, through the determination of sugar recovery and ratio. Whilst these tests correlate significantly (van Wijck et al., 2013), urinary concentrations are approximately 100-fold higher than plasma concentrations (Travis and Menzies, 1992). The higher concentrations from urine are argued to make analysis easier and more reliable (Travis and Menzies, 1992). Urine analysis requires the frequent collection of urine from periods of five to twenty-four hours post-exercise in a fasted state. This drawback increases the difficultly of sugar probe assessment before and immediately after exercise, as well as at shorter intervals after the exercise. Inaccuracies in the timing or quality of sample provision and collection (pooling) can also lead to errors in sugar probe concentration (Fleming et al., 1996). However, a longer period of sample collection increases the exposure of the gut to the test sugars, which can contribute to its permeability; this may explain the larger magnitude of permeability demonstrated from urinary analysis (Brunetto et al., 1990). An alternative to urinary analysis is through plasma/serum-based permeability analysis which facilitates logistically easier and more frequent sampling over time. Blood samples taken two hours post ingestion of the sugar-probe solution can be analyzed for the ratios of L/R, therefore reducing the total amount of time for sample collection (Fleming et al., 1996).

2.4.2: Sugar-probe assay dosage and timing

Currently there are no specific standards or guidelines towards the optimal dose of sugar probes to quantify intestinal permeability. Instead, previous research reports the use of a variety of dosages and combinations of probes. For example, in their landmark exercise study, Pals et al. (1997) utilized a test solution composed of 5 g sucrose, 5 g lactulose and 2 g of L-rhamnose diluted in 50 mL of water; this is similar to a test solution used by Lambert et al. (2008) however these authors diluted their probes in 150 mL of water. van Wijck et al. (2011a) used a test solution containing 1 g lactulose, 1 g sucralose, 1 g erythritol, 1 g sucrose and 0.5 g L-rhamnose, diluted in 150 mL tap water; whilst Snipe and Costa (2018a) recently utilized a test solution containing 5 g lactulose and 1 g of L-rhamnose. Together, the different compositions of these test solutions could display different absorption characteristics (Gisolfi et al., 1998; Gisolfi, Lambert and Summers, 2001; Tokuda and Yu, 2019) and therefore affect the appearance of the sugars for subsequent analysis; however, research is yet to investigate this hypothesis.

Similarly, previous research shows discrepancies in the timing of the provision of sugar-probe solution. Test solutions are generally provided acutely around exercise i.e., within thirty minutes preceding and following exercise, or at a point during exercise performance; however, there is no

consistency between studies in terms of administration. Table.3 demonstrates some of the variation in both the composition of the sugar-probe test solution and the point of provision/ingestion.

Table 3: A summary of exercise studies showing variations in sugar probe composition; timing of ingestion; analysis method; and L/R ratio

Authors	Participants	Exercise Protocol	Solution specifics (composition; timing; analysis)	Lactulose/ L-rhamnose ratio
Ryan, Chang and Gisolfi (1996)	*n* = 7 healthy, active males. With or without aspirin ingestion.	60 min treadmill running at ~70% $\dot{V}O_{2\,max}$	10 g lactulose, 5 g mannitol and 10 g sucrose. Immediately prior to exercise. 6-h urinary recovery.	N/A. Data reported as ratio of lactulose/mannitol
Lambert et al. (2001)	*n* = 17 (*n* = 13 male; *n* = 4 female), healthy, recreational runners.	60 min treadmill run at 70% $\dot{V}O_{2\,max}$	5 g lactulose, 5 g sucrose, 2 g rhamnose in 50 mL solution. . Immediately prior to exercise. Urinary recovery	Data not reported
(Lambert et al., 2007)	*n* = 8 (*n* = 6 male; *n* = 2 female), healthy, recreational runners	60 minutes running at 70% $\dot{V}O_{2\,max}$ 24°C, 33% RH	5 g lactulose, 5 g sucrose, 2 g rhamnose in 50 mL solution. . Immediately prior to exercise. Urinary recovery	Exercise 0.66 (0.31-0.81)*+
Lambert et al. (2008)	*n* = 20 male and female endurance runners	60 minutes running at 70% $\dot{V}O_{2\,max}$. 24°C, 33% RH	5 g sucrose, 5 g lactulose and 2 g L-rhamnose dissolved in 150 mL water. Immediately prior to exercise or rest. 5-h urinary recovery.	Rest: 0.035 (0.01-0.11) Exercise: 0.063 (0.02-0.17)*
(Morrison, Cheung and Cotter, 2014)	*n* = 15 males, (*n* = 7 trained, *n* = 8 untrained)	15 min cycling at 50% heart rate reserve > 60 min running (30 min at 80% HRR, 30 min	15 lactulose, 3 g rhamnose in 50mL of water. Immediately prior to exercise. 5-h urine recovery	Data below detection levels.

		distance trial) > 15 min cycling at 50% heart rate reserve. 30°C, 50% RH. With or without bovine colostrum		
Shing et al. (2014)	*n* = 10 male runners	Time to fatigue at 80% ventilatory threshold at 35 °C 40%RH	5 g lactulose, 5 g rhamnose and 5 g sucrose in 100 mL water. Immediately prior to exercise. Urinary excretion.	Data presented as percentage change. Ratios not expressed
Pugh et al. (2017b)	*n* = 11 male endurance trained runners	18 x 400-m intervals at 120% $\dot{V}O_{2\,max}$	5 g lactulose, 2 g rhamnose, 1 g sucrose, 0.5 g D-xylose in 50 mL of water. Urine and serum analysis. 15 minutes pre-exercise	Serum analysis Rest: 0.031 ± 0.021 Exercise: 0.051 ± 0.015.* Urinary Rest: 0.030 ± 0.005 Exercise: 0.032 ± 0.005
(Pals et al., 1997)	*n* = 6 healthy (*n* = male, *n* = 1 female) volunteers.	60 min treadmill running at 40, 60	5 g sucrose, 5 g lactulose, 2 g rhamnose in 50 mL water. 30 minutes during exercise. 5-h urinary recovery.	Rest: 0.048 ± 0.01 40%: 0.056 ± 0.01 60%: 0.064 ± 0.01 80%: 0.107 ± 0.02*

Van Wijck et al. (2012b)	*n* = 9 healthy male cyclists and triathletes	60 minutes cycling at 70 % maximal power output	1 g lactulose, 1 g sucralose, 1 g erythritol, 1 g sucrose, 0.5 g L-rhamnose in 150 mL of tap water. 30 minutes during exercise. 2h urinary recovery	Rest: 0.01 (0.02-0.04) Exercise: 0.03 (0.00-0.20)
Zuhl et al. (2014b)	*n* = 8 endurance trained adults. (*n* = 5 men and *n* = 3 women).	60 min treadmill run at 65-70% of $\dot{V}O_{2\,max}$	5 g lactulose and 2 g rhamnose in 50-mL water. 20 minutes during exercise. 5-h urinary recovery	Rest: 0.0218 $\pm$ 0.008 Exercise: 0.0603 $\pm$ 0.047*
Zuhl et al. (2015)	*n* = 7 endurance trained adults (*n*= 2 male, *n* = female)	60 min treadmill run at 65-70% of $\dot{V}O_{2\,max}$ at 30°C	5 g lactulose and 2 g rhamnose in 50-ml water. 20 minutes during exercise. 5-h urinary recovery	Rest: 0.02 $\pm$ 0.01 Exercise: 0.06 $\pm$ 0.01*
Pugh et al. (2017c)	*n* = 10 recreationally active males	60 min treadmill run at 70% $\dot{V}O_{2\,max.}$ 30 ºC, 40-45% RH	5 g lactulose, 2 g rhamnose. 15 minutes during exercise. Serum analysis	Data not provided
Snipe and Costa (2018a)	*n* = 24 (*n* = 13 male and *n* = 11 female) endurance trained runners.	120 minutes treadmill running at 60% $\dot{V}0_{2\,max}$ in 35°C.	5 g lactulose, 1 g L-rhamnose dissolved in 100 mL water. 90 minutes into exercise. 5-h urine recovery	Post-exercise: males 0.030 (0.017-0.044)$^{+}$ Females (0.028 (0.011-0.046)$^{+}$
Smetanka et al. (1999)	*n* = 34 (n =20 men, *n* = 14 women).	1996 Chicago Marathon	5 g sucrose, 5 g lactulose and 2 g rhamnose dissolved in 40 mL of water. Urinary analysis. 15 minutes	Control: 0.022 $\pm$ 0.01 Runners: 0.019 $\pm$ 0.01*

March et al. (2017)	*n* = 18, healthy males	20 min Treadmill run at 80% $\dot{V}O_{2peak.}$ With or without 14 days colostrum supplementation	Post-race. 5-h urine recovery 5 g lactulose, 2 g of mannitol, 1 g of rhamnose. Immediately post-exercise. 5-hour urinary analysis.	Baseline: 0.35 ± 0.06 Exercise: 0.95 ± 0.12

*= Significant difference, $^{+}$ = data is median (range). Data is shown from placebo arms where a study utilized multiple research interventions

2.4.3: Summary

Differences in the composition and timing of sugar-probe ingestion, exercise stimulus, environmental conditions and analysis methodology makes study comparison difficult. The 'classic' dual-sugar probe assay has been developed to include additional probes to facilitate analysis of permeability to specific regions of the gastrointestinal tract (van Wijck et al., 2011b). Research has shown that dehydration (Lambert et al., 2008), hyperthermia (Pires et al., 2017), exercise intensity (Pals et al., 1997) and NSAIDs (Lambert et al., 2007; Van Wijck et al., 2012b; Zuhl et al., 2014b) can exacerbate intestinal permeability. However, research is warranted to investigate how the timing of sugar-probe assay administration, in relation to exercise performance, impacts the reported magnitude of intestinal permeability.

2.5: Assessment of intestinal injury: Novel biomarkers.

In assessing GI dysfunction there is a subtle distinction to be drawn between GI permeability and GI injury with the latter not being synonymous with the former. In response to enterocyte tissue injury, cellular proteins known as biochemical markers are released into the plasma. One of these biomarker proteins is fatty acid-binding protein (FABP), a cytosolic protein with an atomic mass of 15 kDa, expressed in abundance within tissues with a dynamic fatty acid metabolism, such as the heart, liver and intestine (Pelsers, Hermens and Glatz, 2005). The cellular expression of FABPs is primarily regulated at the transcriptional level, responsive to alterations in lipid metabolism. Whilst the primary function of FABPs is to facilitate transport of intracellular long-chain fatty acids, secondary functions include the regulation of gene expression through mediation of fatty acid signal translocation to peroxisome proliferator activated receptors (PPARs) (Wolfrum et al., 2001; Pelsers, Hermens and Glatz, 2005).

Exercise induced ischemia causes disruption to the integrity of the mature enterocytes located at the apical region of the intestinal villi, as these are most affected by hypoxia since they are already in a state of low oxygen. (Barberio et al., 2015; Grootjans et al., 2016). The further reduction in oxygenation results in dysfunction to the epithelial membrane and subsequent release of I-FABP into the plasma (van Wijck et al., 2011a), making I-FABP a convenient marker of intestinal damage (Derikx et al., 2009). Research has shown that both steady state (van Wijck et al., 2011a; Barberio et al., 2015) and high-intensity interval (Kashima et al., 2017) endurance exercise can result in a release of I-FABP into the plasma. Interestingly, ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) can exacerbate this response, causing an augmented release of I-FABP when compared to exercise alone and even at rest (Van Wijck et al., 2012b).

Whilst there is currently no normative data regarding resting levels of I-FABP in healthy individuals, the majority of exercise-based studies report resting levels of I-FABP ranging from

approximately 200 to 300 picograms per milliliter (pg·mL^{-1}) (van Wijck et al., 2011a; Van Wijck et al., 2012b; Sessions et al., 2016; Karhu et al., 2017). However, resting values of over 600 pg.mL^{-1} have also been reported (Barberio et al., 2015; March et al., 2018), suggesting that a standardized protocol for I-FABP collection and analysis is warranted, as cross-comparison between studies is difficult. Post-exercise values of I-FABP seem to be approximately two-fold of resting values, despite differences in exercise prescription (running vs. cycling and steady state vs. high intensity interval training) and duration. For example, thirty minutes of intermittent cycling returned similar changes to pre- and post-exercise I-FABP values than ninety minutes of treadmill running (Karhu et al., 2017; Kashima et al., 2017). There also appears to be a large amount of deviation from the mean values. Furthermore, comparison between studies is difficult due to differing reporting methods, for example, some researchers report changes in mean values in pg·mL^{-1} (Barberio et al., 2015; Karhu et al., 2017), whilst some report changes in percentage from baseline (Snipe and Costa, 2018b), median and interquartile range (March et al., 2018), or magnitude-based inferences (Pugh et al., 2017c).

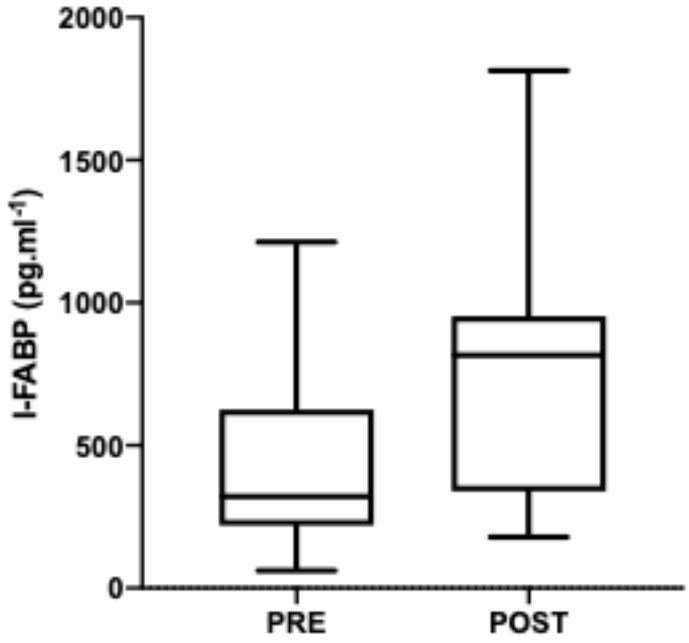


Figure 5: Summary data showing median changes in I-FABP concentrations (pg·mL-1) between pre-exercise (with SDs) and post-exercise (with SDs) across exercise studies *(Lieberman et al., 1997; Pelsers et al., 2003; Pelsers, Hermens and Glatz, 2005; van de Poll et al., 2007; Funaoka, Kanda and Fujii, 2010; van Wijck et al., 2011a; Van Wijck et al., 2012b; Ishimura et al., 2013; Guzel et al., 2014; Morrison, Cheung and Cotter, 2014; Uzun et al., 2014; Barberio et al., 2015; Lis et al., 2015; Grootjans et al., 2016; Sessions et al., 2016; Sun et al., 2016; Karhu et al., 2017; Kashima et al., 2017; March et al., 2017; McKenna et al., 2017; Pugh et al., 2017c; Snipe et al., 2017; Jonvik et al., 2018; Sheahen et al., 2018; Snipe and Costa, 2018b; Snipe and Costa, 2018a).*

Plasma D-lactate, one of two stereoisomeric forms of lactate (D and L), is a byproduct of bacterial fermentation in the GI tract, and another biomarker which has been identified as a potential indicator

of intestinal injury (Evennett et al., 2009). Both forms of lactate are produced from and metabolized to pyruvate by the action of the enzyme lactate dehydrogenase (LDH). However, the enzyme is isomer-specific so that production and metabolism of D-lactate requires D-LDH and L-lactate requires L-LDH. Metabolic production of D-lactate in humans is possible despite not having D-LDH and may occur as a result of the methylglyoxal pathway, a pathway of glycolysis that results in nanomolar concentrations of methylglyoxal, a neurotoxic product that is converted to D-lactate (Uribarri, Oh and Carroll, 1998; Ewaschuk, Naylor and Zello, 2005). In healthy individuals, a low level of circulating D-lactate is normal (5-20 μmol/L in healthy adults), however, under circumstances of intestinal barrier dysfunction circulating levels increase due to augmented translocation from within the GI tract into the plasma (Grootjans et al., 2010b). Indeed, plasma D-lactate has been shown to increase in response to exercise in trained participants (Kondoh, Kawase and Ohmori, 1992) but correlation with exercise duration or intensity has been inconsistent (see Chapter 5). Additionally, the test is also sensitive to precision detection methods, with the enzyme-linked immunosorbent assay technique returning a higher degree of range and precision than a rapid assay UV colour-metric technique (see Chapter 5). Therefore, further research is required to investigate the methodologies and efficacy of D-lactate as a marker of intestinal permeability and injury.

Table 4: Summary of studies demonstrating the response in I-FABP concentrations to exercise

Authors	Participants	Protocol	Outcome
van Wijck et al. (2011a)	*n* = 15 healthy, endurance trained males	60 min cycling at 70% maximum workload capacity	I-FABP increased from 309 ± 46 $pg \cdot mL^{-1}$ to 615 ± 118 $pg \cdot mL^{-1}$
Van Wijck et al. (2012b)	*n* = 9 healthy males, cyclists or triathletes	60 minutes cycling at 70 % maximal power output. With or without ibuprofen	I-FABP increased from 295 ± 46 $pg \cdot mL^{-1}$ to 474 ± 74 $pg \cdot mL^{-1}$. Ibuprofen resulted in higher I-FABP levels (baseline: 328 ± 32 $pg \cdot mL^{-1}$ to 875 ± 137 $pg \cdot mL^{-1}$)
Morrison, Cheung and Cotter (2014)	*n* = 15 males, (*n* = 7 trained, *n* = 8 untrained)	15 min cycling at 50% heart rate reserve > 60 min running (30 min at 80% HRR, 30 min distance trial) > 15 min cycling at 50% heart rate reserve. 30°C, 50% RH. With or without bovine colostrum	Trained: baseline: 143 ± 59 $pg \cdot mL^{-1}$; post-exercise: 949 ± 423 $pg \cdot mL^{-1}$ Untrained: baseline: 160 ± 93 $pg \cdot mL^{-1}$; post-exercise: 443 ± 260 $pg \cdot mL^{-1}$ No effect from bovine colostrum on I-FABP levels
Barberio et al. (2015)	*n* = 8 healthy, endurance trained males	5 consecutive days of exercise and heat exposure (40°C, 40% RH). Intensity at 4 mM blood lactate until exhaustion	I-FABP significantly increased from 640.2 $pg \cdot mL^{-1}$ ± 125.0 (rest) to 936.7 $pg \cdot mL^{-1}$ ± 149 (post-exercise). Mean data from Days 1-3
Lis et al. (2015)	*n* = 13 competitive cyclists (*n* = 8 male, *n* = 5 female)	45 min cycle at 70% peak power, followed by 15-minute time-trial. 7-day gluten or gluten-free diet	I-FABP increased from 94 ± 83 and 99 ± 57 $pg \cdot mL^{-1}$ to 304 ± 191 and 301 ± 252 $pg \cdot mL^{-1}$ in the gluten and gluten-free trials, respectively.

Sessions et al. (2016)	*n* = 7 healthy, endurance trained athletes. (*n* = 5 male, *n* = 2 female)	60 min treadmill run at 70% $\dot{V}O_{2\ max}$. 30°C, RH 12-20%. With or without carbohydrate gel	Carbohydrate: baseline: 261.74 ± 160.27 pg·mL^{-1}; post-exercise: 524 ± 381.25 pg·mL^{-1} Placebo: baseline: not-reported; post-exercise: 337.96 ± 207.38 pg·mL^{-1}
Karhu et al. (2017)	*n* = 17 active runners, 9 asymptomatic and 8 symptomatic for GI symptoms.	Treadmill run at 80% of 10-km race speed. Thermo-neutral conditions	Asymptomatic runners showed significantly high increase from baseline to post-exercise (314 ± 152 pg·mL^{-1} vs. 804 ± 599 pg·mL^{-1}). Symptomatic (baseline: 389 ± 327 pg·mL^{-1}; post: 961 ± 949 pg·mL^{-1})
March et al. (2017)	*n* = 18, healthy males	20 min Treadmill run at 80% $\dot{V}O_{2peak.}$ With or without 14 days colostrum supplementation	Placebo: baseline: 578 (399) pg·mL^{-1}; post-exercise: 928 (382) pg·mL^{-1} Colostrum: baseline: 672 (394) pg·mL^{-1}; post-exercise: 684 (481) pg·mL^{-1}. Data presented as median and (interquartile range)
McKenna et al. (2017)	*n* = 10, active, healthy males	46 ± 7.75 min run at 95% ventilator threshold. 40°C, 50% RH. 14-day supplementation with bovine colostrum or placebo	No significant difference between conditions. Relative increase from baseline to post-exercise: Col 162 ± 50%, Pla 162 ± 56%
Pugh et al. (2017c)	*n* = 10, recreationally active, healthy males	60-min treadmill run at 70% $\dot{V}O_{2\ max}$ (30°C). Placebo, 0.25, 0.5 and 0.9 g kg^{-1} fat-free mass.	Glutamine supplementation was possible or likely to reduce pre-exercise I-FABP. Glutamine at 0.5 g kg^{-1} and 0.9 g kg^{-1} was likely to lower post-exercise I-FABP.

Jonvik et al. (2018)	*n* = 16, well-trained male athletes	60 min cycling at 70% Watt-max.	~250% increase in plasma I-FABP levels in the placebo trial. Sucrose ingestion reduced I-FABP elevation (180% from baseline).
Sheahen et al. (2018)	*n* = 12, physically active males.	45-min cycling at 70% $\dot{V}O_{2\,max}$.30°C , 40%RH or 20°C, 40%RH. Rest, 30°C, 45% RH.	I-FABP levels significantly increased in both exercise trials, but not the resting trial (*n* = 5 participants). Pre: temp 571 ± 175 pg·mL^{-1}vs hot 585 ± 188 pg·mL^{-1} Post: temp 852 ± 317 pg·mL^{-1}vs hot 954 ± 411 pg·mL^{-1}
Snipe and Costa (2018b)	*n* = 12, endurance trained runners (*n* = 6 male, *n* = 6 female)	2h treadmill running at 60% $\dot{V}O_{2\,max}$ in hot (35°C) conditions. Participants received water at COLD (0.4 ± 0.4°C), COOL (7.3 ± 0.8°C), or TEMP (22.1 ± 1.2°C).	I-FABP increased by 419% between pre- and post-exercise. COLD and COOL water reduced I-FABP, mean reduction 460 pg·mL^{-1} and 430 pg·mL^{-1}, respectively.
Snipe and Costa (2018a)	24 endurance trained runners (*n* = 13 male, *n* = 11 female).	2h treadmill running at 60% $\dot{V}O_{2\,max}$ in hot (35°C) conditions.	I-FABP increased 479% pre- to post-exercise. No observed difference between sexes.

2.6: Effects of exercise on gastrointestinal permeability

2.6.1: Reduced splanchnic blood flow and oxygen availability

The release of norepinephrine close to the alpha-sympathetic nervous system during exercise promotes vasoconstriction and thus increasing vascular resistance in the splanchnic area. Thus, blood flow is rapidly redistributed away from the gut in favor of the periphery, working muscle and cardiovascular system so-as-to facilitate thermoregulation and muscle metabolism. The most pronounced decrease in perfusion occurs within the first ten minutes of exercise, returning to baseline within one hour of exercise (Qamar and Read, 1987; Matheson, Wilson and Garrison, 2000; Rehrer et al., 2001; van Wijck et al., 2011a). This hypoperfusion leads to decreased villus oxygenation and subsequent mesenteric ischemia. As a consequence of ischemia, mesenteric acidosis increases along with a local depletion of ATP, and this cascade of events ultimately results in dysfunction of the tight junction proteins leading to mucosal/enterocyte injury, dysfunction of the GI barrier and increase in intestinal permeability (van Wijck et al., 2012a; Grootjans et al., 2016).

The magnitude of intestinal injury and permeability has been correlated with that of exercise-induced hypoperfusion (van Wijck et al., 2011a; ter Steege et al., 2012). Intestinal injury is predicated upon an initial ischemia and a subsequent reperfusion stress which sees upregulated NF-κB gene expression within the epithelial cells, which signals the release of pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ, and a subsequent local inflammatory cascade (Costa et al., 2017b). Interestingly, the reperfusion of the organs can also cause an inflammatory response, further intestinal damage and impaired gastrointestinal integrity (Derikx et al., 2008b; Grootjans et al., 2010a). This inflammatory response and subsequent injury to the epithelial lining has been observed following forty-five (Grootjans et al., 2010a), but not thirty (Matthijsen et al., 2009) minutes of ischemia in a section of the jejunum during surgical procedures. These findings suggest that the duration of ischemia could be an important factor as relates to the extent of intestinal barrier dysfunction induced by transient hypoperfusion. However, questions remain regarding the underlying mechanism between these two timeframes, furthermore these trials were conducted in clinical situations, with complete blood flow reduction and not during exercise trials. Whether the same response is witnessed following similar duration of exercise remains to be investigated, yet, research of this nature would have to also consider the contribution of exercise-induced hyperthermia which rises in accordance with exercise duration (Gleeson, 1998).

2.6.2: Exercise intensity

Current literature regarding the influence of exercise intensity on intestinal permeability is inconsistent. It would appear, however, that for exercise to have an influence on intestinal permeability, a minimum

intensity of 70 % $\dot{V}O_{2\,max}$ is required (Pals et al., 1997; Dokladny, Zuhl and Moseley, 2016; Costa et al., 2017b). Only one study has purposefully investigated a possible relationship between exercise intensity and intestinal permeability (Pals et al., 1997). These authors compared the effects of sixty minutes of treadmill running at 40, 60 and 80 % $\dot{V}O_{2peak}$ in six (*n = 5* males and *n = 1* female) healthy participants (mean $\dot{V}O_{2peak}$ = 57.7±6 2.1 $mL \cdot kg^{-1} \cdot min^{-1}$). They reported a significant increase in L/R appearance in urine during the 80 % $\dot{V}O_{2peak}$ trial, but no linear association between exercise intensity and intestinal permeability. The results of this study appear contradictory however, as running at 60 % $\dot{V}O_{2peak}$ returned a lower increase in L/R appearance than running at 40 % $\dot{V}O_{2peak}$. Furthermore, other research has shown a dose-response effect with other factors such as heat stress (Dokladny, Moseley and Ma, 2006).

The relationship between exercise intensity and intestinal permeability (and injury) is further contradicted by some studies (Lambert et al., 2008; Yeh, Law and Lim, 2013) showing both an increase and no change in permeability whilst running for 60 minutes at 70 % $\dot{V}O_{2\,max}$. These studies demonstrated that additional physiological stress, such as dehydration (Lambert et al., 2008) or environmental (hyperthermia) challenge (Yeh, Law and Lim, 2013) may be required for intestinal permeability to increase, at least whilst exercising at 70 % of $\dot{V}O_{2\,max}$.

Despite these observations, exercise above 80 % $\dot{V}O_{2\,max}$ consistently shows an increase in intestinal permeability relative to rest (Pals et al., 1997; Marchbank et al., 2011; Pugh et al., 2017b). However, studies utilizing intensities at or above 80 % $\dot{V}O_{2\,max}$ are limited, perhaps due to the difficulty in maintaining this exercise intensity for a prolonged duration. However, (Pugh et al., 2017b), utilized a high-intensity intermittent treadmill running protocol (18 x 400m runs at 120% 80 % $\dot{V}O_{2\,max}$). The results demonstrated a ~60 % increase in L/R appearance (permeability), and a 72 % increase in I-FABP appearance (injury) when compared to rest.

The contributory role of exercise intensity towards intestinal permeability has thus far returned contradictory results and requires further clarification. Research has suggested that a minimum exercise intensity threshold of 70 % of $\dot{V}O_{2\,max/peak}$ is required, combined with other stress factors, such as heat or dehydration, to elicit an increase in intestinal permeability. Specifically, research is warranted investigating a possible dose-response relationship between exercise intensity and intestinal dysfunction.

2.6.3: Exercise duration

Recent years have seen an increase in participation in endurance sports such as running or cycling; furthermore, the prevalence of ultra-events, which are classified as races over the standard marathon distance, has also increased (Nikolaidis and Knechtle, 2018). Endurance athletes frequently report the appearance of GI symptoms and, indeed, the majority of research has utilised an endurance exercise model consisting of treadmill running or cycling (de Oliveira, Burini and Jeukendrup, 2014; Costa et

al., 2017b). Yet, exercise duration *per se* has received little focus with regard to its effect on intestinal dysfunction. Little is known about the effects of exercise duration on GI dysfunction; indeed, no research exists directly comparing the effects of varying durations of exercise on intestinal dysfunction.

Current literature examining intestinal dysfunction has implemented a range of exercise protocols (Table 3), including short, high-intensity interval bouts (Pugh et al., 2017b) and marathon running (Buchman et al., 1999; Smetanka et al., 1999; Pugh et al., 2019). Furthermore, (Gill et al., 2015) explored the endotoxin and cytokine response to ultra-marathon running but did not assess intestinal permeability or injury. However, the majority of laboratory-based research has employed a protocol of sixty minutes of exercise, typically at an intensity between 60-70 % $\dot{V}O_{2\,max}$ (Pals et al., 1997; Lambert et al., 2007; Lambert et al., 2008; van Wijck et al., 2011a; Van Wijck et al., 2012b; Yeh, Law and Lim, 2013). Results from field-based studies of ultra-events have reported an increase in endotoxin concentrations and cytokine profiles, and a high incidence of athletes (>75%) reported symptoms of GI discomfort (Gill et al., 2015; Stuempfle and Hoffman, 2015). Pugh et al. (2017b) investigated the intestinal permeability response to 18 repeats of 800m treadmill running. Whilst resting values are similar to those observed from other studies (Zuhl et al., 2014b; March et al., 2017), they observed a ~60 % increase in *serum* L/R ratio when compared to rest (0.051 $\pm$ 0.016 vs. 0.031 $\pm$ 0.031 $\pm$ 0.021). However, urinary analysis showed no difference (0.032 $\pm$ 0.005 vs. 0.030 $\pm$ 0.005), The *serum* post-exercise values from this study are similar to those observed from 60 minutes of treadmill running at 70 % $\dot{V}O_{2\,max}$ (Lambert et al., 2008; Zuhl et al., 2014b), yet higher than those following a marathon race (Smetanka et al., 1999).

Research is lacking into the response in splanchnic hypoperfusion and ischemic response to exercise, particularly in running, most likely due to the difficulty in measurement with ultrasonography (van Wijck et al., 2011a). As such, research has typically used cycling as an exercise model and only quantified the response in intestinal blood flow (Rehrer et al., 2001; Hayashi, Ikemura and Someya, 2011). One study demonstrated that fifteen minutes of treadmill walking at 5 km/h (20 % incline) was able to reduce mesenteric blood flow by 43 %, (Qamar and Read, 1987), however, no effects on intestinal permeability or injury were investigated. Effectively, longer periods of ischemia may increase the likelihood of intestinal injury, clinical studies have shown that 45 minutes of ischemia resulted in an increased inflammatory response and epithelial injury, whilst 30 minutes of ischemia did not (Matthijsen et al., 2009; Grootjans et al., 2010a). Furthermore, (van Wijck et al., 2011a) were able to show that 60 minutes of cycling at 70 % $\dot{V}O_{2\,max}$ was able to induce splanchnic hypoperfusion and causes GI dysfunction. Together, it could be hypothesised that exercise duration influences gastrointestinal dysfunction due to the longer periods of reduced blood flow and consequently reduced oxygen availability in the mesenteric organs. However, future research should aim to investigate the effects of exercise duration alone on intestinal dysfunction and symptoms.

2.6.4: Hyperthermia

Functionally, blood circulation helps the transfer and exchange of metabolic heat to other body tissues for dissipation via evaporative cooling, thus facilitating maintenance of homeostasis and regulation of core temperature (T_{core}). The core temperature of humans is maintained within a fine range at approximately 37°C and will only deviate outside of this range under certain conditions such as during exercise (Gisolfi and Wenger, 1984; Pires et al., 2017), environmental stress (Cramer and Jay, 2016) or pathophysiological conditions such as illness or heat stroke (Lambert, 2004). During exercise, core temperature can rise by 1°C every five minutes if unmitigated, but temperature regulatory mechanisms work to establish a balance between heat production and loss (Nadel et al., 1977). Such mechanisms are efficient, as core temperature during a marathon can be maintained between 39 and 40°C (Cheuvront and Haymes, 2001). However, environmental conditions (such as hot temperatures), dehydration, obesity, or clothing which limits the body's evaporation capabilities, can cause a significant challenge to human thermoregulation. In these conditions, the temperature gradient required for radiative and convective heat loss becomes compromised, leading to an increased storage of heat within the body (Nadel et al., 1977; Coris, Ramirez and Van Durme, 2004). The progression of heat accumulation within the body begins the process of heat-illness, which can manifest into a cascade of physiological events that, if not addressed, can lead to severe heat stroke and death. Following heat-stress, symptoms of heat illness can develop, indicated by an elevated core temperature up to 40.5°C, such as heat cramps, syncope, mild confusion, nausea and vomiting. If heat strain is allowed to progress, heat stroke may occur, whereby core temperature exceeds 40.5°C (Coris, Ramirez and Van Durme, 2004). Indeed, heat stroke can develop into a life-threatening medical condition, and increased intestinal permeability is thought to be a contributing factor. Augmented permeability facilitates increased transport of harmful endotoxin contained within the gut into the portal blood, which subsequently causes a systemic inflammatory response, which can manifest into multiple organ failure and death (Ogden et al., 2020).

Passive heat stress is defined as "heat stress that occurs through increases in heat gain via exogenous sources at a time when metabolism remains at relatively basal levels" (Crandall and Wilson, 2015). Protocols typically involve methods such as immersion in hot water, environmental chambers or water-perfused suits to increase core temperature (Miwa et al., 1994; Sheahen et al., 2018). A review by Crandall and Wilson (2015) explains that passive heat stress results in a plethora of physiological responses including increases in catecholamines, cardiac output and heart rate. Further responses to passive heat stress, specifically HWI, include increases in subcutaneous and cutaneous blood flow, whilst blood flow through the muscle may be decreased (Bonde-Petersen, Schultz-Pedersen and Dragsted, 1992). This is in contrast to immersion in thermoneutral water, where an increase blood flow through muscle beds may be observed due to a decrease in peripheral resistance with an accompanied increase in cardiac output (Wilcock, Cronin and Hing, 2006). Splanchnic blood flow has also been shown to decrease by approximately 40% in response to passive heat stress (Rowell, 1974).

Alternatively, exertional heat stress studies use an exercise model with the addition of heat stress, induced by clothing (McLellan, Boscarino and Duncan, 2013) or elevated environmental temperatures (Ogden et al., 2020). The addition of clothing or hot water immersion (HWI) effectively attenuate loss by limiting radiative and convective processes, thereby creating a condition of uncompensable heat stress (UHS) (Cheung, McLellan and Tenaglia, 2000). Interestingly, examination of exertional heat stress studies reveals a lack of resting control conditions, instead, the majority of passive hyperthermia studies are found from HWI in cardiac research (An, Lee and Yi, 2019). Furthermore, research has failed to investigate the effects of passive hyperthermia on intestinal permeability in humans (Dokladny, Zuhl and Moseley, 2016).

Animal and *in vitro* studies have indicated that passive hyperthermia, induced by environmental heat stress, can increase intestinal permeability (Dokladny, Zuhl and Moseley, 2016). Yet, research is lacking as to the effects of passive hyperthermia on intestinal permeability in humans; likely due to ethical restrictions of increasing core temperature purposefully towards the levels of heat stress/stroke (Dokladny, Zuhl and Moseley, 2016). Sheahen et al. (2018) observed no increase in core temperature or plasma I-FABP levels (injury biomarker) following 45 minutes of passive heat stress at 30°C in twelve physically active men. Unfortunately, intestinal permeability was not quantified. However, the addition of exercise at 70% $\dot{V}O_{2\ max}$ in temperatures of 30°C and 20°C significantly increased I-FABP and core temperature. Exercise in 20°C and 30°C resulted in a peak core temperature of 38.2°C and 38.3°C, respectively; whilst post-exercise levels of plasma I-FABP were similar in both conditions (852 $\pm$ 317 pg·mL^{-1}and 954 $\pm$ 411 pg·mL^{-1}, respectively).

Pires et al. (2017) recently conducted a systematic review to investigate a possible relationship between the exercise-induced rise in core temperature and augmented intestinal permeability. They concluded a direct association between increased intestinal permeability and the magnitude of rise in core temperature. They explained that a core temperature of 38.5°C was associated with augmented intestinal permeability in some individuals, but when core temperature exceeded 39^{o}C, increased intestinal permeability was universal amongst participants (Pires et al., 2017). This finding was in agreement with a previous estimate that a core temperate range of 38.2-39.6 °C, together with exercise performed at an intensity equivalent to 70 % of $\dot{V}O_{2\ max}$, resulted in augmented intestinal permeability (Dokladny, Zuhl and Moseley, 2016). Further, in vitro studies have also reported a dose dependent effect of heat stress on tight-junction integrity, with greater permeability being reported with increasing heat stress (Dokladny, Moseley and Ma, 2006; Yang, He and Zheng, 2007; Davison et al., 2016).

Interestingly Pals et al. (1997) also reported a correlation between increased intestinal permeability and rising core temperature whilst investigating the role exercise intensity has on GI permeability. Running at 40, 60 and 80 % of $\dot{V}O_{2\ max}$ for 60 minutes resulted in mean peak core temperatures of 38.0 $\pm$ 0.11^{o}C; 38.7 $\pm$ 0.11 ^{o}C and 39.6 $\pm$ 0.15 ^{o}C, respectively. In this study, increased permeability was only significant following running at 80 % of $\dot{V}O_{2peak}$ which also correlated with the highest peak core temperature.

Passive hyperthermia has been shown to reduce splanchnic blood flow and induce intestinal barrier dysfunction in rodents (Hall et al., 2001b), but to date, no studies have investigated the response in intestinal permeability to passive heat stress in humans. Currently data is only available from case studies of humans suffering from heat stroke that co-present with other morbidities or are elderly (Ogden et al., 2020). As such, it is presently unclear whether the increase in intestinal permeability associated with increased core temperature occurs independent of exercise-induced splanchnic ischemia. Research has shown an increase in blood flow to the periphery under passive heat stress (Ogoh et al., 2013) which would indicate a reduction in splanchnic blood flow by proxy. However, research is warranted to investigate a possible correlation between a non-exertional rise in core temperature and thus minimal influence of splanchnic hypoperfusion, and intestinal permeability.

2.6.5: The effect of glutamine on intestinal permeability

Glutamine is the most abundant amino acid in human muscle and plasma, and the preferred fuel source of the enterocytes of the GI system (Newsholme et al., 2003). Plasma glutamine levels are maintained at approximately 550 - 750 µmol/L, but can be significantly reduced following exercise and conditions such as shock or trauma. The gastrointestinal tract plays a pivotal role in the response to infection and injury, with prolonged periods of stress or trauma seeing a reduction in the glutamine pool, and subsequent mucosal injury (Rao, Baker and Baker, 1999). However, oral glutamine supplementation has been shown to offset mucosal injury and, furthermore, attenuate gastrointestinal permeability and injury (Soares et al., 2014; Zuhl et al., 2014b; Pugh et al., 2017c). Glutamine provides a major source of energy for the rapid proliferation of the epithelial cells of the GI mucosa. As such, glutamine may be an effective mitigation strategy against stressors which may compromise GI barrier integrity and injury (Rao and Samak, 2012). Animal studies have demonstrated glutamine supplementation to attenuate GI injury in response to ischemia/ reperfusion (Mondello et al., 2010), whilst human studies have shown glutamine to attenuate GI permeability in response to exercise and heat stress (Zuhl et al., 2015; Pugh et al., 2017c). The underlying mechanisms behind the protective effect of glutamine on the GI tract is complex and beyond the scope of this review, and has been explained elsewhere (Rao and Samak, 2012).

Over twenty years ago, Hond et al. (1999) demonstrated that an acute dose of 7 g of glutamine was able to attenuate GI permeability in response to non-steroidal anti-inflammatory drug (NSAID) ingestion in healthy people. Fourteen years later, (Zuhl et al., 2014b) showed that 7 days of glutamine supplementation at 0.9g.kg^{-1} fat free mass was able to prevent an increase in intestinal permeability in response to 60 minutes of treadmill running at 65-70% $\dot{V}O_{2\,max}$, when compared to placebo. Glutamine supplementation has since been shown to offset GI permeability in a dose-response manner; where doses of 0.25 g.kg^{-1}, 0.5 g.kg^{-1} and 0.9 g.kg^{-1} of glutamine were provided two hours before 60 minutes of treadmill running at 70% $\dot{V}O_{2\,max}$ in 30°C heat (Pugh et al., 2017c). However, this study failed to

include an exercise only condition, so the relative contribution of heat is difficult to conclude. Research is scarce investigating GI permeability and injury in humans, in response to passive heat stress. Rodent models (rats) subjected to hyperthermia by increasing core temperature to 42°C, after five days of glutamine supplementation at 0.9 $g.kg^{-1}$ observed enhanced the heat shock protein (HSP) response, significantly reduced intestinal permeability, plasma endotoxin, and decreased mortality. Given the promising scope of glutamine to ameliorate GI permeability and injury in response to heat stress, further consideration is warranted.

2.6.6: Summary

Despite an abundance of research investigating the characteristics and mechanisms of GI dysfunction, the complex nature of the gastrointestinal tract and differing methodologies used in previous research leave many questions still unanswered. Whilst researchers have explored the response in GI permeability and injury to different exercise stresses such as intensity (Pals et al., 1997) or modality e.g. running versus cycling (van Nieuwenhoven, Brouns and Brummer, 2004; Karhu et al., 2017), we do not yet know at which point (in relation to exercise) that maximum permeability occurs. Further investigation is also required to understand the mechanistic contributions of exercise-induced hypoperfusion and hyperthermia, as we do not yet know which factor is responsible for the largest magnitude of intestinal barrier dysfunction. It has been hypothesised that exercise-induced intestinal injury could be due to hypoperfusion alone, or in combination with the subsequent reperfusion of the epithelium (van Wijck et al., 2012a). However, this hypothesis fails to consider the contribution of hyperthermia to intestinal barrier dysfunction. No data are available that have compared the contribution of increased core temperature or hypoperfusion to intestinal dysfunction. An investigation of this hypothesis would prove difficult as an increase in core temperature and a reduction in mesenteric blood flow would have to occur independent of each other, or to an extent where one factor would not impact on the other. This is technically difficult as exercise induces a mesenteric hypoperfusion as well as elevated core temperature. Similarly, questions remain regarding intestinal damage in response to passive hyperthermia. Increased I-FABP concentrations have been recorded in patients with heat stroke (Zhang et al., 2015) but data is lacking regarding whether passive hyperthermia results in intestinal tissue injury. The use of passive heating methods, however, provides a controlled methodological approach by allowing elevation of core temperature through environmental stress whilst maintaining exercise at an intensity which is known to elicit a level of hypoperfusion that does not result in gastrointestinal permeability. Furthermore, since glutamine has shown positive outcomes on intestinal permeability and injury in response to exercise and heat stress, more research is required investigating the effects of glutamine on passive heat stress, such research could provide scope for nutritional interventions for athletes exercising in the heat.

Chapter 3: General Methods

3.0 Introduction

The following section provides and overview of the core philosophies behind the methods employed in the subsequent research studies and a rationale for their use. Where appropriate, unique methods and protocols are described in the specific chapter.

3.1 Ethical Approval and list testing locations

All of the research studies conducted for this thesis received ethical approval by the local committee of Liverpool John Moores University. All participants received written and verbal descriptions of the testing procedures and provided informed consent prior to participation. Participants were made aware of the risks of participation, with written inform consent being given in the presence of a third-party witness. All exercise testing, including maximal oxygen uptake ($\dot{V}O_{2\ max}$.) and experimental trials were completed within in the Sport and Exercise Laboratories at the University. Analysis of serum lactulose and L-rhamnose was completed at Royal Cornwall Hospital Trust, Cornwall, UK by an accredited laboratory.

3.2 Participants

Participants were male and female, healthy, non-smokers who were free from gastrointestinal and neurological disease, and muscoskeletal abnormality. Inclusion criteria also included being recreationally active, training for a minimum of four hours per week. Participants were excluded if they reported using nutritional supplements at the time of recruitment. Further exclusion criteria was that participants were currently not engaged in another research study. Whilst heat acclimation status was not an inclusion/exclusion factor, none of the participants reported recently returning from a hot environment. Participants were asked to abstain from NSAID ingestion, exercise, alcohol and spicy foods in the 24 hours preceding laboratory visits. A minimum of three, and maximum of 7 days were scheduled between tests. These procedures were in place in light of the turnover rate of the small intestine enterocytes; whereby whole intestinal enterocyte turnover is complete within 72 hours.

Table 5: Summary of participant characteristics

	Chapter 4	Chapter 5	Chapter 6	Chapter 7
Participants (*n)*	9	10	6	6
Age (years)	34 ± 7	29 ± 6	31 ± 16	23 ± 4
Height (cm)	177.8 ± 2	173.0 ± 7.9	175.2 ± 5.4	-
Body Mass (kg)	77.2 ± 7.1	69.9 ± 10.1	74 ± 4.5	69.9 ± 4
$\dot{V}O_{2\,max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	57.8 ± 5.8	55 ± 6	53.6 ± 2.6	64.3 ± 5.9

3.3 Anthropometry

Following the provision of informed consent, each participant underwent assessment of anthropometry during the first visit of his respective trial. Using an audiometer (SECA 213, SECA, UK), height was measured and recorded in centimetres (cm) to one decimal point. Weight was also recorded using digital column scales (SECA 704, SECA, UK) and recorded in kilograms (kg) to one decimal point.

3.4 Cardiorespiratory Measurements

3.4.1 Maximal oxygen uptake

All participants completed a graded exercise test on a motorised treadmill (HP Cosmos, Germany) for determination of maximal oxygen uptake. Prior to beginning the test, a facemask (with attached volume sensor) was fitted to the participant using an adjustable head-strap; a safety harness was also fitted. The treadmill running test began at 8.0 $km \cdot h^{-1}$ and increased by 2.0 $km \cdot h^{-1}$ every 2 minutes until 16 km·h-1 thereafter the gradient increased by 2 % until volitional exhaustion. Breath-by-breath gas analysis was performed throughout the duration of the test using an online gas analysis metabolic cart (Oxycon Pro, Jaeger, Germany). The metabolic cart was calibrated with a 5 % C02 / 16 % Oxygen / Nitrogen mixture, whilst the volume transducer was calibrated with a three-litre calibration syringe (Jaeger, Wuerzberg, Germany). Laboratory environmental conditions were recorded during all exercise testing procedures and remained constant with a temperature at 22 + 1ºC and relative humidity at 48 + 3%. Maximal oxygen consumption was assessed by the attainment of the following criteria: (1) a plateau in $\dot{V}O2$ despite increases in external work, (2) maximal respiratory exchange ratio (RER) > 1.1, and (3) maximal HR within 10 b/min of the age-predicted maximum (220 – age). Peak $\dot{V}O_2$ was determined from the mean of the last 15 seconds of each 2-minute interval.

3.4.2 Heart Rate

Heart rate (HR) ($b \cdot min^{-1}$**)** was continually monitored during all pre- and experimental trials by

electrocardiography (Polar FT1, Polar UK). A heart rate monitoring strap was fitted to the participant's chest at V5 level, with the transmitter positioned centrally on the sternum, then adjusted to prevent any movement during trials. Through all chapters, heart rate was recorded every three minutes.

3.4.3 Rating of Perceived Exertion (RPE)

RPE was recorded, using the BORG 6-20 (Borg, 1974) scale, at the end of each stage during the maximal oxygen uptake assessment. In Chapter 4, RPE was recorded every three minutes during the exercise trial.

Rating	Description
6	No Exertion at All
7	Extremely Light
8	
9	Very Light
10	
11	
12	
13	Somewhat Hard
14	
15	Hard
16	
17	Very Hard
18	
19	Extremely Hard
20	Maximal Exertion

Figure 6: Borg scale for reporting subjective RPE (Borg, 1974).

3.4.4 Thermal Comfort

Subjective rating of thermal comfort was obtained at the same time-points as heart rate using a 9-point VAS (Figure 7), adapted from Snipe et al. (2018a).

Thermal Comfort Scale	
1	Very Cold
2	Cold
3	Cool
4	Slightly Cool
5	Neutral
6	Slightly Warm
7	Warm
8	Hot
9	Very Hot

Figure 7: Thermal comfort scale for quantification of subjective thermal strain.

3.5 Thermoregulatory Measurement

3.5.1 Core temperature

Core temperature was measured via rectal thermistor inserted 10 cm past the external anal sphincter (Mead and Bonmarito, 1949; Miller et al., 2017) which was connected to a portable multi-channel data logger (SQ2010, Grant Instruments, UK).

3.6 Assessment of gut permeability, injury and symptoms

3.6.1 Intestinal Permeability

To determine GI permeability, serum samples from participants underwent a permeability assay. Each participant ingested a solution containing a mixture of two mono- and disaccharide sugar probes. The probes consisted of 10 g lactulose and 2 g L-rhamnose, diluted in 230 mL of tap water (Fleming et al., 1996). Timing of ingestion of the probe solution is explained in each specific study chapter. Timing of collection of blood samples is outlined in each specific chapter. Analysis of sugar probe permeability was determined across all chapters through serum collection, two hours post-ingestion of the sugar probe solution. High-performance liquid chromatography (HPLC) was used to determine serum sugar concentrations using a previously published protocol (Fleming et al., 1996), with the modification of using L-rhamnose instead of mannitol as the monosaccharide probe. The various sugars were separated using high-pressure liquid chromatography (HPLC) and quantitated by use of a pulsed

electrochemical detector using a gold working electrode and silver/silver chloride reference electrode. The detection potential was -0.01 V (0-0.5 s), the oxidation potential was +0.75 V (0.51-0.64 s), the reduction potential was -0.75 V (0.65 0.75 s), and the integration period was 0.05 to 0.5 s. Retention times were 2.7 min for L-rhamnose and 6.1 min for lactulose. The coefficient of variation for the samples in the trial in this thesis using this method has been found to be between 1.8 – 8.5 %. Quantification of lactulose, L-rhamnose, and the ratio of lactulose to L-rhamnose was used to evaluate small intestinal permeability.

3.6.3 Gastrointestinal symptoms

An assessment of gastrointestinal symptoms was performed using a 10-point Likert questionnaire at time-points to coincide with those of blood sample collection. Participants completed a questionnaire detailing 16 symptoms with a visual analogue scale (VAS; 100 mm lines) (Pugh et al., 2017b). Participants were asked to indicate by marking the scale, indicating the magnitude of different symptoms, with 0 indicating no symptoms to 10 indicating severe symptoms. These data were quantitated in terms of percentage, with 0% indicating no symptoms, and 100% representing severe symptoms). Symptoms of GI discomfort included: side stitch; nausea; bloating; urge to burp; urge to vomit; urge to defecate; diarrhoea; stomach cramps; stomach upset; intestinal cramps; dizziness; shivering; and heart burn.

3.7 Blood sample collection and biochemical analysis

All blood samples were collected by the principal researcher. Blood samples were drawn from an antecubital vein using standard venepuncture procedures. In all studies, three separate tubes were collected, one serum separating tube (SST), one containing ethylenediaminetetraacetic acid (EDTA) and one containing sodium heparin. Sample tubes were given ten gentle inversions before being stored in ice until further processing, for later analysis. SST sample tubes were stored at room temperature for thirty minutes before being stored in ice. Following the final blood collection, samples were centrifuged at 1500 relative centrifugal force for fifteen minutes at 4^{o}C using a Sigma 3-18 k centrifuge (Scientific Laboratory Supplies). Plasma was then pipetted into sterile Eppendorf tubes before being frozen at -80^{o}C.

3.8 Heat Stress

In chapters six and seven, heat stress was induced through passive hyperthermia by submerging participants to the sternal notch in a thermostatically controlled bath. Following assessment of baseline measures, all participants were lowered into the water bath wearing swimwear, whilst secured in a safety harness. The bath was thermostatically controlled at 40°C, with water temperature being recorded

every fifteen minutes. Participants were encouraged to submerge all limbs for the duration of the sixty-minute protocol. Whilst participants were informed that they could be removed from the bath at their request, all participants completed the protocol. Due to ethical reasons, a ceiling core temperature of 39.5°C was in place. It was hypothesised that core temperature would continue to increase beyond the participant being removed from the bath. Hence, pilot work was completed prior to the main studies, which indicated a time-lag of approximately ten minutes between the participants being removed from the bath to reaching peak T_{core}, before decreasing. Figure 9 demonstrates the core temperature response of three participants to the heat stress protocol. Two participants were assigned to a higher core temperature threshold of 39°C, and one participant was assigned to a threshold of 38°C. When their T_{core} reached the designated value, the participant was removed from the bath.

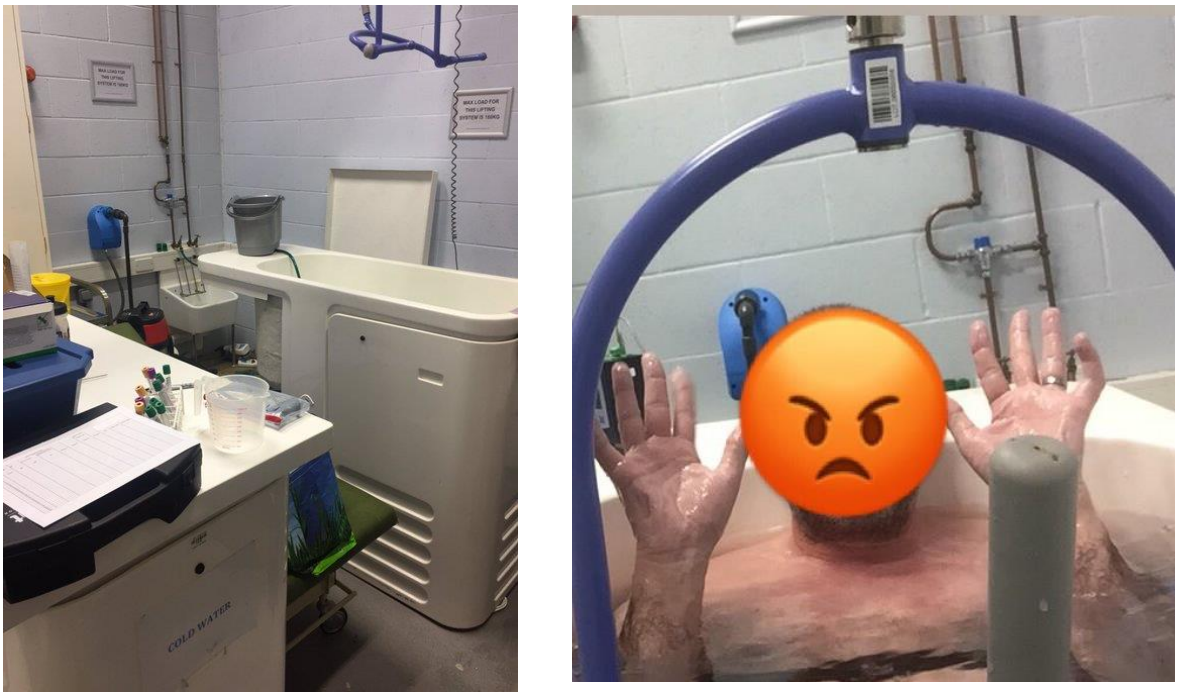


Figure 8: Experimental equipment set up for passive hyperthermia by hot water immersion

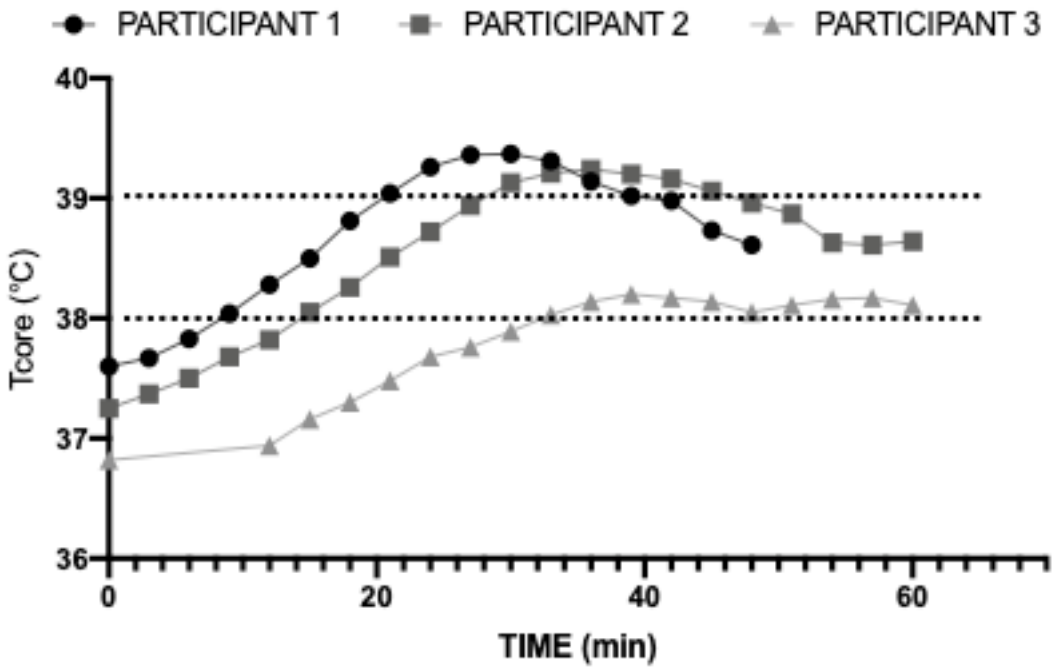


Figure 9: Core temperature response of three participants during pilot work to establish core temperature response to perceived passive heat stress methods

Chapter 4 – Determining GI barrier Dysfunction in Exercise: Does the Timing of Lactulose/L-rhamnose (L/R) Sugar-Probe Ingestion Affect Subsequent L/R Recovery Profile?

4.1: Abstract

Purpose: It is unknown if the timing of dual-sugar probe test solution ingestion, in relation to exercise, impacts the subsequent ratio of L/R ratio in the serum. *Methods:* Nine healthy, recreationally active males participated in the study [age 34 $\pm$ 7 years; height 177.8 $\pm$ 2 cm; body mass 77.2 $\pm$ 7.1 kg]. Participants completed three experimental trials of 60 minutes of treadmill running at approximately 80 % $\dot{V}O_{2\,max}$. Timing of the sugar probe solution was administered to represent pre- (PRE), during (MID), or post-exercise (POST) ingestion. Small intestinal permeability was determined through appearance of L/R in serum and expressed as a ratio. *Results:* Ingestion of the dual sugar probe solution pre- or during exercise returned a higher L/R ratio than post-exercise ingestion, however no significant difference was observed between conditions. *Conclusion:* The timing of sugar-probe test solution does not significantly affect the subsequent serum L/R ratio.

4.2: Introduction

Exercise, if of sufficient intensity and duration, can cause an increase in intestinal permeability whereby the tight junctions of the enterocytes become dysfunctional, allowing the transport of otherwise impassable molecules through the mucosa into the portal circulation (Grootjans et al., 2010b; Costa et al., 2017b). Potentially, this barrier dysfunction is mediated through a sustained reduction in mesenteric blood flow and hyperthermia in the small intestine (van Wijck et al., 2011a). Small intestinal permeability is typically determined by the appearance of a dual-sugar test solution containing lactulose and L-rhamnose or mannitol, in urine or plasma (serum) (van Wijck et al., 2011b; Costa et al., 2017b). Typically, urine analysis requires the collection of urine for five to twenty-four hours post sugar probe ingestion, whereas a two-hour post ingestion blood sample is required for plasma (serum) analysis. The total recovery and ratio of the sugars is thus utilised as a determinant of intestinal permeability (Fleming et al., 1996).

Whilst there is a considerable body of research investigating the exercise-induced response in intestinal permeability, comparisons between studies is difficult due to the large variance in methods

between the studies (Table 3). Researchers have employed a wide range of sugar-probe solutions containing different doses of lactulose and L-rhamnose, whilst in some cases adding further sugars such as sucralose, mannitol or erythritol (Van Wijck et al., 2012b; March et al., 2017). Comparisons are further complicated by the wide range of exercise protocols, analysis methods used (5-24 hr. urine, or serum), how the data was reported i.e., mean or median, and the timing of ingestion of the test solution (Table 3). Test solutions have been predominantly ingested prior to exercise (Lambert et al., 2008), however some researchers have provided the solution during (Pals et al., 1997) or post-exercise (Smetanka et al., 1999). Considering these variations, it is difficult to determine whether the timing of sugar-probe ingestion impacts the subsequent appearance and ratio of the sugars in urine or serum. Furthermore, these differences between studies complicate the contextualization of the specific exercise protocol or intervention, such as the provision of NSAIDs (Lambert et al., 2012), in terms of the magnitude of intestinal permeability reported. Current research (Table 3) demonstrates notionally similar levels of L/R recovery when test solutions are provided before or during exercise (Lambert et al., 2008; Zuhl et al., 2014a; Zuhl et al., 2015). Yet, research is currently lacking investigating how the timing of the sugar-probe test solution post-exercise affects the recovery of L/R. Indeed, the post-exercise period could represent the highest point of intestinal permeability. Since blood flow is rapidly reduced at the onset of exercise (Rehrer et al., 2001), unless carbohydrate is provided (Rehrer et al., 2005), the gut exists in a state of hypoperfusion and hyperthermia for a greater amount of time than if the test solution had been provided pre- or during exercise providing an extended window of opportunity for barrier disruption and for translocation to occur. (Otte et al., 2001).

It must be considered that fluids, for example the lactulose/L-rhamnose test solution, are not made immediately available for small intestine absorption upon ingestion, but initially stored in the stomach and subject to gastric emptying. Individual variation exists in gastric emptying, which can be further affected by the volume, osmolality and chemical composition of solutions (Leiper, 2015). When provided during exercise, weak glucose solutions have shown no difference in gastric emptying when compared to rest, however, emptying can be significantly delayed following ingestion of stronger solutions (Costill and Saltin, 1974). Once absorbed, it is difficult to comment on factors which may affect subsequent appearance of lactulose and rhamnose in the urine or serum. Considering the large variation in both the timing of ingestion, and osmolality of test solutions, research is warranted to investigate how the timing of ingestion of a standardized solution reflects the reported magnitude of intestinal permeability. If research were to compare intestinal permeability in response to a similar exercise stimulus, this data would highlight when the small intestine is most permeable. Furthermore, this research could demonstrate the recovery in intestinal permeability following the cessation of exercise. Therefore, the aim of the present study was to investigate the dynamics of intestinal permeability in response to a moderate intensity bout of treadmill running, and investigate whether altering the timing of sugar-probe ingestion affects the subsequent recovery of L/R.

4.3 Methods

4.3.1 Participants

Nine healthy, recreationally active males volunteered to participate in the study. All participants provided written consent prior to the commencement of testing. Participant requirements included that they were male, training three or more hours per week of endurance-type exercise, free from gastrointestinal syndrome and willing to abide by the restrictions implemented by the study (24h rest prior to each trial, overnight fast, no caffeine or alcohol in the 24 hours prior to each trial.)

Table 6: Descriptive characteristics of participants displaying means ± SD for age (yrs.); body mass (kg); height (cm) and $\dot{V}O2$ max. ($mL \cdot kg^{-1} \cdot min^{-1}$).

	Age (yrs.)	Body Mass (kg)	Height (cm)	$\dot{V}O_{2\,max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)
Mean ± SD	34 ± 7	77.2 ± 7.1	177.8 ± 2.4	57.8 ± 5.8

4.3.2 Assessment of maximal oxygen uptake

During the first visit, $\dot{V}O_{2\,max}$ was determined as per section General Methods 3.4.1. Following the assessment, the estimated running speed for the experimental trials was determined using a linear regression equation. Running speed corresponding to 80% was then determined.

4.3.3 Experimental design

In a repeated measures cross-over design, each participant completed a baseline assessment of maximal oxygen uptake and three test conditions comprising of 60 minutes of running on a motorised treadmill at approximately 80% of their predetermined $\dot{V}O_{2\,max}$. The duration was chosen to align with previous literature to facilitate comparison, whilst the intensity was also determined from literature as being above the minimal intensity needed to elicit an increase in permeability (Costa et al., 2017b). Participants arrived at the laboratory between 07:00 and 9:00 h following an overnight fast, as well as abstaining from strenuous exercise and alcohol consumption for the preceding twenty-four hours. All preliminary measures and experimental trials were conducted in standard laboratory conditions, at approximately 22°C, 45% RH. Participants were asked to ingest at least 500 mL of water prior to arriving at the laboratory to promote hydration.

Upon arrival at the laboratory, participants were measured for height (cm) and weight (kg), before completing a questionnaire of gastrointestinal symptoms and being fitted with a heart rate monitor strap as described in the General Methods section. A pre-exercise blood sample was then collected. The participant then inserted a rectal thermometer 10 cm past the anal sphincter, which was connected to a portable data logger (SQ210, Grant Instruments, UK.) Following preliminary

assessments, participants completed a five minute self-paced warm up followed by two minutes of complete rest. The self-paced warm up was always completely 'free' for the participants to complete as desired. The participant then re-mounted the treadmill before the speed was increased to their estimated velocity within ten seconds of the start, at an incline of one percent (Jones and Doust, 1996). Treadmill speed ($km \cdot h^{-1}$) was also recorded every three minutes. Breath-by-breath measurements of oxygen consumption ($\dot{V}O_2$) and carbon dioxide ($\dot{V}CO_2$) production were recorded via online gas analysis (Oxycon Pro, Jaeger) every six minutes to record the expired gases equivalent to the required estimate of appropriate exercise intensity. Treadmill speed was adjusted in response to the recorded measurements so-as-to maintain the desired intensity. Participants were provided with 500 mL tap water throughout the trial which they ingested both ad-libitum and, or, within ten-minute intervals.

4.3.4 Sugar probe administration

Participants completed three experimental trials, informed by the timing of ingestion of the dual-sugar probe solution. Gastrointestinal permeability was measured with a solution containing dual-sugar probes, as described in section 3.6.1. Participants ingested the solution 15 minutes before (PRE), 30 minutes during (MID), or immediately post (POST) exercise; figure 10 demonstrates a schematic representation of the study.

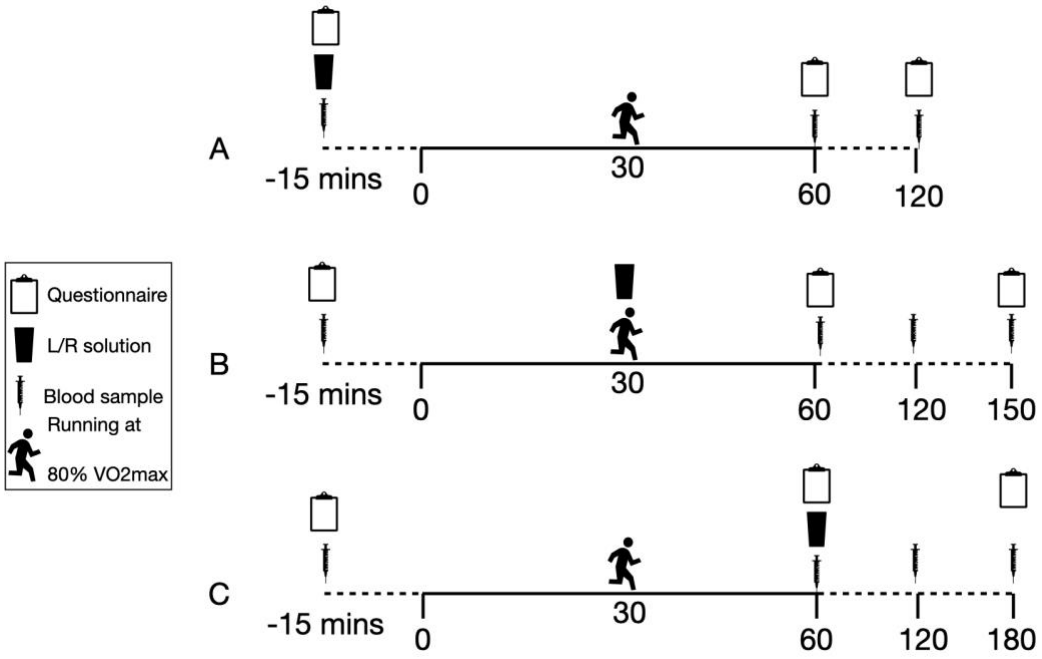


Figure 10: Protocol schematic for PRE (A), MID (B) and POST (C) exercise trials. Syringe graphics indicate timing of bood sample collection, the clipboard indicates the timing of gut symptom questionnaire collection and timing of the sugar-probe solution is indicated by the drink graphc. Dashed lines indicate the time period before and after the exercise (solid line.)

4.3.5 Thermal comfort, HR, RPE, and core temperature

Core temperature (T_{core}), heart rate (HR), RPE, thermal comfort (TC) were measured as described in the General Methods, and recorded every three minutes.

4.3.6 Gastrointestinal symptoms

Symptoms of gastrointestinal discomfort were collected in line with the timing of blood-sample collection (Figure 10) and collected as described in 3.6.3.

4.3.7 Blood analysis

Blood samples were collected fifteen minutes before (-15), within three minutes post-exercise (60) and sixty minutes post-exercise (120) as well as 120 minutes post-ingestion of the dual sugar probe solution. Samples were collected and analysed for L/R as per section 3.7. Blood sample analysis samples were prepared according to the methods outlined in the General Methods section.

4.3.8 Statistical analysis

Data was analysed with the use of the statistical software package SPSS (Version 26.0, SPSS Inc., Armonk, NY: IBM Corp), with significance accepted at $p \leq 0.05$. Values found to be below three decimal places of 0 were adjusted to P<0.001. Changes between variables throughout conditions (PRE, MID, POST) were tested using a repeated measures ANOVA. To correct violations of sphericity, the degrees of freedom were corrected in a normal way, using Huynh-Feldt ($\varepsilon > .75$) or Greenhouse- Geisser ($\varepsilon < .75$) values for ε, as appropriate (Field 2007). A Bonferroni pairwise comparison was applied when main effects were present.

4.4 Results

4.4.1 Physiological Responses

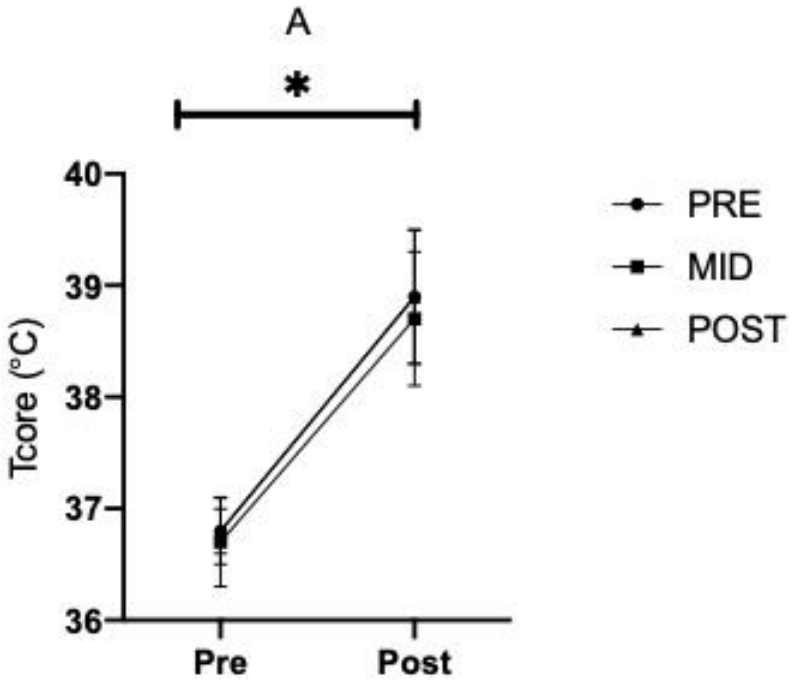
One participant did not complete the study, leaving nine ($n = 9$) for analysis. Body mass (BM) decreased in response to exercise, with a significant main effect for time ($F_{1, 6} = 49.894$, $P < 0.001$). The decrease in BM was similar between all conditions. There was no significant main effect observed for condition ($F_{2, 12} = 0.443$, $P = 0.652$). No significant interaction was observed between condition ($F_{1.29, 7.72} = 0.179$, $P = 0.774$). No significant interaction was observed between condition and time ($F_{1.29, 7.71} = 0.79$, $P = 0.774$). There was an increase in HR in response to exercise, with a significant main effect for time ($F_{1, 6} = 254.075$, $P < 0.001$). HR increased by similar amounts between all conditions. There was no significant main effect observed for condition ($F_{1.317, 7.9} = 2.064$, $P = 0.191$). No significant interaction was observed between conditions ($F_{1.33, 7.95} = 0.567$, $P = 0.521$). No significant interaction was observed between condition and time ($F_{1.33, 7.95} = 0.567$, $P = 0.521$). RPE showed no significant main effect between conditions ($F_{2, 12} = 0.087$, $P = 0.917$). No significant interaction was observed between condition and time ($F_{1.29, 7.9} = 0.567$, $P = 0.521$). Mean oxygen uptake represented ~79% of maximum with no difference between conditions.

4.4.2 Core temperature

There was an increase in core temperature in response to exercise, with a significant main effect for time ($F_{1, 5} = 184.81$, $P < 0.001$). Core temperature increased by similar amounts across all conditions. There was no significant main effect observed for condition ($F_{2, 10} = 0.856$, $P = 0.454$). No significant interaction was observed between conditions ($F_{1.05, 5.26} = 0.242$, $P = 0.65$).

Table 7: Post-exercise values for physiological responses. Showing heart rate (b·min^{-1}); body mass loss (%); percent of $\dot{V}O_2$ for the exercise; RPE (AU); core temperature (°C); and thermal comfort (AU). Data are mean ± SD.

	Pre	Mid	Post	Main Effect	Significance
HR (b·min^{-1})	173 ± 10	169 ± 11	170 ± 10	$F_{1.9, 11.5}$ =1.905	*P* = 0.288
Body mass loss (% BM)	1.1 ± 0.2	0.7 ± 0.6	0.6 ± 0.7	$F_{2, 12}$ =1.873	*P* = 0.196
$\dot{V}O_{2\ max}$ (%)	79 ± 6	79 ± 4	80 ± 4	$F_{1.5, 9.2}$ =0.333	*P* = 0.677
RPE (AU)	13 ± 1	13 ± 1	13 ± 1	$F_{1.9, 11.9}$ =0.087	*P* = 0.936
Speed (km/h)	12 ± 1	12 ± 1	13 ± 1		
Tcore (°C)	39.1 ± 0.7	38.8 ± 0.7	39.1 ± 0.4	$F_{1.8, 7.1}$ =0.505	*P* = 0.564
TC (AU)	7 ± 1	7 ± 1	6 ± 1	$F_{2, 12}$ =1.560	*P* = 0.250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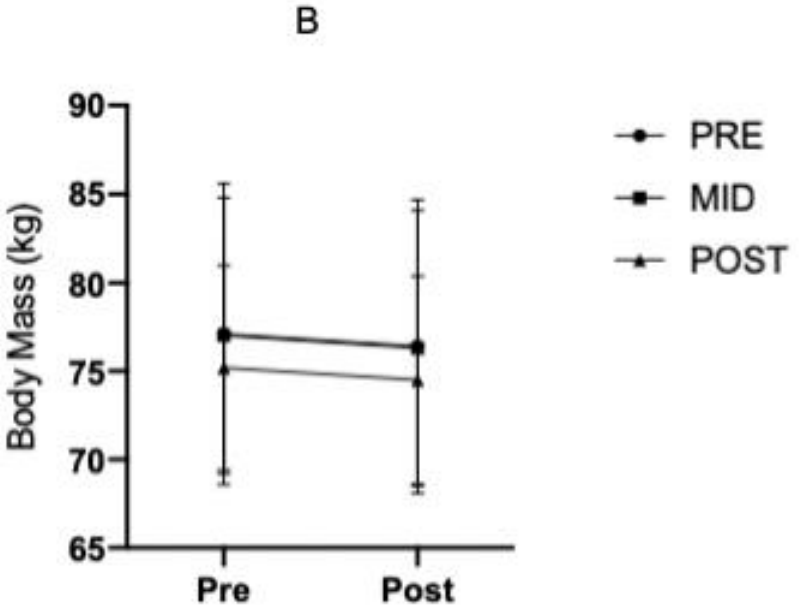
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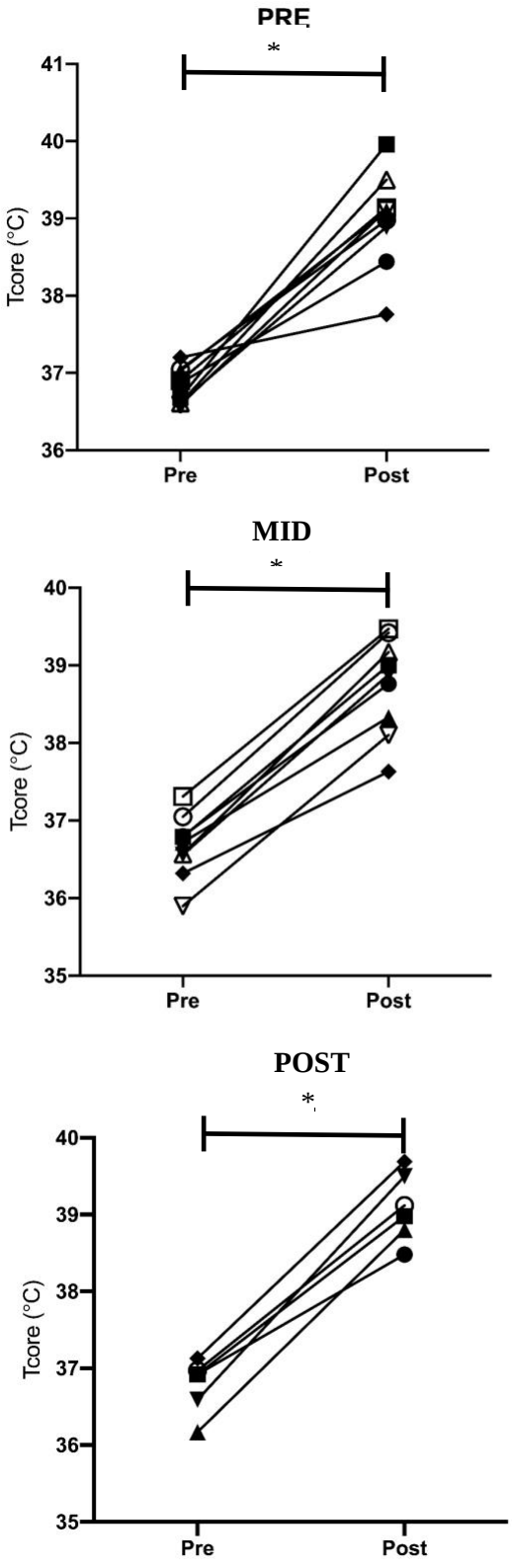


Figure 11: showing mean (A) body mass and core temperature (B) pre- and post-exercise. Also showing individual values for each condition (PRE, MID, POST) in core temperature and body mass at pre- and post-exercise. * significant effect of time (P<0.001).

4.4.3 Small Intestinal Permeability

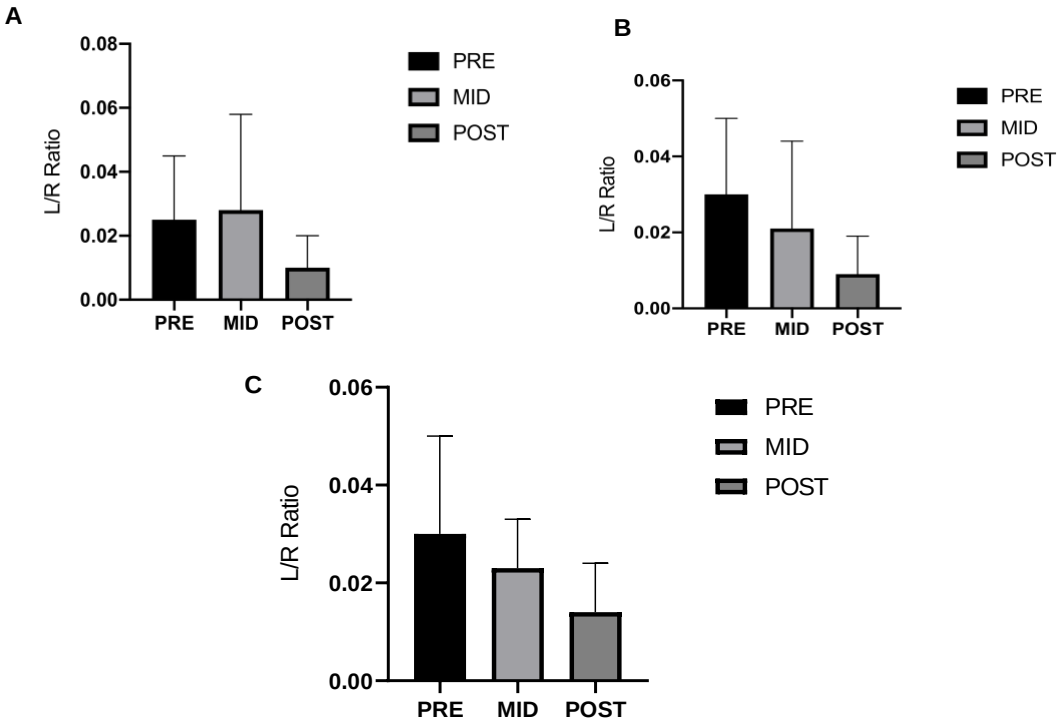
No significant main effect was observed between conditions at any time point (Table 8). L/R values at two-hour post-ingestion of the dual sugar probe solution are shown in Table 9. Immediately post-exercise, the highest L/R ratio was reported in the MID condition. At sixty minutes post-exercise, the highest L/R ratio was observed in the PRE condition (Table 8). At two-hour post exercise, the highest L/R ratio was observed in the PRE condition. Although non-significant, post-exercise ingestion of the dual-sugar probe solution returned the lowest ratios of lactulose/ rhamnose at all time points.

Table 8: The L/R ratio (+ SD) at time points 0, 60 and 120, for the PRE, MID, and POST conditions. Data are Mean ± SD. Where 0 represents immediately post exercise, 60 = 60min post exercise and 120 = 120 mins post-ingestion of the dual sugar probe solution.

	Pre	Mid	Post	Main Effect	Significance
0	0.026 ± 0.020	0.030 ± 0.032	0.012 ± 0.011	$F_{1.13,5.63}$= 1.553	*P* = 0.268
60	0.032 ± 0.024	0.024 ± 0.016	0.010 ± 0.005	$F_{1.23,8.57}$= 4.008	*P*= 0.073
120	0.032 ± 0.024	0.026 ± 0.015	0.015 ± 0.006	$F_{1.35, 9.45}$= 2.374	*P* = 0.154

Table 9: The L/R ratio mean ± SD and 95% CI at all time points for the PRE, MID and POST conditions.

0			
	L/R (AU)	**Lower Bound**	**Upper Bound**
PRE	0.026 ± 0.020	0.005	0.047
MID	0.030 ± 0.032	-0.004	0.063
POST	0.012 ± 0.011	0.000	0.023
60			
	L/R (AU)	**Lower Bound**	**Upper Bound**
PRE	0.032 ± 0.024	0.012	0.052
MID	0.024 ± 0.016	0.013	0.038
POST	0.010 ± 0.005	0.011	0.020
120			
	L/R (AU)	**Lower Bound**	**Upper Bound**
PRE	0.032 ± 0.024	0.012	0.052
MID	0.025 ± 0.015	0.011	0.038
POST	0.015 ± 0.006	0.006	0.014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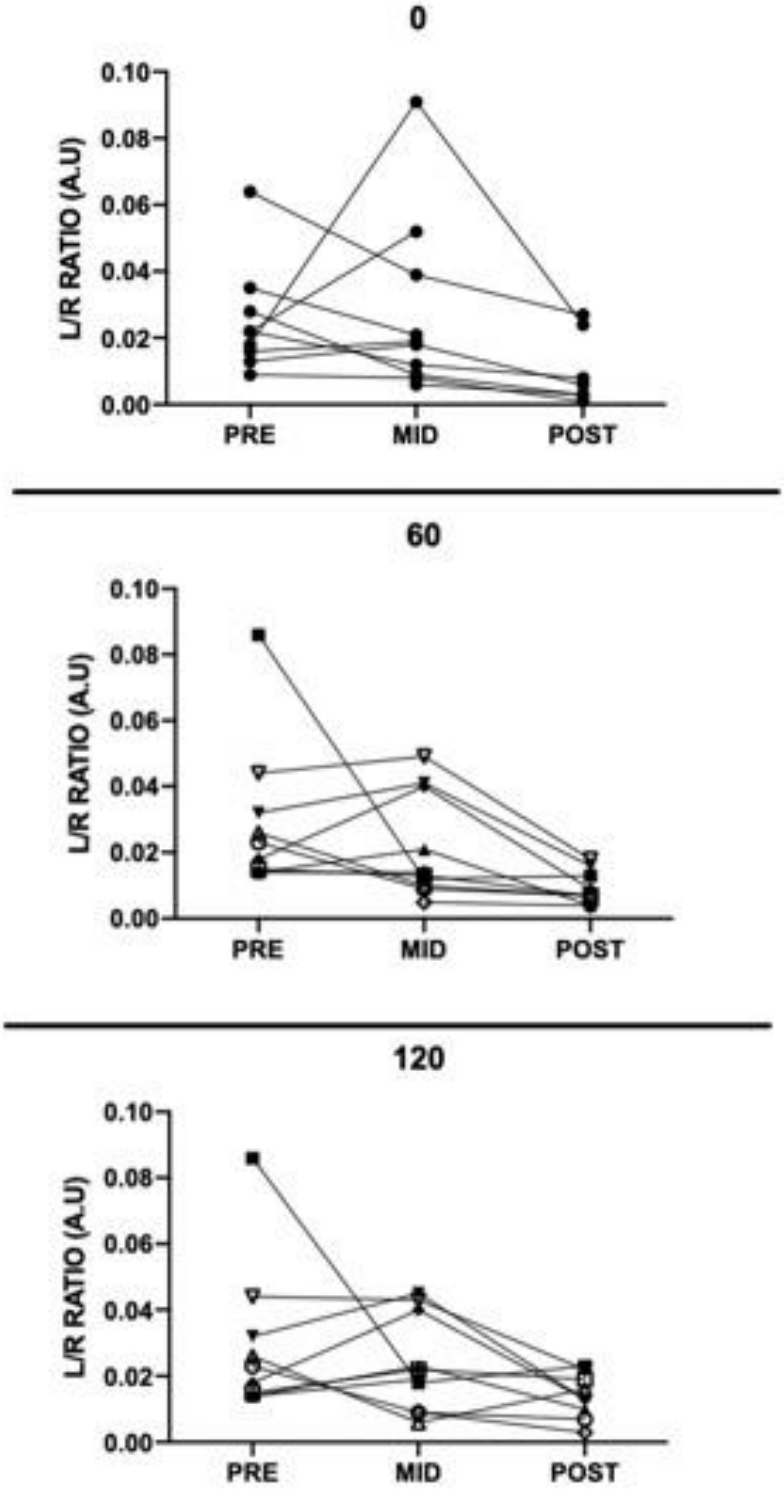

Figure 12: The L/R ratio at immediately post-exercise (A); 60 minutes post-exercise (B); and two hours post ingestion of the test solution (C). Also showing individual participant responses for PRE, MID and POST conditions at 0 (immediately post-exercise), 60 minutes post exercise and 120 post L/R solution ingestion time points.

4.4.4 GI symptoms

Assessment of gastrointestinal symptoms was completed through a questionnaire which ranked several symptoms on a scale of zero (no symptoms) to ten (severe symptoms). Symptoms included: side stitch; nausea; bloating; urge to burb; urge to vomit; urge to defecate; diarrhoea and flatulence. We observed only mild symptoms (mean of 1) in the PRE condition for nausea, urge to burp, urge to defecate and flatulence. Very mild symptoms (mean of 1) we reported during the MID condition for bloating and flatulence. No symptoms were reported during the POST condition (Table 10).

Table 10: Post-exercise subjective gastrointestinal symptoms between conditions. Data are presented as mean values indicated by participants from a 0-10 VAS.

	Condition		
	Pre	**Mid**	**Post**
Gut symptoms		**Severity (%)**	
Side Stitch	0	0	0
Nausea	1	0	0
Bloating	0	1	0
Urge to Burb	1	0	0
Urge to Vomit	0	0	0
Urge to defecate	1	0	0
Diarrhoea	0	0	0
Flatulence	1	1	0

4.5 Discussion

The aim of the current study was to investigate whether the timing at which the dual-sugar probe test solution was administered, in relation to a bout of moderate treadmill exercise, affects the subsequent ratio of L/R recovered. To the author's knowledge, present data has shown for the first time that, whilst non-significant, ingestion of the dual-sugar probe solution pre or during exercise returns a higher L/R ratio than post-exercise ingestion. However, providing the dual-probe solution pre-or during exercise, consistently returns higher L/R estimates than when the solution is ingested post-exercise.

Previously, the L/R solutions to measure GI permeability have been administered before (Pugh et al., 2017b), during (Van Wijck et al., 2012b; Snipe et al., 2017) or post-exercise (Marchbank et al., 2011; Davison et al., 2016; March et al., 2017; March et al., 2018). Additionally, the dose of sugar-probes in these test solutions has varied widely (Table 3). These differences make cross-study comparisons of changes in small intestine permeability difficult, which are further complicated by the chosen method of sample collection, for example urine or serum analysis, exercise modality, environmental conditions, exercise intensity and reporting of results (mean vs. median). To align our protocol with current literature, an exercise intensity of ~80 % maximal oxygen uptake was utilised to

promote an increase in small intestinal permeability (Pires et al., 2017), with sixty minutes continuous treadmill running in thermoneutral conditions (Pals et al., 1997; Lambert et al., 2001; Lambert et al., 2007; Lambert et al., 2008).

Ingesting the L/R dual probe solution pre-exercise returned the highest magnitude of permeability. These results are similar to those of resting participants, but much lower than those following 60 minutes of treadmill running at 70% $\dot{V}O_{2\,max}$ in 30°C heat (Pugh et al., 2017c). However, comparisons between studies is difficult, despite using a similar assay, these authors used only 5 g of lactulose, whereas 10 g of lactulose was used in the present study. At present, research is lacking investigating the effects of different doses of sugar-probes on the L/R ratio, but some researchers argue that larger doses of lactulose increases the passage through the GI system, effectively influencing the sensitivity of the analysis (van Wijck et al., 2011b).

The ratios of L/R from the present study are lower than the urinary L/R values reported by Lambert et al. (2008) for participants at rest (0.035 (0.011-0.107), or with placebo (0.049 (0.017-0.124) following sixty minutes treadmill running at seventy % of maximal oxygen uptake. These values are also lower than another study by Lambert et al. (2007), which returned a L/R ratio of 0.065 (0.04 – 0.08), again following sixty minutes treadmill running at 70% $\dot{V}O_{2\,max}$. Utilising a similar exercise protocol, but providing the L/R dual probe solution at 30 minutes during a 60 minute exercise protocol, Pals et al. (1997), reported mean L/R values of 0.048 $\pm$ 0.009 at rest, 0.056 $\pm$ 0.005 at 40% $\dot{V}O_{2\,max}$., 0.064 $\pm$ 0.010 at 60% $\dot{V}O_{2\,max}$., and 0.107 $\pm$ 0.021 at 80% $\dot{V}O_{2.\,max}$. Given that no statistical difference was observed between the L/R ratio at any time point in the present study, and these authors used a similar test-solution, the results of these studies (Pals et al., 1997; Lambert et al., 2007; Lambert et al., 2008) are, arguably, comparable. However, the data from Lambert et al. (2007) is inconsistent with the conclusions made by Pals et al. (1997), who argued a positive relationship between exercise intensity and intestinal permeability. Lambert et al. (2007) returned L/R values of 0.065 (0.04 – 0.08) following sixty minutes treadmill running at 70% $\dot{V}O_{2\,max}$, compared to 0.064 $\pm$ 0.010 at 60% $\dot{V}O_{2.\,max}$ reported by Pals et al. (1997).

Smetanka et al. (1999) investigated intestinal permeability in thirty-four runners in the 1996 Chicago Marathon, providing the dual-sugar test solution within 15 minutes of race completion, and utilising a five-hour urinary excretion analysis. Taking values from runners who did not ingest ibuprofen or aspirin, the L/R ratio was 0.019 $\pm$ 0.01; interestingly, these values were lower than resting controls (0.022 $\pm$ 0.01). Whilst not significantly different, it would be expected that intestinal permeability be higher following a marathon than an equivalent time of rest since the gut may have experienced an extended period of hypoperfusion, core temperature would have also been elevated (Adams et al., 1975). Interestingly, the results from the present study report an L/R ratio of 0.012 at the 0 time point during the post-condition. These findings are difficult to explain as the pre-exercise serum samples returned an undetectable level of L/R; since the test-solution had not been provided to participants at the 0 time point, similar or undetectable of lactulose and rhamnose should have been

reported. Indeed, these results were questioned by the present researcher with the analysis laboratory, but were confirmed correct. As such, it cannot be out-ruled that some mislabelling of samples may have occurred, leading to samples containing L/R being analysed at this time point. However, labelling procedures were strict during all studies, so these results remain difficult to explain.

Nevertheless, despite similar exercise models and test-solutions, comparisons with present study are difficult, since these authors utilised urinary excretion of the sugar-probes. Urine analysis requires a higher dosage of sugar-probes to be used compared to serum analysis (Fleming et al., 1996), since the assay is less sensitive. Serum analysis facilitates a faster detection of changes in L/R ratio and the convenient collection of more sample points as collection is not limited by the reliance on the urination of participants (van Wijck et al., 2011b). The dose of 10 g lactulose and 2 g rhamnose was advised, as lower doses of lactulose are reported to be on the limit of detection of the analysis (Fleming et al., 1996)(Fleming Personal Communications 2018). The increased sensitivity of serum analysis permits an easier and more efficient method of sample collection, as a blood sample is required two-hours post ingestion of the test solution, as opposed to 5 – 24 hour urine collection (Fleming et al., 1996). However, a limitation to this method is the reduced time of exposure between the sugar probes and the GI tract before the cessation of sample collection. A longer period of sample collection may provide increased passage time for the sugar-probes, potentially affecting the reported L/R ratio.

Despite no significant difference, the L/R ratio values during the PRE condition were consistently higher than those in the POST condition. Serum analysis of the L/R ratio requires a sample to be collected two hours post-ingestion of the sugar-probe test solution. Therefore, PRE conditions samples were collected approximately sixty minutes post-exercise, whereas POST condition samples were collected two hours following the cessation of exercise. Given that mesenteric blood flow, and thus oxygen supply, may return to pre-exercise levels within ten minutes (Rehrer et al., 2001) there may have been an increased recovery of tight junction protein integrity before the sugar-probes reached the intestinal tract (Grootjans et al., 2016). Grootjans et al. (2016) demonstrated that intestinal ischemia results in the appearance of sub-epithelial spaces, whereby the basal membrane contracts below the (damaged) epithelial cells; the damaged cells, located at the most apical point of the villus, are subsequently shed. Once shed, the less mature cells pull together, creating a new epithelial lining, thus potentially limiting the translocation of bacteria and other particles and reducing permeability.

The results of the present study indicate no significant occurrence of gastrointestinal symptoms in any of our experimental conditions. Whilst the appearance of GI symptoms is common amongst athletes (Pugh et al., 2017a), less is understood about the governing factors underlying these appearances. Current literature suggests that the appearance of GI symptoms are likely due to a combination of physiological, psychological and environmental factors (ter Steege and Kolkman, 2012; Costa et al., 2017b; Snipe et al., 2018a). Snipe et al. (2017) reported increased incidence of GI symptoms during running in the heat (35°C) compared to temperate (22°C) conditions. Those running in the heat demonstrated significantly higher core temperatures and heart rate, suggesting a higher

physiological strain (Moran, Shitzer and Pandolf, 1998). Mean peak core temperature in the heat was 39.6°C, compared to 38.5°C in the temperate condition. The peak core temperatures observed in the present study lie between these values at a mean 39°C between conditions. These comparisons suggest that the environment of the present study may not have imposed sufficient physiological strain to induce GI symptoms. As such, the lack of appearance of GI symptoms in the present study are aligned with those of other studies which have utilised a similar exercise and environmental protocol (Pals et al., 1997; Lambert et al., 2008; Karhu et al., 2017).

The present study aimed to establish whether the time at which the L/R solution was administered effected the resultant L/R ratio in the plasma, and thus the magnitude of permeability noted. These results suggest that studies which have provided the test solutions pre- or during exercise, when protocols and test solutions are similar, can be compared. Despite no significant difference, post-exercise provision of the L/R solution consistently returned lower values of plasma L/R ratio. However, despite these methodological considerations for future research in intestinal permeability, it is important to consider some limitations. Firstly, a resting control trial was not included, so comparisons between the changes in intestinal permeability from baseline values could not be completed. Furthermore, the potential implications of a relatively small sample size (n = 9), cannot be overlooked, particularly since large deviations in L/R ratios were observed. A trend towards significance was observed at 60 minutes post exercise, and a larger sample size may have established a significant difference. Moreover, despite strict pre-testing procedures being followed and all participants reported compliance, it is possible that changes in diet, ingestion of NSAIDs or intense exercise sessions may have occurred in the twenty-four hours preceding exercise trials; factors which could have affected the participants gastrointestinal permeability (Pals et al., 1997; Lambert et al., 2007).

In summary, the results of present study suggest that the time at which the dual-sugar probe test solution is ingested (in relation to the exercise bout) does not significantly affect the resultant ratio of serum L/R. However, given that L/R values were higher in the PRE and MID conditions, compared to POST, future studies utilising a serum assay analysis should consider providing the test solution either pre- or during-exercise to estimate small intestinal permeability. In response to a sixty-minute bout of running within a heavy intensity domain (see Chapter 6), ingesting the dual-sugar test solution pre- or during exercise, reports a higher amount of gastrointestinal permeability than ingesting the test solution post-exercise. These results suggest that, when using a similar exercise protocol, the test solution should be provided prior to the participant commencing exercise, or sometime at or before the mid-point during exercise.

Chapter 5 – Effect of Exercise in Different Intensity Domains on Gastrointestinal Permeability and Symptoms in Runners and Triathletes

Chapter 4 investigated methodological considerations surrounding the timing of sugar probe ingestion in relation to exercise, and whether this affected the subsequent serum L/R ratio. Given the importance of exercise intensity as a contributing factor towards intestinal permeability (Pals et al., 1997; Costa et al., 2017b), Chapter 5 will address the influence of increasing exercise intensity domains on intestinal permeability and symptom expression.

5.1 Abstract

Aim: The aim of this study is to determine the effects of running exercise intensity on GI dysfunction, GI permeability and biomarkers of GI damage. *Methods:* A total of ten participants (Male = 7, Female = 3) [age = 29 $\pm$ 6 years , body mass = 69.9 $\pm$ 10.1 kg, height = 173.0 $\pm$ 7.9 cm, $\dot{V}O_{2\ max}$ = 55 ± 6 mL·kg^{-1}·min^{-1}] volunteered to take part in the study. After assessment of maximal oxygen uptake and familiarisation, in a counterbalanced experimental design, participants completed four experimental trials (rest, moderate, heavy and severe intensity based on CP estimation). GI permeability was determined by serum lactulose/ rhamnose ratio whilst translocation of D-lactate into portal circulation was taken as a marker of GI permeability. Subjective GI symptoms were assessed via 10-point Likert scale (Lambert et al. 2008). *Results:* Exercise in the severe intensity domain returned higher L/R ratio values than exercise completed in the moderate and heavy intensity domains. *Conclusion:* Intestinal permeability demonstrates a stepwise linear relationship with exercise intensity. The appearance of GI symptoms does not correlate with the step wise increase in exercise intensity within the ranges utilised within this study.

5.2 Introduction

Exercise results in a intergated range of physiological events, particularly involving the muscoskeletal and cardiovascular systems. Some biological functions are upregulated to meet the increased demand for oxygen by the muscles and the need for an augmented rate of ATP synthesis. During exercise, an increase in oxygen consumption, heart rate, heat and lactate production can be observed which positively correlate with exericse intensity (Hill, 1923). Furthermore, the nature of exercise adaptions can be dictated by the exercise intensity of the training session, leading to athletes and coaches

incorporating a range of intensities into the training program. Athletes engaged in endurance sports such as running, cycling or rowing have previously used heart rate to dictate the intensity of their training sessions; this method required the athlete to increase their effort to raise their heart rate into the specific zone. Technological advances have since facilitated athletes and coaches with power meters to monitor the exercise intensity, either through monitoring the force applied through the pedal or through the rowing-machine flywheel (Jeukendrup and VanDiemen, 1998). However, more recently, power meters have also been developed for running, whereby the athlete attaches a device to the shoe and can relay their power output to their training watch.

An athlete's training zones are typically developed in accordance with their notional critical power or the sometime interchangeably utilised functional threshold power; an intensity correlating to a steady-state of lactate production, where lactate production is met by lactate clearance (Burnley and Jones, 2018). Research has typically utilised a fixed % maximal oxygen uptake to prescribe exercise intensity, however, the relative physiological strain experienced by the participant may be influenced by their training status. Thus, variations in the level of physiological strain experienced by participants within a study may exist. An alternative method for determining exercise intensity is critical power (CP), which represents a threshold where an athlete transverses from steady to non-steady exercise intensity domains. CP zones are defined as moderate, heavy and severe and represent intensities utilised to elicit specific physiological adaptions (Vanhatalo, Jones and Burnley, 2011). Exercise below CP represents an intensity corresponding to maximal utilisation of oxidative metabolism, without progressive accumulation of metabolites (Poole et al., 2016), however above CP (W'), the athlete is considered to be on 'borrowed time', whereby exercise above this threshold quickly leads to exhaustion (Jones et al., 2010).

During exercise, the gastrointestinal system also displays an array of physiological events. Specifically, blood is directed away from the gut towards the working muscle to aid thermoregulation and nutrient delivery. As such, blood flow to the gut can be reduced by as much as eighty percent, which can lead to a state of intestinal ischemia (Rehrer et al., 2001). These disturbances can lead to an increase in intestinal permeability, whereby the enterocyte tight junctions become dysfunctional. As a result, large particles such as sugars, or harmful bacteria within the gut which cannot normally be absorbed by paracellular transport, can now enter the portal circulation (van Wijck et al., 2011a).

Intestinal permeability is typically quantified by measurement of the ratio of dual-sugar probes (lactulose and L-rhamnose) in the urine or plasma (Fleming et al., 1996; Camilleri et al., 2010). Under conditions of increased intestinal permeability, lactulose can now be transported across the intestinal mucosa, allowing subsequent detection in the urine or plasma. More recently, plasma D-lactate has been identified as another potential biomarker of intestinal injury. This isoform of lactate is normally produced by the bacteria of the GI tract through fermentation (Ewaschuk, Naylor and Zello, 2005). Healthy individuals display low levels of circulating D-lactate (5-20 μmol/L); however, GI dysfunction causes a greater concentration of D-lactate to translocate the intestinal mucosa to systemic circulation.

Whilst this biomarker has typically been used in clinical studies, a limited amount of research has investigated the response in plasma D-lactate to exercise (Kondoh, Kawase and Ohmori, 1992). Since, increased plasma D-lactate has been linked to intestinal ischemia (Li et al., 2017), together with the limited research in exercising healthy individuals, further research is warranted investigating the role of D-lactate and its suitability as a marker of intestinal permeability.

Previous research has demonstrated that intestinal permeability increases in a dose response manner with exercise intensity (Pals et al., 1997). Current reviews suggest that intestinal permeability occurs above an exercise intensity of ~70% $\dot{V}O_{2\,max}$, with core temperature also demonstrating a dose response relationship (Costa et al., 2017b; Pires et al., 2017). However, these studies have utilised an exercise intensity calculated as a percentage of $\dot{V}O_{2\,max}$. Furthermore, discrepancies exist in the results of the only study investigating a dose response relationship between exercise intensity and GI permeability (Pals et al., 1997), and there is a need for additional research into the suitability of D-lactate as a biomarker for intestinal damage. As such, the aim of the present study was to investigate the effects of increasing exercise intensity domains on GI permeability, GI injury and the appearance of gastrointestinal symptoms.

5.3 Methods

5.3.1 Participants

Ten participants (Males = 7, Females = 3) [age = 29 $\pm$ 6 years , body mass = 69.9 $\pm$ 10.1 kg, height = 173.0 $\pm$ 7.9 cm, $\dot{V}O_{2\,max}$ = 55±6 $mL \cdot kg^{-1} \cdot min^{-1}$] volunteered to take part in the study. All participants provided written consent prior to the commencement of testing. Participant requirements included that they were training three or more hours per week of endurance-type exercise, free from gastrointestinal syndrome, not taking NSAIDs or nutritional supplements and willing to abide by the restrictions implemented by the study (24h rest prior to each trial, overnight fast, no caffeine or alcohol in the 24 hours prior to each trial.)

5.3.2 Experimental Design

Following consent, participants completed a familiarisation of the critical power protocol, to familiarise themselves with the pace and intensities required for each stage of the test. Seven days after familiarisation, participants returned to the laboratory to complete the pre-experimental phase of the study, to determine the critical power (CP) and critical speed (CS). Power was determined with a running power meter pod (Stryd, Boulder, CO, USA), which has previously been validated (Imbach et al., 2020) attached to the dominant foot and synchronised in accordance with the height and body mass

values of the participants during each trial. The data was recorded using the manufacturer's software (Stryd, Boulder, CO, USA), with power being displayed every sixty seconds. Pulmonary gas exchange, heart rate, RPE, thermal comfort and rectal temperature (n = 3 participants) were measured every three minutes as outlined in the General Methods section. Aural temperature was utilised in the remaining participants using a digital tympanic infrared thermometer (Genius™ 2 Tympanic Thermometer and Base, Covidien, UK). Measures of aural temperature were taken pre-exercise, post-exercise and one-hour post-exercise. Haematology parameters, including blood lactate, haemoglobin and haematocrit were collected at pre, post and 2 hours post-exercise, to quantify changes in concentration between different intensities and times. Assessment of GI symptoms was completed pre- and immediately post-exercise, and one-hour post-exercise.

The order of experimental trials was determined in a counterbalanced design. In both studies, during each experimental trial, participants were asked to verbally verify their adherence to the pre-experimental conditions as expressed in the General Methods section. All testing sessions were conducted in the morning under the laboratory conditions outlined in the General Methods. Exclusion criteria followed the guidelines as outlined in the General Methods section.

5.3.3 Experimental Protocol

Pre-experimental protocol: The test to determine CP and CS consisted of three consecutive time trials of varying distances (800, 1200 and 2400 meters, respectively), which were separated by different resting periods, as per the manufacturer's instructions (Stryd 2017) (Fig.2). Participants were familiarised with the test during their first visit.

Running Protocol: Each participant completed three experimental trials, each consisting of a treadmill run. Run speed was determined by power output (Watts), equivalent to three intensity domains: moderate (80-85% CP/CS); heavy (90-95% CP/CS) and severe (above CP/CS). Run time for each experimental trial was determined through completion of approximately 42000 to 43000 joules of work. Upon completion of the desired time, the trial was stopped. Running commenced 15 minutes following ingesting of the dual-sugar probe solution. Throughout each running test, participants ingested tap water at a rate of 1 $mL \cdot kg^{-1} \cdot BW$ of fluid every 15 minutes. During the rest arm of the study, participants were asked to rest, either sitting or supine for 120 minutes.

5.4 Data analysis

Determination of time and intensity: Critical power was determined by linear regression of the average power (watts) in each running trial, against the inversed time (seconds). The gradient of the regression line determined the CP, while the point of interception determined capacity available to accumulate work above this threshold W' (Vanhatalo, Jones and Burnley, 2011). Power was predicted using Equation 1, where P is power and T_{lim} represents time-to-exhaustion. The result of *Tlim* was divided against 1 to get the legitimate time to exhaustion.

$$P = W' \cdot \left(\frac{1}{Tlim}\right) + CP \qquad \text{[Equation 1]}$$

To determine CS, a linear regression was performed, plotting completed distance (meters), against time to complete the distance (seconds). The gradient of the regression line provided the distance (D') whilst the intercept provided the CS, and T_{lim} represents time-to-exhaustion (Vanhatalo, Jones and Burnley, 2011).

$$D = CS \cdot Tlim + D' \qquad \text{[Equation 2]}$$

To facilitate a comparison between the participants, it was necessary to standardise the amount of work (J). It is estimated that the minimum time required to augment intestinal permeability is approximately 20 minutes (Costa et al., 2017b). Therefore, the CP equation was modified to calculate the required amount of work (Vanhatalo, Jones and Burnley, 2011)

$$P = \left(\frac{\mathrm{W}}{(Tlim)}\right) + CP \qquad \text{[Equation 3]}$$

The power displayed at 20 minutes was used to establish T_{lim}, outlining the power that could be sustained for 20 minutes or above. Work in joules was calculated by multiplying power by time in seconds. A range of 42 – 43 kJ was established to warrant a similar amount of work between participants whilst ensuring a minimum of twenty minutes was completed in each of the intensity domains. To predict the time required to attain the desired work (42 – 43 kJ), the following equation was utilised:

$$T = \left(\frac{\mathrm{Joules}}{(Watt)}\right) / 60 \text{[Equation 4]}$$

The power at each intensity domain was 81 ± 2%, 93 ± 3% and 104 ± 1% of CP, for moderate, heavy and severe, respectively. Working within these power zones is reported to elicit different physiological response (Poole et al., 2016). Initial treadmill speed during every experimental trial was determined by the Critical Speed, in accordance with the specific power zone. Speed was calculated as moderate and

heavy at 80 – 85 % and 90 – 95 %, respectively, as well as the %CS equivalent to the power above CP, which is sustainable for twenty minutes or longer. Despite the determination of CS for each power zone, intensity was primarily determined by power. As such, running speed was modified to attain the desired power. $\dot{V}O_{2\,peak}$ was determined during the test as the highest mean $\dot{V}O_2$ value over ten seconds. The gas exchange threshold (GET), was established during each test from the first disproportionate increase in VCO_2, determined by visual inspection of the individual $\dot{V}CO_2$ by $\dot{V}O_2$ plots (Vanhatalo et al., 2011).

5.6 Assessment of GI permeability, injury and symptoms

Gastrointestinal permeability was quantified by the dual-sugar probe method as outlined in the General Methods section (Fleming et al., 1996). Blood samples were collected at baseline, immediately post-exercise and 120 minutes post-ingestion of the test solution.

D-lactate was determined by a rapid assay ultra violet (UV) colour metric technique, in 100 µl of EDTA, at 340 nm in plasma (Meagazyme, Ireland). Briefly, 100 µl of plasma was reacted for 5 minutes with sample reagents with the change in NADH release proportional to D-lactate present. The detection threshold for D-lactate of this micro-plate approach translates to a D-lactate lower concentration threshold of $0.21 mg \cdot L^{-1}$.

The appearance of GI symptoms was quantified by a 10-point Likert scale using 100 mm lines, as described in the General Methods section. Briefly, participants would indicate the appearance of any symptoms by marking on the scale. Symptoms being assessed included side stitch, nausea, bloating, urge to burp, urge to vomit, urge to defecate, diarrhoea, fluctuation, stomach cramps and stomach upset.

5.7 Statistical analysis

Data are presented as the mean ± the standard deviation (SD) unless otherwise stated. Ninety-five percent confidence intervals (95% CIs) are presented where appropriate. Changes between different variables throughout intensity domains were examined using a repeated-measures ANOVA. To correct violations of sphericity, the degrees of freedom were corrected in a normal way, using Huynh-Feldt ($\varepsilon > .75$) or Greenhouse-Geisser ($\varepsilon < .75$) values for ε, as appropriate (Field 2007). A Bonferroni pairwise comparison was made when 10 main effects were present. Pearson's product-moment correlation analysis was used to assess associations between variables respectively. Statistics were analysed using Statistical Package for Social Science (SPSS; 26.0, Armonk, NY: IBM Corp) with significance accepted at $p \leq 0.05$. Values found to be equal to P=0.000 were adjusted to P<0.001.

5.8 Results

5.8.1 Physiological responses to treadmill running

There was no significant main effect observed in work completed (42.5 ± 0.1 kilojoules) or distance covered (5.9 ± 0.1 km), indicating no difference in load between intensity conditions and participants. We observed no difference (P = 0.77) in body weight loss between exercise conditions (0.7 ± 0.1 kg). Mean power for each intensity domain was 192±37, 227±44 and 252±49 watts for moderate, heavy and severe intensity respectively. Table 11 displays power, %CP, HR, % of maximal HR, AND the relative $\dot{V}O_2$, % of $\dot{V}O_{2peak}$ associated at every intensity domain.

Blood lactate concentrations showed a significant increase between conditions in a stepwise manner, with the severe domain returning higher lactate concentrations than the heavy (P = 0.015), moderate (P <0.001) and rest (P <0.001) conditions. The heavy intensity domain returned higher lactate levels than moderate (P = 0.026) and rest (P = 0.011), but lower concentrations than severe (Fig 5.2). A significant effect for condition was reported for HR and RPE, with the severe domain returning higher values than heavy (P<0.05), moderate (P<0.001) and rest (P<0.001) (Table 11).

Table 11: Physiological responses to treadmill running at rest and within the moderate, heavy and severe exercise intensity domains.

	Rest	Moderate	Heavy	Severe	Main effect	Significance
Power (W)	-	192±37	227±44	252±49	$F_{1.3,12.1}$=96.9	**P<0.001**
Percentage CP (%)	-	81±2	93±3	104±1		
Work (kilojoules)	-	42.54±2.48	42.40±3.13	42.58±1.59	$F_{1.4,11.2}$=1.12	P=0.34
Distance (Km)	-	5.9±1.0	5.8±0.9	6.0±1.0	$F_{1.2,11.5}$=0.38	P=0.60
Running time (min)	-	37 ± 7	32 ± 6	28 ± 4	-	-
Speed (km/h)	-	9.8 ± 0.8	11.4 ± 1.2	12.5 ± 1.5	-	-
$\dot{V}O_2$ (mL·kg^{-1}·min^{-1})	9±2	39±4	44±6	49±6	$F_{1.2,10.4}$=490.9	**P<0.001**
$\dot{V}O_2$ (L.min^{-1})	625±155	2720±437	3094±584	3435±609	$F_{1.2,10.7}$=48.9	**P<0.001**
$\dot{V}O_{2\ max}$ (%)	16±2	71±3	81±5	90±4	-	-
HR (b·min^{-1})	58±9	150±11	163±11	174±10	$F_{1.7,15.3}$=541.5	**P<0.001**
HR (%)	31±5	78±5	85±6	91±5	-	-
RPE (AU)	6	11±1	14±2	16±1	$F_{2.1,18.9}$=196.7	**P<0.001**

5.8.2 Core Temperature

A significant main effect was observed in core temperature between exercise intensities (Table 12). Severe intensity domain returned a significantly higher core temperature response than the heavy (P = 0.003), moderate (P = 0.002) and rest (P< 0.001) intensity domains. Whilst an increase in rectal temperature was observed between intensity domains (Fig. 5.1), the limitations in sample size prevent rigorous conclusions being possible. A significant difference was observed in thermal comfort between severe and moderate (P=0.019) and rest (P<0.001). TC also showed a significant difference between heavy and moderate (P = 0.008), and rest (P<0.001). A significant difference between moderate and rest was also observed for TC (P=0.001). A significant correlation between core temperature and intestinal permeability was observed (r = 0.55, P<0.001) with core temperature accounting for 30 percent of the changes in L/R ratio. Furthermore, a significant positive correlation was observed between core temperature and subjective GI symptoms (r=0.54, P<0.001) with core temperature accounting for 29 percent of the changes in GI symptoms.

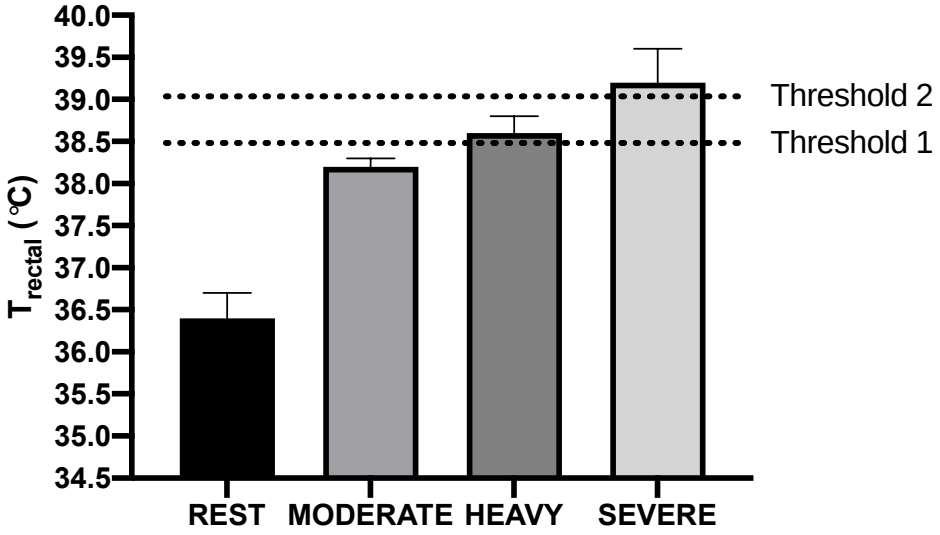

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Figure 13: Post-exercise core-temperature (°C) values for all experimental conditions.

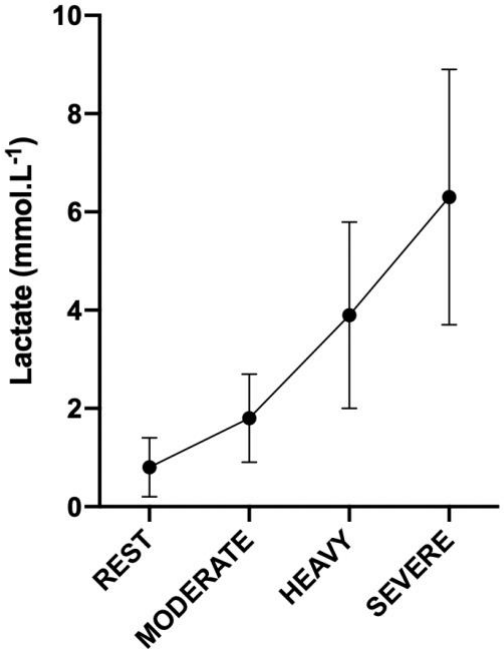

Figure 14: Post-exercise lactate (mmol.L-1) values for all experimental conditions

Table 12: Response in L/R ratio (AU); lactate (mmol.L-1); GI symptoms; core temperature (ºC); rectal temperature (ºC); and thermal comfort (AU) for all experimental conditions.

	Rest	Moderate	Heavy	Severe	Main effect	Significance
L/R ratio (AU)	0.031±0.011	0.039±0.011	0.052±0.012	0.055±0.011	$F_{3,27}$=13.3	**P<0.001**
Lactate (mmol.L^{-1})	0.8±0.6	1.8±0.9	3.9±1.9	6.3±2.6	$F_{1.8,16.2}$=26.6	**P<0.001**
GI symptoms (%)	1±2	0±1	5±3	12±6	$F_{1.5,13.8}$=34.0	**P<0.001**
T_{core} (ºC)	36.0±0.4	36.9±1.0	37.2±0.9	37.9±1.0	$F_{2.1,19.6}$=29.2	**P<0.001**
T_{rectal} (ºC)	36.4±0.3	38.2±0.1	38.6±0.2	39.2±0.4	$F_{1.9,3.8}$=115.1	**P<0.001**
TC (AU)	5.1 ± 0.3	6.7 ± 0.9	7.4 ± 0.8	7.8 ± 1	$F_{2.3,21.50}$=45	**P<0.001**

5.8.3 GI permeability and injury

A significant main effect for condition was observed (P<0.001) in serum L/R ratio, as the ratio increased in a stepwise manner in response to increasing exercise intensity (Table 12). L/R increased in the severe condition by 75% relative to rest. The severe condition also returned significantly higher L/R ratio values than moderate (P=0.041) and rest (P=0.05). However, no significant difference was observed between the severe and heavy exercise intensity domains. L/R in serum increased in the heavy condition by 65 percent when compared to rest, a significant effect (P=0.05), with no difference between heavy and moderate (Fig. 5.3). A significant positive correlation was observed between L/R ratio and GI symptoms (r=0.48, P=0.002), with L/R accounting for 23 percent of the changes in GI symptoms. D-lactate concentrations returned inconsistent data throughout the different conditions, with 89 % of data being under levels of detection (<0.21 mg·L).

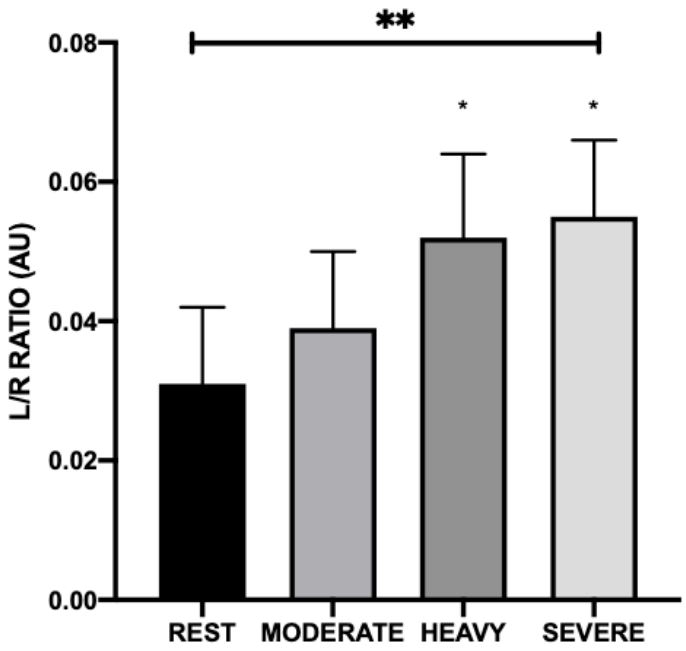

Figure 15: Two-hour lactulose/L-rhamnose ratio values for all experimental conditions. Data are mean ±SD.

5.8.4 GI symptoms

Despite a low appearance of GI symptoms, a significant main effect for condition was observed (Table 13). The severe intensity domain returned significantly higher appearance of GI symptoms compared to heavy (P=0.006), moderate (P=0.001) and rest (P=0.001). The heavy intensity domain also showed a significant higher appearance of symptoms than moderate (P=0.005) and rest (P=0.007), but

significantly lower symptoms than severe. Post-exercise subjective GI symptoms are displayed in Table 13.

Table 13: Post-exercise subjective gastrointestinal symptoms across different intensity domains, presented as mean + SD. Data are presented from a 0-10 VAS, whereby 0 represents no symptoms and 10 represents severe symptoms.

Gut symptoms	Rest	Moderate	Heavy	Severe
Side Stitch	0 ± 0	0 ± 0	1 ± 1	2 ± 2
Nausea	0 ± 0	0 ± 0	0 ± 0	0 ± 1
Bloating	0 ± 0	0 ± 0	1 ± 1	1 ± 2
Urge to Burb	0 ± 0	0 ± 0	0 ± 0	2 ± 3
Urge to Vomit	0 ± 0	0 ± 0	0 ± 0	1 ± 3
Urge to defecate	0 ± 0	0 ± 1	1 ± 2	2 ± 3
Diarrhoea	0 ± 0	0 ± 0	0 ± 1	0 ± 0
Flatulence	0 ± 0	0 ± 0	1 ± 2	1 ± 2
Stomach Cramps	0 ± 0	0 ± 0	1 ± 2	1 ± 3
Stomach upsets	0 ± 0	0 ± 0	1 ± 2	1 ± 2

5.9 Discussion

The aim of the present study was to investigate the effect of exercise intensity on GI permeability, biomarkers of GI injury and subjective GI symptoms in trained runners. We report that GI permeability increases in a stepwise manner with exercise intensity, when intensity is based upon running Critical Power. Furthermore, despite the appearance of only mild symptoms of GI discomfort, symptomology also increased in relation to exercise intensity, thus indicating a positive relationship between exercise intensity and GI symptoms. Core temperature and lactate also displayed a positive correlation with exercise intensity; suggesting a possible relationship between exercise intensity associated physiological disturbance and GI integrity. Due to inconsistencies and lack of detection of D-lactate, we cannot in our hands conclude that this biomarker is a reliable indicator of disruption to the GI barrier.

Exercise may result in a disturbance to the integrity of the gastrointestinal barrier, leading to increased permeability of the enterocyte tight junctions and damage to the mucosal layer (van Wijck et al., 2011a). These disturbances are influenced by intestinal ischemia as blood is directed away from the gut towards the periphery (Rehrer et al., 2001; van Wijck et al., 2012a). Furthermore, it has been hypothesised that the rise in core temperature in response to exercise may also contribute mechanistically to GI dysfunction (Pires et al., 2017). Following high intensity interval running at 120% $\dot{V}O_{2\,max}$, whereby average $\dot{V}O_{2\,max}$ for the bout was 90%, Pugh et al. (2017b) reported serum L/R ratio to be approximately 0.051. The results of the present study indicate similar concentrations in the heavy (0.052) and severe (0.055) conditions, where average $\dot{V}O_{2\,max}$ was 81 and 90 %, respectively. The present data therefore indicates that exercise above 93 % of critical power, corresponding to below 80

% of $\dot{V}O_{2\ max}$ and 93 % of Gas exchange threshold is able to induce a significant increase in GI permeability despite a low volume of running (5.9 km and 32 ± 5 min).

To date, only one study exists exploring the relationship between exercise intensity and gastrointestinal permeability (Pals et al., 1997). However, this study represented a relatively small sample (n = 6), and presented anomalies in their data; furthermore, methodological changes have developed the means of assessing gut permeability from urine analysis to a serum based biomarker method allowing the detection of smaller fluctuations in permeability which may not be detected with urine analysis (Fleming et al., 1996). These authors investigated the response in intestinal permeability to repeated bouts of sixty minutes of treadmill running at intensities corresponding to 40, 60 and 80 % of $\dot{V}O_{2\ max.}$ Whilst their analysis indicated a stepwise increase in GI permeability with exercise intensity, when compared to rest, the only significant increase was observed in response to running at 80 % of $\dot{V}O_{2\ max}$. Whereby, their results indicate a 123 % increase in L/R when compared to rest. This is distinctively higher than the increases reported in the present study, where we observed increases of 68 and 77 % in the *heavy* (81% $\dot{V}O_{2\ max)}$ and *severe* (90% $\dot{V}O_{2\ max}$) conditions, respectively. The results of the present study also report a smaller increase in L/R ratio of 26 % in the moderate condition (71 % $\dot{V}O_{2\ max}$). Similarly, data from the current study indicates lower resting L/R values of 0.031, compared to 0.048 reported by Pals et al. (1997). Collectively, despite methodological differences in permeability assessment between studies, a threshold of exercise intensity indicative of a *significant* increase in GI permeability appears to exist at or above 80 % $\dot{V}O_{2\ max}$; higher than the 70 % $\dot{V}O_{2\ max}$ threshold previously proposed, and used widely, in the literature (Dokladny, Zuhl and Moseley, 2016; Costa et al., 2017b). The differences between these two identified thresholds could be accountable to variance in the protocols and analysis methods of the studies; methodological differences such as the composition of the sugar-probe test solution, timing of ingestion of the solution, serum versus urine analysis where serum samples return higher sensitivity (van Wijck et al., 2011b; Pugh et al., 2017b) and variations in the length of urine collections (from one to twenty-four hours), collectively make comparisons between studies difficult (Costa et al., 2017b). Together, the results of the present study suggest that exercise intensity is a determining factor in the dynamics of gastrointestinal permeability.

A recent review identified a strong correlation between GI permeability and an increase in core temperature (Pires et al., 2017). Specifically, two key thresholds were identified: 38.5 °C, whereby GI permeability increases in some, but not all cases; and a core temperature of 39 °C or above, which corresponds to a universal increase in GI permeability. These results are similar to those of the present study, where a significant positive correlation was observed. However, in the present study, core temperature accounted for just 30 % of the variations in L/R, questioning the robustness of the findings by Pires et al. (2017). Rectal temperature is regarded as the most accurate way of recording core temperature (Moran and Mendal, 2002). With only three of our participants volunteering for this assessment, comparisons with the previous literature and that of core temperature thresholds is limited (Pires et al., 2017). Despite these limitations, a positive trend between core temperature and L/R was

observed and we report significant increases in GI permeability, compared to rest, from exercise performed in the *heavy* and *severe* intensity domains only. The mean peak core temperatures from these experimental conditions were above the thresholds outlined by Pires et al. (2017), suggesting a positive association between core temperature and GI permeability. These observations are further supported by our data, whereby exercise performed within the *moderate* intensity domain, where peak core temperature remained below 38.5 °C, did not induce a significant rise in GI permeability. Nonetheless, further research is warranted investigating the relationship between core temperature and GI permeability since clear associations from the present study are limited by the small sample size.

D-lactate is an alternative isoform of lactate, produced through fermentation by bacteria within the GI tract (Ewaschuk, Naylor and Zello, 2005). Studies in critical care patients have demonstrated a positive relationship between D-lactate and intestinal ischemia (Poeze et al., 2003); as such, this metabolite has been recognised as a potential biomarker for GI dysfunction (Li et al., 2017). To date, only Kondoh, Kawase and Ohmori (1992) have explored the D-lactate response in exercising humans. These researchers investigated the changes in plasma D-lactate in trained, and untrained runners, in response to approximately five minutes of stair running and thirty minutes of running around an athletics field. Whilst no control on exercise intensity was reported, D-lactate showed a greater increase following the stair running when compared to longer duration running. As such, this was the first study to investigate a potential relationship between exercise, D-lactate, GI permeability and symptoms within a controlled laboratory setting. However, data from the current study failed to show any change in D-lactate as the majority of results were below the detection limit of our assay. On face value these results would suggest D-lactate to be an unsuitable biomarker for detecting GI damage in healthy, exercising adults. However, the current study utilised a rapid assay UV colorimetric technique at 340 nm in plasma (Meagazyme, Ireland) to detect changes in plasma D-lactate. This method may have influenced the results of the present study as precision and detection levels are lower when compared to other more sensitive enzyme-linked immunosorbent assay (Shi et al., 2015). Furthermore, the body of research investigating D-lactate has been conducted in clinical patients experiencing severe GI ischemia or conditions (Poeze et al., 2003; Shi et al., 2015). Together, the results of the current study suggest that further research is warranted investigating the suitability of D-lactate as a marker of GI damage, but attention should be paid towards the method of analysis, with regard to detection threshold sensitivity.

The appearance of gastrointestinal symptoms is common amongst athletes, particularly runners (de Oliveira, Burini and Jeukendrup, 2014) and, indeed, a mild expression of GI symptoms (12 % of participants) was observed in the present study. Yet, the underlying mechanisms governing GI symptomology in response to exercise are not clear and current research remains contradictory (Costa et al., 2017b). In the present study, a positive relationship between GI symptoms and exercise intensity, with a significant increase in symptomology occurring in response to exercise in the *severe* domain. A further positive correlation was evident between the appearance of GI symptoms and the L/R ratio, yet

L/R ratio accounted for only 23 % of the presence of symptoms. Together with previous research, these results suggest a very weak relationship between intestinal permeability and GI symptoms (Karhu et al., 2017; Pugh et al., 2017b; Snipe et al., 2017). Participants of our previous studies (Chapter 4) reported no appearance of GI symptoms in response to treadmill running at 80 % $\dot{V}O_{2\ max,}$ yet in the present study, symptoms started to appear during the *heavy* (81% $\dot{V}O_{2\ max)}$ condition and significantly increased during the *severe* (90% $\dot{V}O_{2\ max}$) condition. The mild appearance of symptoms during the *heavy* condition is particularly interesting as the mean running time (32 $\pm$ 6) is almost half of that from our previous study (Chapter 3), where participants exercised for sixty minutes at a similar intensity. This is despite similar conditions between studies including environment, hydration and participant characteristics. One important difference between the studies remains that the current study was of mixed gender design including three female participants. The literature is equivocal with regards to whether female athletes are more likely to develop GI symptoms and only one study exists whereby confounding factors such as menstrual cycle phase were controlled (Snipe and Costa, 2018a). Despite similar control measures being in place in the present study, one female participant accounted for 56 % of the appearance of GI symptoms in the *heavy* condition and 47 % in the *severe* condition. One other (male) participant accounted for a distinct contribution to the expression symptoms in both the *heavy* (37 %) and *severe* (17 %) conditions. The remaining female participants reported almost no symptoms in any condition (2 %). Given the relatively small sample size of participants, it is difficult to draw conclusions as to whether females are more likely to develop GI symptoms, but instead, individual susceptibility may be a more critical determining factor. Together, these results may suggest that in thermoneutral conditions, exercise intensity may be a governing factor of GI symptoms. Core temperature has been shown to display a positive correlation with exercise intensity (Pals et al., 1997), furthermore, when compared to thermoneutral conditions, exercise in hot environments has shown to result in both higher core temperatures and increased incidence of GI symptoms (Snipe et al., 2017). These results agree with the present study, whereby the highest occurrence of GI symptoms and peak core temperatures were observed in the severe exercise domain trial. Indeed, Snipe et al. (2017) reported a significant increase in GI symptoms in response to exercise in the heat, where core temperature averaged 39.6°C, similar to the peak core temperature during the *severe* condition (39.5°C). Collectively, these data suggest that a relationship between core temperature and the appearance of gastrointestinal symptoms may exist and, thus, further research is warranted investigating this relationship.

Previous research has typically utilised $\dot{V}O_{2\ max}$ to prescribe exercise intensity, such as running speed or cycling power, with the majority of studies prescribing an intensity relative to 70 % of $\dot{V}O_{2\ max}$ (Costa et al., 2017b). Yet, a participant's training status may influence the physiological strain they experience when exercise intensity is determined by $\dot{V}O_{2\ max}$, meaning some participants may experience greater physiological disturbances than others (Poole et al., 2016). Critical power identifies a threshold within the power-duration relationship where energy supply reaches a steady-state, being

provided through oxidative metabolism without a progressive accumulation of blood lactate. This threshold thereby provides the determination of exercise intensity zones within which physiological reactions may or may not be stabilised, specifically below or above critical power, respectively (Poole et al., 2016). As such, when exercise intensity is prescribed in relation to critical power, within the *moderate, heavy,* or *severe* domains, the physiological and biochemical responses represent a specific stimuli to the participant (Burnley and Jones, 2018). Indeed, research has described a variety of physiological responses which increase when exercise is performed at an intensity which coincides with the *severe* domain, such as blood lactate accumulation, heart rate and faster decline of PCr stores; yet these physiological responses achieve a more steady-state at lower exercise intensities (Burnley, Vanhatalo and Jones, 2012; Vanhatalo et al., 2016). There are a number of similarities between the results of the present study and the literature, as exercise in the *severe* domain produced significantly higher responses in blood lactate, core temperature, HR and RPE when compared to the *moderate* and *heavy* intensity domains (Pals et al., 1997). Incidentally, this data provides further evidence of a potential association between exercise intensity and GI dysfunction, whereby higher exercise intensities contribute to a greater physiological strain which includes augmented intestinal permeability. However, a key weakness of the present study and indeed the existing literature, is a lack of quantification of mesenteric blood flow, a key contributor to GI dysfunction (ter Steege and Kolkman, 2012). Measurement of mesenteric blood flow and its relationship with exercise intensity, intestinal permeability and the appearance of GI symptoms, would strengthen our understanding of the mechanisms underlying GI dysfunction, thus, future research is warranted. Nonetheless, the association between running critical power and the alterations in physiological disturbances suggest CP to be an applicable method in prescribing a more individualised exercise intensity in future research. In particular the ecological application of CP determination and measurement through running power meters allows for refinement of field and lab based studies.

The results of the present study show that GI permeability displays a dose-response relationship with exercise intensity. The current study also demonstrated that exercise above CP compromises gastrointestinal integrity. Whilst an increase in GI symptoms was observed following exercise in the severe domain, the majority of this data was attributed to two participants, and therefore it cannot be concluded that a relationship between exercise intensity and subjective GI symptoms exists. Furthermore, the inconsistent data observed for D-lactate warrants further investigation into this biomarker as an indicator of GI dysfunction in exercising individuals.

Chapter 6– Effects of Passive Hyperthermia on Gastrointestinal Permeability, Injury and Symptoms

Chapter 5 demonstrated that intestinal permeability increases in a stepwise manner with exercise intensity, which correlated with core temperature. As such, Chapter 6 will investigate how an increase in core temperature with no physical exercise (passive heating) affects intestinal permeability.

6.1 Abstract

This study tested the hypothesis that increased intestinal permeability and injury are related to hyperthermia, independent of exercise-induced ischemia. *Methods:* Six healthy males participated in the study. After familiarisation, each participant completed three trial conditions (CONTROL, WARM and HOT), whereby core temperature was increased through hot water immersion (40°C). GI permeability was determined by serum L/R ratio. *Results:* Intestinal permeability increased in all conditions but there was no difference between conditions $P = 0.566$. No correlation between increased gastrointestinal permeability and core temperature was observed ($r = 0.019$). *Conclusion:* Hyperthermia independent of exercise-induced intestinal hypoperfusion does not augment gastrointestinal permeability or symptoms.

6.2 Introduction

Under normal physiological conditions, the passage of molecules across the enterocytes in the GI barrier is well controlled. However, under certain physiological conditions such as hyperthermia or ischemia (caused by a reduction in blood and oxygen supply to the gut), the tight junctions become dysfunction and intestinal permeability increases (Costa et al., 2017b). Indeed, hyperthermia has been shown to increase the permeability of the intestinal tract in both human studies and *in vitro* (Dokladny, Moseley and Ma, 2006; Dokladny, Zuhl and Moseley, 2016). However, it is not yet clear which contributing factor, hyperthermia or hypoperfusion, plays the predominant role in augmenting intestinal permeability.

The physical barrier between the contents of the intestine and the portal blood consists of an epithelial layer connected by protein structures known as tight-junctions (Grootjans et al., 2016). During exercise, blood is redirected away from the GI tract towards the periphery and working muscle, reducing the blood and oxygen supply to the GI tract (Rehrer et al., 2001). This state of intestinal ischemia causes

a cascade of events which ultimately leads to the dysfunction of the tight junctions and an increase in intestinal permeability. These events have been discussed in Chapter 2.2.

Normal core temperature is maintained at approximately 37℃, however this can increase during exercise (exertional) or in hot environments (passive) (Tyler et al., 2016). The temperature regulatory systems of the body can be compromised by clothing or submersion in water, preventing the loss of heat by radiation and creating an environment of uncompensable heat stress (McLellan, Boscarino and Duncan, 2013). As heat accumulates in the body, core temperature continues to rise and symptoms of heat illness may develop, such as nausea or syncope, posing a serious threat to the health of athletes, military personnel and some workers (Coris, Ramirez and Van Durme, 2004; McLellan, Boscarino and Duncan, 2013). If these symptoms are not addressed and core temperature continues to rise, heat stroke may occur. Indeed, heat stroke is a serious, life-threatening, medical condition, which is characterised by an elevation in core temperature above 40℃, which sees a cascade of events leading to an inflammatory response, multiple organ failure and death (Epstein and Yanovich, 2019). Hall et al. (2001b) also demonstrated in rats, that elevating core temperatures above 40℃ reduces splanchnic blood flow, leading to intestinal barrier dysfunction and subsequent endotoxemia. Whilst research into the effects of passive hyperthermia on the intestinal permeability of humans is currently lacking, a recent review by Pires et al. (2017) identified an association between exercise induced hyperthermia and intestinal permeability. These researchers identified two key thresholds of core temperature: 38.5℃ where intestinal permeability is augmented but not universal amongst participants; and 39℃ where an increase in intestinal permeability is universal. This review fails to investigate the effects of passive hyperthermia on intestinal permeability, likely due to the lack of research in this area given the ethical restrictions towards increasing the core temperature of humans towards levels associated with heat stroke (Dokladny, Zuhl and Moseley, 2016). Therefore, the aim of the present study was to investigate whether increasing core temperature via passive hyperthermia to the two identified core temperature thresholds augments intestinal permeability. A secondary aim of the present study was to quantify the response in subjective GI symptoms in response to passive hyperthermia.

6.3 Methods

6.3.1 Participants

Eight healthy, recreationally active males volunteered to participate in the study. One participant withdrew from the study following familiarisation. All participants provided written consent prior to the commencement of testing. Participant requirements included that they were training four or more hours per week, which included endurance or resistance-type exercise, free from gastrointestinal syndrome and willing to abide by the restrictions implemented by the study (24h rest prior to each trial, overnight fast, no caffeine, alcohol or non-steroidal anti-inflammatory drugs in the 24 hours prior

to each trial.)

Table 14: Descriptive characteristics of participants displaying means ± SD for age (yrs.); body mass (kg); height (cm) and $\dot{V}O2$ max ($mL \cdot kg^{-1} \cdot min^{-1}$).

	Age (yrs.)	Body Mass (kg)	Height (cm)	$\dot{V}O_{2\ max.}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)
Mean ± SD	31 ± 16	74 ± 4.5	175.2 ± 5.4	53.6 ± 2.6

6.3.2 Pilot Measures

To investigate the physiological responses to our proposed methods of passive hyperthermia, we performed a series of pilot procedures prior to the main experimental study. The aims of the pilot study were to establish an estimate of the time required for core temperature to increase to the two critical values previously identified (38°C and above 39°C). Furthermore, we aimed to observe the core temperature response following removal of the heat stress i.e., once the participant was removed from the bath. Three healthy, trained males participated in the pilot study. Following resting measurements of weight (kg), heart rate (bpm) and core temperature (rectal thermometer, °C) each participant sat, resting, in a thermostatically controlled bath set at 40°C. Core temperature and heart rate were recorded every three minutes.

6.3.3 Assessment of Maximal Oxygen Uptake

During the first visit, $\dot{V}O_{2\ max}$ was determined as per section General Methods 3.4.1.

6.3.4 Experimental Design

In a crossover, repeated-measures design, participants completed a familiarisation trial and three experimental trials: control (CON); warm (WARM) and hot (HOT). Familiarisation trials included rectal probe insertion and introduction to heat stress induced by hot water immersion until T_{core} reached 39°C. Familiarisation trials were performed at least seven days before the first experimental trial to limit the acute effects of heat acclimation. All experimental trials began between 7.00 h and 9.00 h at the Sport & Exercise Science laboratory. During all experimental conditions, upon arrival at the laboratory, participants rested in a supine position for two minutes before a resting measure of blood pressure and a blood sample were taken. During WARM and HOT, participants then changed into swimwear before having their nude mass (kg) recorded. During CON, participants would wear light sports clothing including a t-shirt, shorts or tracksuit bottoms, socks and shoes. Participants then inserted a calibrated rectal thermometer 10 cm past the anal sphincter and provided a urine sample to

determine hydration status, before being fitted with a heart rate monitor strap around the chest (Polar, H10, Finland). During CON, participants sat, resting, in the laboratory under ambient environmental conditions. During the WARM and HOT conditions, participants were secured in a safety harness and, fifteen minutes post sugar-probe ingestion, lowered into a thermostatically controlled bath, set at 40°C, to the manubrium, so-as-to to induce a rise in core temperature. Participants remained submerged until their T_{core} reached specific values: WARM (38-38.5°C) and HOT (39°C). When T_{core} reached the specific value, participants were raised out of the bath to attenuate any further increase in core temperature. Cooling was implemented via cold water misting and a fan in the WARM condition to attenuate T_{core} increasing beyond 38.5°C. In each experimental condition, exposure was set at 60 minutes. After 60 minutes, the participant was raised out of the bath and lowered onto a bed where they remained, resting, for thirty minutes. During all experimental conditions, measures of heart rate (BPM), core temperature (°C), RPE (6-20), thermal comfort (0-10) and gastrointestinal symptoms (Likert scale 1-10) were recorded every three minutes.

During the WARM and HOT conditions, participants received tap water at approximately 37°C, at a rate of 250 mL every fifteen minutes. We provided water to prevent the possibility of augmented tight-junction permeability due to dehydration (Lambert et al., 2008). The temperature of the water was selected to represent typical core temperature, and prevent any attenuation of thermal strain or intestinal injury which may occur with water of a lower temperature (Snipe and Costa, 2018b). During the CON trial, participants were provided with tap water set at room temperature and instructed to drink ad libitum.

6.3.5 Sugar probe administration

During each experimental visit, participants ingested a solution containing 10 g of lactulose and 2 g of L-rhamnose in 230 mL tap water, at 15 minutes prior to be submerged in the bath or rest.

6.3.6 Thermal comfort, HR, RPE, and core temperature

Core temperature (T_{core}), heart rate (HR), RPE, thermal comfort (TC) were measured as described in the General Methods, and recorded every three minutes.

6.3.7 Gastrointestinal symptoms

Symptoms of gastrointestinal discomfort were collected in line with the timing of blood-sample collection and every three minutes during the experimental trials. Data was collected as described in 3.6.3.

6.3.8 Blood analysis

Blood samples were collected fifteen minutes before (Pre), within three minutes post-PHS (Post) and 120 minutes post-ingestion (120) of the dual sugar probe solution. Samples were collected and analysed for L/R as per section 3.7. Blood sample analysis samples were prepared according to the methods outlined in the General Methods section.

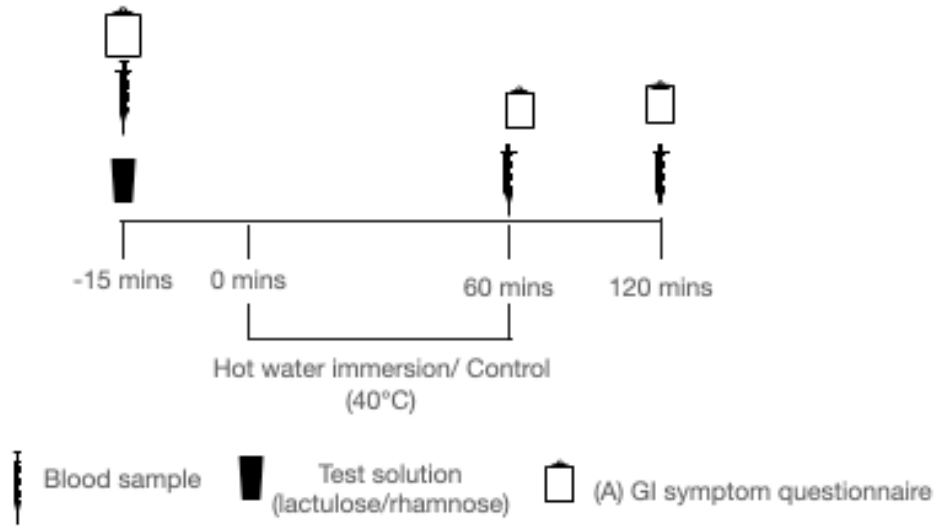

Figure 16: Schematic representation of the hot-water immersion protocol.

6.3.9 Statistical analysis

Data was analysed with the use of the statistical software package SPSS (Version 26, SPSS Inc., Armonk, NY: IBM Corp), with significance accepted at $p \leq 0.05$. Values found to be equal to P=0.000 were adjusted to P<0.001. Where appropriate, ninety-five percent confidence intervals (95% CIs) are presented. Changes between variables throughout conditions (CON, WARM, HOT) were tested using a repeated measures ANOVA. To correct violations of sphericity, the degrees of freedom were corrected in a normal way, using Huynh-Feldt ($\varepsilon > .75$) or Greenhouse- Geisser ($\varepsilon < .75$) values for ε, as appropriate (Field 2007).

6.4 Results

6.4.1 Physiological Responses

No change in body mass was observed in any condition (see Figure 17), with no significant main effect for time ($F_{1, 6}$ = 4.134, P = 0.88) or condition ($F_{2, 12}$ = 0.181, P = 0.837). No significant interaction was observed between conditions ($F_{2, 12}$ = 1.138, P = 0.353). There was an observed increase in HR in response to heat stress, with a significant main effect for time ($F_{1, 5}$ = 14.329, P =0.013). A similar increase in HR occurred between WARM and HOT conditions. A significant main effect was observed for condition ($F_{2, 10}$ = 4.618, P = 0.042). There was also a significant interaction observed ($F_{2, 10}$ = 9.380, P = 0.005). RPE increased between pre- and post-exposure in the WARM and HOT conditions, with a significant main effect observed for condition ($F_{2, 12}$ = 4.263, P = 0.04) and time ($F_{1, 6}$ = 6.280, P = 0.05). A significant interaction was also observed ($F_{1.80, 12}$ = 4.263, P = 0.04). TC increased between pre- and post-exposure in the WARM and HOT conditions; significant main effect was observed for condition ($F_{2, 10}$ = 11.023, P = 0.003) and time ($F_{1, 5}$ = 14.412, P = 0.013), however no significant interaction was observed ($F_{1.76, 8.80}$ = 3.824, P = 0.068) (Table 15).

Table 15: Peak physiological values: heart rate (b·min-1); thermal comfort (AU); core temperature (°C); blood pressure (mmHg); and RPE (AU). Data are mean ± SD.

	CON	WARM	HOT	Main Effect	Significance
HR (b·min^{-1})	58 ± 5	89 ± 15	96 ± 24	$F_{1.87, 9.33}$= 4.618	**P = 0.042**
TC (AU)	5 ± 1	7 ± 2	8 ± 2	$F_{2,10}$ = 11.023	**P = 0.003**
T_{core} (°C)	36.50 ± 0.23	38.15 ± 0.20	39.20 ± 0.10	$F_{1.78, 12.4}$= 136.648	***P* = 0.001**
Systolic Blood Pressure (mmHg)	131 ± 6	132 ± 8	131 ± 9	$F_{1.58, 7.91}$= 0.254	P = 0.732
Diastolic Blood Pressure (mmHg)	75 ± 4	65 ± 4	64 ± 9	$F_{1.87, 7.49}$= 8.432	P = 0.013
RPE	6 ± 0	8 ± 4	12 ± 6	$F_{2, 12}$ = 4.263	**P = 0.04**

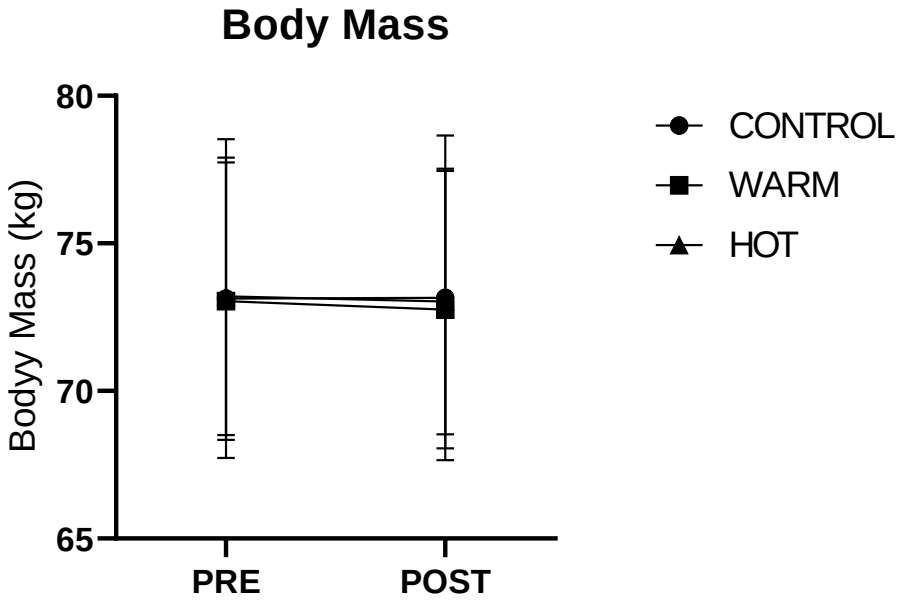

Figure 17: Changes in body mass (kg) between pre- and post-hot water immersion for control (CON), warm (WARM) and hot (HOT) conditions.

6.4.2 Core Temperature

There was an observed increase in core temperature in response to heat stress (Figure 18), with a significant main effect for time ($F_{1, 7}$ = 373.998, P < 0.001). Mean water temperature was 40 ± 0.3°C across all bath conditions. Core temperature increased to the greatest degree in the HOT condition (39.2 ± 0.2°C), compared to the WARM condition (38.1 ± 0.2°C). There was a significant main effect observed for condition ($F_{2, 14}$ = 136.648, P = <0.001) and a significant interaction was observed ($F_{2, 14}$ = 180.222, P = <0.001). In the WARM condition, participants' core temperature was elevated above 38°C for 33 ± 6 minutes. In the HOT condition, participants' core temperature was elevated above 39°C for 24 ± 8 minutes (Figure 18 (B)).

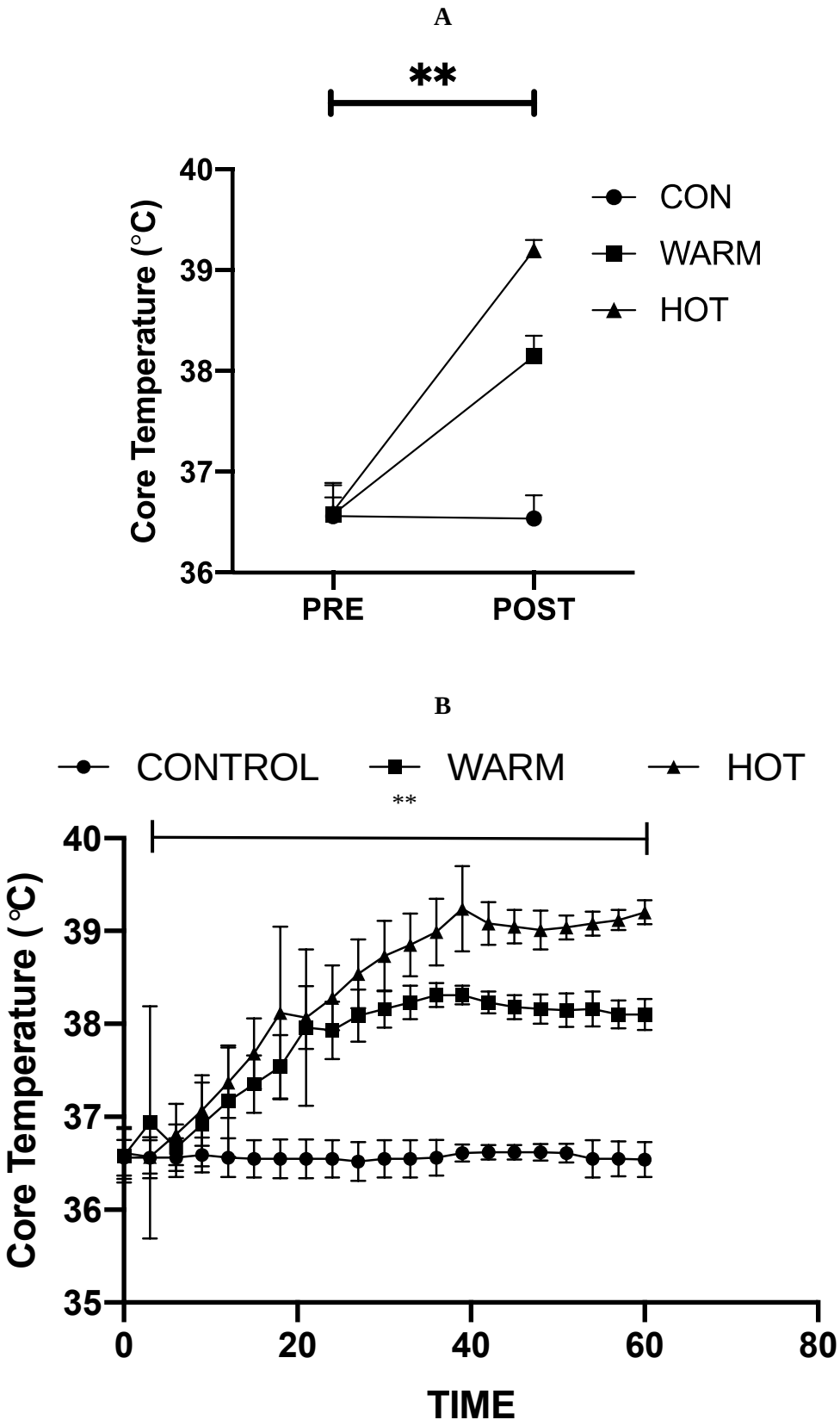

Figure 18: (A) Change in core temperature between pre- and post-hot water immersion for all conditions; (B) showing the core temperature response of participants during hot-water immersion. ** representing a significant effect between time-points

6.4.3 Small Intestinal Permeability

An increase in intestinal permeability was observed in all conditions, indicated by the ratio of lactulose to rhamnose in the serum. There was a significant effect of time on permeability ($F_{1, 7}$ = 22.286, P = 0.002). There was no significant difference between the mean L/R ratios for all conditions ($F_{2, 14}$= 0.132, P = 0.0878). There was also no significant interaction ($F_{1, 56,}$ 10.89= 2.234, P = 0.159). There was no significant main effect between conditions (P = 0.566). The WARM condition resulted in the highest observed permeability (0.018 ± 0.006). Interestingly, CON displayed a higher L/R ratio (0.017 ± 0.004) than HOT (0.015 ± 0.007). There was no correlation observed between small intestinal permeability and core temperature (r = 0.019, P = 0.517).

Table 16: Showing the L/R ratio at PRE and POST HWI, and two hours post sugar-probe ingestion (120). nd represents values below the detection limit of the assay.

PRE					
	CON	**WARM**	**HOT**	**Main Effect**	**Significance**
L/R ratio (AU)	nd	nd	nd		
POST					
	CON	**WARM**	**HOT**	**Main Effect**	**Significance**
L/R ratio (AU)	0.012 + 0.005	0.012 + 0.004	0.013 + 0.007	F2,14= 0.144	P = 0.867
120					
	CON	**WARM**	**HOT**	**Main Effect**	**Significance**
L/R ratio (AU)	0.017 ± 0.004	0.018 ± 0.006	0.015 ± 0.007	$F_{2,14}$ = 1.083	P = 0.365

6.4.4. Gastrointestinal symptoms

Symptoms of *regurgitation, stomach fullness, cramps, flatulence* or *urge to defecate* were non-existent to mild (< 2). Peak values for symptoms were higher in the HOT condition than WARM. In the WARM trial, there were no reported symptoms other than nausea. In the HOT trial, one participant reported a peak of 2 for *regurgitation.* Three participants reported symptoms of fullness, with peaks of 7, 3 and 4, respectively. One participant reported a peak of 2 for cramps, two participants reported a peak of 2 for flatulence. No participants reported the *urge to* defecate. No GI symptoms were reported in the CON trial.

In the WARM trial, nausea accounted for the highest occurring symptom, with three participants reporting peak values of 6, 8, and 1, respectively. However, a strong correlation over time was observed between core temperature and nausea (r = 0.856). During the HOT trial, participants reported symptoms

of nausea and stomach fullness. Four participants reported symptoms of nausea, with peak values of 10, 8, 6 and 10, respectively. A strong correlation over time between core temperature and nausea was observed in the HOT condition (r = 0.856). Of the eight participants, four reported symptoms of nausea, two of which reported a peak score of ten. Three participants reported symptoms of stomach fullness, with peak scores of seven, three and four, respectively.

6.5 Discussion

The aim of the current study was to quantify the intestinal permeability responses and symptoms to non-exercise induced (passive) hyperthermia in a healthy, active population. Whilst variations in gastrointestinal permeability were observed, as indicated by a change in L/R ratio in the serum, we report no difference between conditions. Furthermore, a weak correlation between core temperature and gastrointestinal permeability is reported. Collectively, passive heat stress did not induce lower gastrointestinal symptoms. However, when taken individually, a strong correlation was observed between core temperature and nausea.

Exercise results in a multitude of physiological responses that can contribute to GI dysfunction, including a rise in core temperature (Gleeson, 1998) and a reduction in mesenteric blood flow (Rehrer et al., 2001). However, the magnitude of contribution from these physiological responses towards augmented intestinal permeability is not fully understood. A systematic review (Pires et al., 2017) identified a strong correlation between increased core temperature and GI permeability. In their review, these researchers proposed two key thresholds of exercise-induced core temperature: 38.5°C (threshold 1), whereby intestinal permeability is augmented in some participants, and 39°C (threshold 2), where increased intestinal permeability is universal amongst participants. Similarly, in their landmark study, Pals et al. (1997) reported a stepwise positive correlation between core temperature and GI permeability. These authors reported elevated GI permeability in participants after running at 80% of $\dot{V}O_{2\ peak}$ for sixty minutes, which also resulted in the highest final rectal temperature (39.6°C).

At two hours post ingestion of the dual-sugar probe solution, a serum L/R ratio of 0.015 was observed in the HOT condition. This ratio was lower than that observed in the WARM condition (0.018). Interestingly, the L/R ratio in our CON trial returned a higher value (0.016) than WARM, although the value equalled only 0.001 (AU). These values represent increases between post-HWI and 120 in the L/R of 0.004, 0.006 and 0.002 for the CON, WARM and HOT trials respectively. According to the hypothesis proposed by Pires et al. (2017), the highest L/R ratio should have been observed in the HOT condition, followed by the WARM condition. The changes in the L/R ratio may thus present the 'normal' transport of the sugar probes across the intestinal barrier, not necessarily an increase in intestinal permeability. The results therefore suggest that passive hyperthermia has no effect on the transport of the sugar probes when compared to a thermoneutral control condition. An alternative explanation behind these results may be that the two-hour plasma assay may not be sensitive enough to

detect a meaningful change or difference in the L/R ratio between time-points. Given the large variation in methodology within intestinal permeability research (Table 3), comparisons between the change in L/R values from the present study and previous literature are challenging. It is therefore difficult to conclude whether these value changes, between sample points, have physiological significance. For example, previous research has observed similar resting L/R ratios to those of the present study, yet these were obtained from urinary measures whilst using a different composition of test solution (van Nieuwenhoven, Brouns and Brummer, 2004; van Wijck et al., 2011a). Another study (Pugh et al., 2017b) observed a mean resting serum L/R ratio of 0.031, almost two-fold of that observed in the current study, with a wider range in their L/R ratio ($\pm$ 0.016). L/R ratio was found to increase following high-intensity intermittent exercise (0.051 $\pm$ 0.016). Whilst these studies observed an increase in L/R ratio following exercise, they failed to record core temperature, making comparisons between the present study and the thresholds proposed by Pires et al. (2017) difficult. Collectively however, it can be assumed that resting L/R values can differ significantly between individuals, and no data is currently available to compare the changes in L/R ratio across time amongst resting participants. Furthermore, it is unclear how the rapid turnover rate of the GI enterocytes, approximately 72 hours (Darwich et al., 2014), affects an individual's intestinal permeability reproducibility.

Mesenteric blood flow decreases during exercise (Rehrer et al., 2001) which can lead to a decreased supply of oxygen to the gastrointestinal organs (Otte et al., 2001). Short periods of hypoperfusion and ischemia can cause erosion of the epithelial lining at the tips of the villi (Grootjans et al., 2016). However, before the epithelial cells shed, a zipper-like mechanism acts to pull the lower epithelial cells together, creating a new barrier and preventing the translocation of endotoxins into the portal blood. Under periods of prolonged ischemia with complete cessation of blood flow of approximately forty-five minutes or more, the zipper-like mechanism becomes dysfunctional; the restorative mechanisms of the membrane fail, leading to a compromised physical barrier through disruption of the epithelial lining and tight-junction protein complexes (Grootjans et al., 2016). Specifically, cytoskeletal structure within the enterocyte may be altered, which begins a cascade of disturbances to the transmembrane proteins, ultimately decreasing their resistance and increasing paracellular permeability(Rodgers and Fanning, 2011). Given that the present author and other researchers (Pals et al., 1997) have observed increased intestinal permeability in response to sixty minutes of exercise, it could be hypothesised that sufficient hypoperfusion occurred to induce dysfunction in the tight-junction protein complexes. However, these exercise studies also observed a rise in core temperature, making it difficult to conclude the relative contribution of increased core temperature or intestinal hypoperfusion to increased intestinal permeability.

The present study utilised sixty minutes of hot-water immersion to elevate core temperature above the thresholds proposed by Pires et al. (2017). However, it was not viable to measure mesenteric blood flow due to logistical restrictions related to the participants being submerged in a hot water bath via the only available method of simultaneous Doppler ultrasound of the femoral and mesenteric

arteries. Heat exposure induces cutaneous vasodilation and skin blood flow to promote heat dissipation away from the core to the environment. To facilitate this process, cardiac output is increased and blood is redirected from areas such as the splanchnic region (Charkoudian, 2003). Indeed, substantial inter-individual differences exist in the response of skin blood flow to heat due to factors such as heat acclimation or training status (Roberts et al., 1977). Again, due to logistical limitations of participants being submerged in water, measurement of skin blood flow was not viable in the present study. Blood pressure was however recorded every fifteen minutes yet showed no change over time ($P = 0.595$). Which would be indicative of an adaptive cardiovascular response to maintain blood pressure (Crandall and Wilson 2015), that could bring about reductions in splanchnic blood flow which has previously been shown to be reduced by 40% in response to passive hyperthermia in rats (Hall et al., 2001b). In humans, reductions of ~20-40% splanchnic blood flow have been observed in response to passive heat stress (Rowell et al., 1970; Rowell, 1974). This wide range in splanchnic blood flow response reflects high intra-and inter individual differences to comparable levels of heat exposure (Crandall and Wilson et al., 2015). However, these values are still considerably below the 80% reductions achieved in during high intensity exercise (Rehrer et al., 2001). Unfortunately it cannot be demonstrated that a reduction in mesenteric blood flow occurred in the participants of the present study; such data would have helped inform the contribution of hypoperfusion to the augmented intestinal permeability observed in the present study. However, the values and changes in L/R ratio between sample time points in the present study are minor compared to those of previous research (Table 3). Together, these results may suggest that hypoperfusion, rather than hyperthermia, may elicit greater increases in intestinal permeability in exercise-related studies in healthy athletes.

Whilst prolonged periods of mesenteric ischemia (over forty-five minutes) may cause disruption to the epithelial barrier (Grootjans et al., 2010b; Grootjans et al., 2016), research has not investigated whether similar periods of hyperthermia alone (i.e., not induced by exercise), result in a similar response. Dokladny, Moseley and Ma (2006) exposed Caco-2 intestinal epithelial cells to temperatures of 37 and 41ºC over a twenty-four hour period. They observed a dose-response effect of temperature on tight-junction permeability, with the higher temperature resulting in increased permeability (Zuhl et al., 2014a). However, it is not clear whether this response would have occurred either at temperatures similar to those achieved in the present study, or with shorter exposures to hyperthermia. Nevertheless, considering the mean exposure of the participants of the present study to the higher core temperature threshold of 39°C was only 24 $\pm$ 8 minutes, this may not have been sufficient for a distinct rise in intestinal permeability to occur. The exposure times of the present were limited due to ethical limitations.

The appearance of GI symptoms during exercise is common amongst athletes (de Oliveira, Burini and Jeukendrup, 2014). However, the literature is inconclusive as to the dominant factor underlying the appearance of symptoms, indeed, it is likely due to a combination of factors including environment, psychological stress or nutrition (Lis et al., 2016; Wilson, 2017; Snipe et al., 2018a).

Nausea is a common symptom of heat illness (Coris, Ramirez and Van Durme, 2004) and whilst it has been correlated to exercise intensity (Keeffe et al., 1984), this is the first study to quantify a correlation between core temperature and nausea. Prevalent amongst athletes and a leading cause for non-completion of events, nausea induces a feeling of light headedness, with a feeling of sickness and urge to vomit (Wilson, 2019). In the present study, the prevalence of GI symptoms was low, however, nausea was common and correlated strongly with core temperature. Indeed, higher core temperatures have also been associated with increased exercise intensity (Pals et al., 1997), further suggesting an association between core temperature and nausea.

It has been previously demonstrated that dehydration of approximately 1.5 % of body mass can increase GI permeability without any difference in final core temperature (Lambert et al., 2008); suggesting dehydration *per se* may contribute to an increase in GI permeability. In the present study, participants were permitted to drink water ad-libitum (CON) or at a rate of 250 mL, every fifteen minutes (WARM and HOT). In the WARM and HOT condition, participants were provided with water heated to approximately 37°C to replicate normal core temperature and prevent any dampening in core temperature increase (Snipe and Costa, 2018b). We observed no difference between pre- and post-weight in any condition, suggesting that euhydration was maintained, and that dehydration did not contribute to the differences observed in gastrointestinal permeability.

Considering a similar response was observed across all conditions, it is difficult to delineate the relative contribution of factors that contributed to the rise in intestinal permeability. Using hot water immersion, an increase in core temperature above those proposed by Pires et al. (2017) was achieved in the present study, yet it was observed that no significant difference between conditions occurred, including CON. Therefore, no significant correlation between the elevation in core temperature and intestinal permeability is evident. Indeed, more research is required to quantify variations in resting levels of intestinal permeability and its reproducibility in a large cohort of participants. Further, passive heating and GI permeability with mesenteric blood flow determined simultaneously will allow further delineation of the relationship between whole body hyperthermia and GI permeability.

Chapter 7 – Effects of Glutamine Supplementation on Gastrointestinal Permeability, Injury and Symptoms in Response to Heat-Stress

Chapter 6 demonstrated that hyperthermia without exercise-induced hypoperfusion may not elevate intestinal permeability. However, the response in intestinal injury to passive heat stress remains unknown. Furthermore, acute glutamine supplementation has demonstrated protective effects on core temperature and intestinal damage, but these effects remain unexplored in response to passive heat stress.

7.1 Abstract

Aim: Investigate the effects of acute glutamine supplementation on markers of gastrointestinal permeability, injury and subjective measures of heat illness in response to heat stress. *Methods:* Six healthy, recreationally active males [age 23 ± 4 yrs., body mass 69.9 ± 4.0 kg , $\dot{V}O_{2\ max}$ 64.3 ± 5.9mL·kg^{-1}·min^{-1}] volunteered to participate in the study. In a randomised, placebo-controlled cross-over design, each participant completed two experimental trials. Participants received either glutamine (0.5g·kg^{-1} BM) or placebo, two hours prior to exposure to heat stress by hot-water immersion at 40°C. Each experimental trial consisted of sixty minutes of heat exposure. Markers of GI permeability and injury were quantified by serum L/R ration and I-FABP, respectively. Subjective measures of heat illness were quantified by questionnaire throughout each experimental trial. *Results:* L/R ratio did not differ between glutamine or placebo trials (0.029 ± 0.011; and 0.025 ± 0.009). Heat stress resulted in a significant increase in GI damage, however no significant difference was observed between trials. Similarly, heat illness resulted in an increase in subjective symptoms, with no difference between trials. *Conclusion:* Passive hyperthermia, induced by hot-water immersion, results in an increase in gastrointestinal injury and subjective symptoms of heat illness, with no effect from glutamine consumption. Passive hyperthermia independent of exercise induced hypoperfusion does not induce increased gastrointestinal permeability.

7.2 Introduction

Heat-related pathology poses a serious threat to the health of athletes and certain workers who are required to wear protective clothing such as the military or firefighters (Coris, Ramirez and Van Durme, 2004; McLellan, Boscarino and Duncan, 2013). Summer heat waves have accounted for approximately 200 deaths per year in the United States (Leon and Helwig, 2010). Certain factors such as obesity, poor

fitness, dehydration, common medications such as antihistamines and widely used stimulants like caffeine, put people at a higher risk of developing heat-illness (Coris, Ramirez and Van Durme, 2004). With global ambient surface temperatures predicted to increase, along with more frequent and intense extreme weather events, heat-illness also poses a future risk to athletes and certain workers (Lucas, Epstein and Kjellstrom, 2014).

Heat illness is a pathological condition which can develop following elevation of core temperature above normal physiological range in response to certain stimuli such as high environmental temperatures or exertion from exercise (Ogden et al., 2020). Heat can disturb many intracellular molecular structures and proteins, and ameliorate protein, DNA and RNA synthesis. Furthermore, heat stress can induce oxidative stress and reactive oxygen species production (Rhoads et al., 2013). These disturbances are similar to those observed in the gut following hypoperfusion/ reperfusion, which sees excess production of reactive oxygen species (Granger and Kvietys, 2015).

Those experiencing heat illness can display symptoms, some of which can be displayed by athletes during training or competition, such as nausea, syncope, muscle cramps and confusion; indeed, nausea has been attributed as the leading cause of drop-outs from endurance events (Barrow and Clark, 1998; de Oliveira, Burini and Jeukendrup, 2014; Wilson, 2019). If left untreated, heat-illness can develop into heat-stroke, a serious pathological condition which can result in organ failure and death. Furthermore, heat stroke patients are often left with permanent impairments to neurological and peripheral tissue systems (Leon and Helwig, 2010).

Considering the potential implications of heat-illness on the health and performance of athletes and the wider population, nutritional interventions to ameliorate the progression of heat-illness has received some attention from researchers. Strategies such as ice-slurry ingestion have been shown lower core temperature and increase performance in runners and cyclists (Siegel et al., 2010; Burdon et al., 2013). Another nutritional strategy that has been explored in the literature as a way to offset heat disturbance and intestinal injury is the ingestion of the amino acid glutamine (Mondello et al., 2010; Soares et al., 2014). Glutamine is the most abundant amino acid within the human body and serves as the primary fuel source for the enterocytes of the intestinal tract, however plasma glutamine concentrations are reduced under conditions of exercise, shock or trauma (Rao and Samak, 2012). Supplementation with glutamine has been shown to reduce intestinal permeability in response to exercise in thermoneutral conditions and the heat (Zuhl et al., 2014b; Zuhl et al., 2015; Pugh et al., 2017c). Research from rodent studies also demonstrates that glutamine supplementation can protect the cells of the intestinal tract from reperfusion injury by offsetting oxidative stress, increase epithelial cell proliferation, as well as increasing villus height (Mondello et al., 2010; Wu et al., 2018). Furthermore, one rodent study demonstrated glutamine supplementation to attenuate a rise in core temperature following acute heat exposure (Soares et al., 2014), however, this effect has not been demonstrated in exercising humans (Pugh et al., 2017c).

One consequence of exercise is a reduction in intestinal blood flow as blood is redirected away from the gut towards the periphery to promote thermoregulation and nutrient delivery (Rehrer et al., 2001). Hypoperfusion of the intestinal tract is thought to be a primary cause of the augmented intestinal permeability and injury observed during exercise (Costa et al., 2017b), with heat being hypothesised as another major contributor (Pires et al., 2017). The gold standard for quantifying intestinal permeability is through the use of sugar absorption test, with subsequent analysis of urine or serum to measure the ratio of the reappearance of the probes (Fleming et al., 1996; van Wijck et al., 2011b). I-FABP, an intracellular protein expressed exclusively in the apical epithelial cells of the small and large intestine, has more recently received attention as a marker of intestinal injury. Briefly, following injury to the mucosal layer of the small intestine, the apical cells of the villus are shed and I-FABP is released into circulation, permitting the detection of the protein in the serum (Lau et al., 2016). Since the half-life of I-FABP is approximately 11 minutes, its detection in the serum is considered a marker of preceding acute intestinal injury (Guzel et al., 2014). Indeed, I-FABP concentrations have been shown to be elevated following exercise in the heat, but this elevation was prevented with acute glutamine supplementation (Pugh et al., 2017c).

However, research is currently lacking distinguishing the effects of hypoperfusion or hyperthermia alone on intestinal permeability and, or injury, in healthy participants. Core temperature can be increased through exertional or passive heat stress, through methods such as exercise and, or, increasing environmental temperatures (Miwa et al., 1994; Dokladny, Moseley and Ma, 2006; Tyler et al., 2016; Sheahen et al., 2018; Snipe et al., 2018a). Physiologically relevant elevations in temperature, induced by passive hyperthermia, have previously been shown to augment permeability in *vitro*, in caco-2 cell lines (Dokladny, Moseley and Ma, 2006) and rodents (Wu et al., 2018), by disrupting the integrity of the tight-junction proteins. However, passive hyperthermia without exercise has not yet been shown to increase intestinal permeability in humans. As such, the present study addressed the following aims: to investigate the effects of passive heat stress on intestinal permeability and injury as expressed by the presence of I-FABP, and furthermore, investigate the effects of acute glutamine supplementation on these responses. Given the association between heat-illness symptoms, particularly nausea, and the effects of these symptoms on athletic performance, a further aim of the present study was to quantify the response of heat-illness symptoms to passive hyperthermia, and whether glutamine supplementation demonstrated an effect on these symptoms.

7.3 Methods

7.3.1 Participants

Six healthy, recreationally active males [age 23 $\pm$ 4 years, body mass 69.9 $\pm$ 4.0 years, $\dot{V}O_{2\,max.}$64.3 $\pm$ 5.9 $mL \cdot kg^{-1} \cdot min^{-1}$] volunteered to participate in the study. Participant requirements included that they

were male, training three or more hours per week of endurance-type exercise, not taking medication (NSAIDs, diuretics) free from gastrointestinal syndrome and willing to abide by the restrictions implemented by the study (24h fast rest prior to each trial, overnight fast, no caffeine or alcohol in the 24 hours prior to each trial.)

7.3.2 Assessment of maximal oxygen uptake

During the first visit, $\dot{V}O_{2\,max}$ was determined as per section General Methods 3.4.1.

7.3.3 Experimental Design

In a double blind, placebo controlled randomised crossover design; each participant completed a baseline assessment of maximal oxygen uptake and familiarisation, and a total of two experimental trials. In both trials, participants were required to remain submerged in a thermostatically controlled bath, set to 40°C, to the sternal notch, for 60 minutes. Participants received either a placebo or glutamine bolus to ingest at a dose of 0.5g·kg body mass. A dose of 0.5g·kg body mass was chosen to both align with previous research from our group (Pugh et al., 2017c), but also due to ethical restrictions imposed by the University ethics committee. Trials were separated by a one-week washout period to minimise any effects of supplementation or heat adaption.

Participants were asked to complete a 24 h food diary prior to the first experimental trial and asked to repeat this diet ahead of the second trial. Participants arrived at the laboratory between 07:00 and 9:00 h following an overnight fast. Upon arrival at the laboratory and two-hours prior to hot water immersion (HWI), a baseline blood sample was taken. Participants were then provided with an opaque bottle containing 400mL of tap water, mixed with either 100mL sugar-free lemon cordial (placebo) or cordial plus glutamine, and asked to consume the solution within five to ten minutes. Participants then sat, resting for two hours, before inserting a rectal thermometer 10 cm past the anal sphincter, which was connected to a portable data logger (SQ210, Grant Instruments, UK) and providing a urine sample. Participants were also fitted with a heart rate monitor strap, followed by another blood sample, before ingesting of the dual-sugar probe solution, which contained 10 g of lactulose and 5 g L-rhamnose. Participants completed an adapted State-Trait Anxiety Inventory questionnaire (State-Trait Anxiety Inventory, 2014)

Participants were then lowered into the bath whilst sat in a safety harness, and remained submerged for sixty minutes. Measures of thermal comfort (TC), RPE, core temperature and heat illness symptoms were collected every three minutes. After sixty minutes, the participants were lifted out of the bath before being transferred to a bed where they rested in a supine position.

7.3.4 Sugar probe administration

During each experimental visit, participants ingested a solution containing 10 g of lactulose and 2 g of L-rhamnose in 230 mL tap water, at 15 minutes prior to be submerged in the bath or rest.

7.3.5 Thermal comfort, HR, RPE, and core temperature

Core temperature (T_{core}), heart rate (HR), RPE, thermal comfort (TC) were measured as described in the General Methods, and recorded every three minutes.

7.3.6 Symptoms of heat-illness and GI discomfort

Symptoms of heat-illness were collected every three minutes during the experimental trials utilising an adapted State-Trait Anxiety Inventory questionnaire Data was collected as described in 3.6.3.

7.3.7 Blood Collection

Blood samples were collected at baseline, immediately prior to the ingestion of the sugar-probe solution (0), immediately post hot water immersion (60), and two hours post-ingestion of the dual sugar probe solution (120). Blood was collected as previously explained in the General Methods section. Following the final sample collection all samples were prepared according to the methods outlined in the general methods section.

7.3.8 Assessment of plasma glutamine

Plasma glutamine was assessed using a quantitative colorimetric enzyme assay kit (EGLN-100, BioAssay Systems, Hayward, CA) sensitive to 0.023 mM glutamine according to manufacturer's instructions. Samples were diluted 1:2 with distilled water. Glutamate was measured in each sample and subtracted from the glutamine absorbance of the respective sample.

7.3.9 Assessment of I-FABP

I-FABP was determined by analysis of EDTA plasma samples by an enzyme-linked immunosorbent assay (ELISA) (Hycult Biotechnology, Uden, the Netherlands; detection window 47 – 3000 $pg \cdot mL^{-1}$) according to the manufacturer's instructions. The ELISA is a solid-phase enzyme-linked immunosorbent assay based on the sandwich principle. Samples were diluted 1:2 with a provided sample dilution buffer and plates were read at 450 nm. The coefficient of variation was 8% for between-

sample duplicates. I-FABP concentrations were analysed from samples taken pre- and immediately post- heat stress. Post heat stress values were corrected for adjustments to plasma volume.

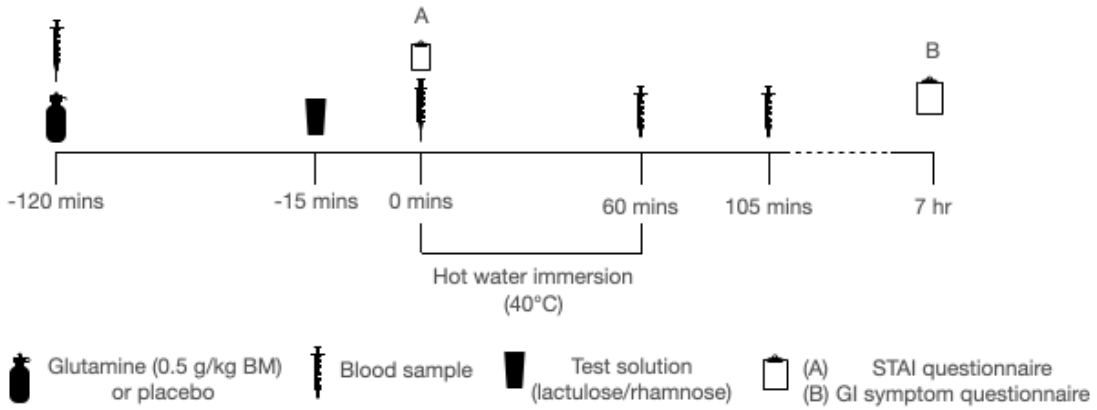

Figure 19: Schematic representation of the experimental procedure.

7.3.10 Statistical analysis

Data was analysed with the use of the statistical software package SPSS (Version 26, SPSS Inc., Armonk, NY: IBM Corp), with significance accepted at $p \leq 0.05$. Values found to be equal to P=0.000 were adjusted to P<0.001. Where appropriate, ninety-five percent confidence intervals (95% CIs) are presented. Changes between variables throughout conditions (PLA, GLU) were tested using a repeated measures ANOVA. To correct violations of sphericity, the degrees of freedom were corrected in a normal way, using Huynh-Feldt ($\varepsilon > .75$) or Greenhouse- Geisser ($\varepsilon < .75$) values for ε, as appropriate (Field 2007). A Bonferroni pairwise comparison was made when main effects were present.

7.4 Results

7.4.1 Glutamine supplementation did not affect the physiological response to passive heat stress

Total hot-water immersion time during the glutamine and placebo trials was 48.0 ± 10.4 and 51.5 ± 9.0 min, respectively. A similar but non-significant increase in HR was observed between conditions (P= 0.668). No difference was observed between conditions for thermal comfort (P= 0.363), peak core temperature (P= 0.885), RPE (P= 0.363) or blood pressure (Table 17)

Table 17: Peak physiological values: heart rate (b·min-1); thermal comfort (AU); core temperature (°C); blood pressure (mmHg); RPE (AU); Data are mean ± SD.

	Placebo	Glutamine	Significance
HR (b·min^{-1})	116 ± 20	114 ± 11	0.668
Thermal comfort (AU)	8.83 ± 0.41	8.67 ± 0.52	0.363
Core temperature (°C)	39.19 ± 0.12	39.27 ± 0.13	0.855
Systolic Blood Pressure (mmHg)	139.7 ± 12.7	133.67 ± 9.07	0.235
Diastolic Blood Pressure (mmHg)	63 ± 8.41	63 ± 5.8	1
RPE	14.67 ± 6.74	14.5 ± 6.66	0.363

7.4.2 Glutamine supplementation did not affect the thermoregulatory response to passive heat stress

There was an observed increase in core temperature in response to HWI, with a significant main effect for time ($F_{1, 5}$ = 283.051, P < 0.001). Core temperature increase across both conditions was similar. There was no significant main effect observed for condition ($F_{1, 5}$ = 0.229, P = 0.652). No significant interaction was observed between conditions ($F_{1, 5}$ = 1.848, P = 0.232). There were no differences in peak core temperature between conditions. GLU peak T_{core} was 39.27 (34.46 - 39.34) °C, whilst PLA peak T_{core} was 39.19 (36.5-39.35) °C.

7.4.3 Gastrointestinal permeability and injury

There was no significant difference in post-HWI ratios of L/R in the GLU and PLA condition (Table 18). HWI resulted in increased IFABP concentrations across both conditions. Baseline values of IFABP were similar in both conditions, however, there was a significant effect of time on serum IFABP concentrations (P = 0.002). There was a larger, non-significant increase in IFABP following glutamine supplementation compared to placebo (Table 17) There was no significant interaction observed (P = 0.234). Similarly, no significant main effect was observed for condition (P = 0.312).

Table 18: Post-HWI values for L/R ratio (AU) and I-FABP (pg·mL-1). Data are mean ± SD. nd represents values below the detection limit of the assay.

	PLA		GLU		Significance
	PRE	**POST**	**PRE**	**POST**	
I-FABP ($pg \cdot mL^{-1}$)	515.9 ± 208.1	1123.0 ± 61.7	511.3 ± 277.5	1554.0 ± 588.3	*P*= 0.002
L/R (AU)	nd	0.029 ± 0.011	nd	0.025 ± 0.009	*P*= 0.503

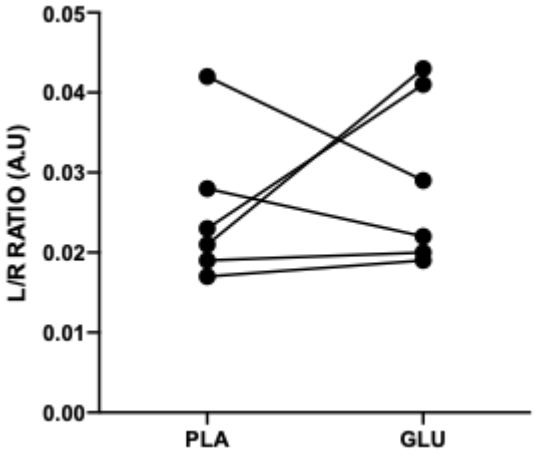

Figure 20: Individual 2-hour L/R ratio between the glutamine (GLU) and placebo (PLA) conditions.

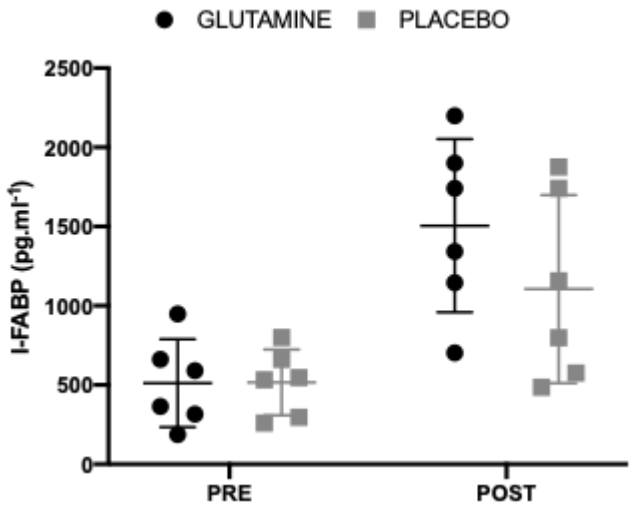

Figure 21: Individual post-HWI I-FABP between the glutamine (GLU) and placebo (PLA) conditions.

7.4.4 Glutamine supplementation augments plasma glutamine concentrations

There was no difference observed in plasma glutamine concentrations at baseline or post-HWI in either condition (0.84 ± 0.31 vs. 0.94 ± 0.47 mM in glutamine and placebo conditions, respectively; P = 0.310). Plasma glutamine concentrations were significantly elevated between baseline and pre- hot water immersion in the GLU condition (1.77 ± 0.66 mM) (P = 0.009). When compared to placebo, pre-HWI plasma glutamine concentrations were significantly higher in the GLU condition (0.87 ± 0.36 mM) (p = 0.007). Plasma glutamine concentrations decreased during HWI, and there was no significant difference in post-HWI plasma glutamine concentrations between conditions.

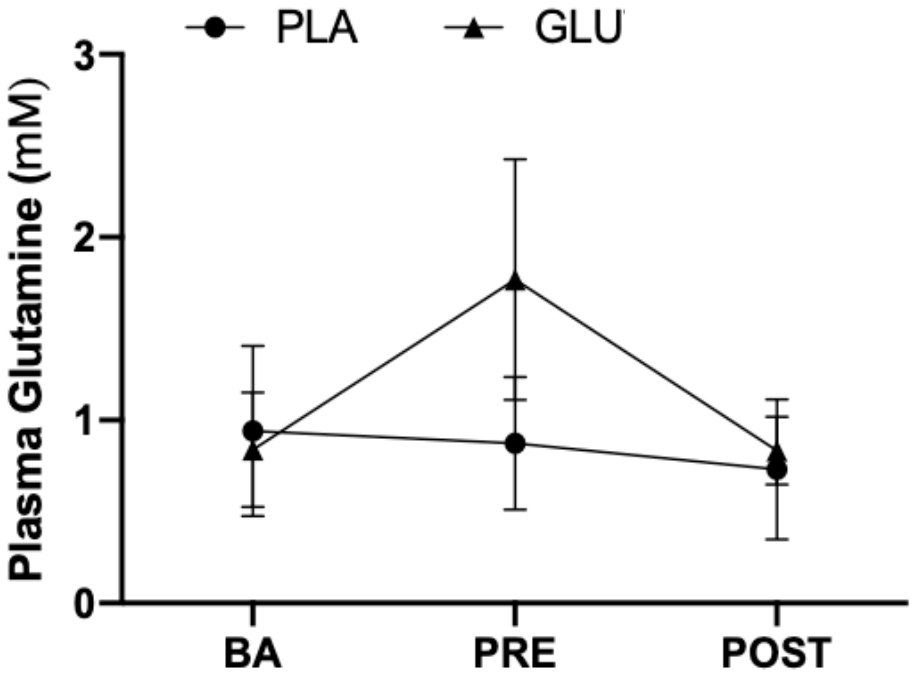

Figure 22: Change in plasma glutamine values between baseline (BA), pre-HWI (PRE) and post-HWI (POST), for the glutamine (GLU) and placebo (PLA) conditions.

7.4.5 GI symptoms were not affected by glutamine supplementation

Participants reported mild to severe occurrence of subjective heat illness symptoms. Only one participant reported symptoms of 'chills' and 'stopped sweating.' Glutamine supplementation did not affect the appearance of subjective symptoms in response to hot-water immersion when compared to placebo. The highest reported symptom was '*feeling lightheaded'*, with feelings of '*tiredness'*, *'dizziness'*, *'nausea'*, and *'heat sensation'* being augmented to a similar magnitude. Participants

reported a high occurrence of "*thirst"* to a similar extent in both conditions.

Table 19: Appearance of subjective heat-illness symptom during HWI.

	Glutamine	Placebo	Wilcoxon sig. value
Tired	8.5 (0-10)	8.5 (0-10)	0.564
Swelling	7 (0-10)	5.5 (0-9)	0.102
Cramps	4 (0-10)	2.5 (0-8)	0.276
Nausea	8 (0-10)	8 (0-8)	0.109
Dizziness	8.5 (0-10)	8 (0-10)	0.180
Thirst	9 (0-10)	8.5 (0-9)	0.180
Vomiting	5.5 (0-7)	6 (0-8)	1
Confusion	6.5 (0-10)	5.5 (0-10)	0.276
Muscle weakness	7 (0-10)	6 (0-9)	0.336
Heat sensation	9 (0-10)	8.5 (0-10)	0.705
Chills	0 (0-0)	0 (0-4)	0.317
Stop sweating	0 (0-0)	0 (0-2)	0.317
Feeling lightheaded	9.5 (0-10)	8.5 (0-10)	0.102

*Data are median and range appearing in parenthesis

7.5 Discussion

The aim of the present study was to quantify the effects of acute glutamine supplementation (0.5 $g.kg^{-1}$ BM) versus a placebo on intestinal permeability, injury and symptoms in response to passive hyperthermia induced by hot water immersion in a healthy, active population. Heat stress increased core temperature and markers of intestinal injury, however we observed no attenuating effect from glutamine supplementation. Similarly, no difference was observed in intestinal permeability between conditions, indicated by the ratio of lactulose to L-rhamnose in the serum. Furthermore, heat stress resulted in a high occurrence of subjective symptoms of heat-illness, yet no attenuation effect was observed from glutamine supplementation. Together, the results of the current study suggest that acute glutamine supplementation does not prevent the appearance of gastrointestinal injury or symptoms of heat illness in response to heat-stress. Furthermore, hyperthermia for the period of ~60 minutes, without exercise mediated reductions in blood flow, may not be sufficient to induce an increase in gastrointestinal permeability.

Fatty acid-binding proteins (FABP) are cytosolic proteins with a low molecular weight (atomic mass of 14-15 kDa), expressed in abundance within tissues with a dynamic fatty acid metabolism, such

as the heart, liver and intestine (Pelsers, Hermens and Glatz, 2005). Cellular expression of FABP is regulated at the transcriptional level, responsive to alterations in lipid metabolism. Whilst the primary function of FABPs is to facilitate transport of intracellular long-chain fatty acids, secondary functions include the regulation of gene expression through mediation of fatty acid signal translocation to peroxisome proliferator activated receptors (PPARs) (Wolfrum et al., 2001; Pelsers, Hermens and Glatz, 2005). FABPs are released into circulation in response to tissue injury and subsequently removed from circulation primarily through renal clearance, facilitating the quantification of both urinary and plasma concentrations of FABP. Approximately 30 % of FABP are removed in a single pass through the kidneys, and the plasma half-life of FABP has been calculated at approximately 11 minutes (van de Poll et al., 2007), suggesting FABP to be a useful indicator of acute tissue injury.

Despite three isoforms of FABP being expressed within the intestine (intestinal (I)-FABP, liver (L)-IFABP and ileal-bile acid binding protein (I-BABP)) (Pelsers, Hermens and Glatz, 2005), I-FABP has primarily been utilized as an indicator of intestinal injury. I-FABP is abundantly expressed within the apical region of the villi, in the most mature enterocytes, with the highest tissue concentrations being found within the jejunum, followed by the duodenum (4.79 $\mu g \cdot g^{-1}$ and 2.22 $\mu g \cdot g^{-1}$, respectively) (Pelsers et al., 2003). These region are primarily affected in response to physiological stressors, which can lead to intestinal injury and disruption of the epithelial lining (Barberio et al., 2015; Grootjans et al., 2016). This disruption can subsequently lead to dysfunction of the tight-junction proteins located between the enterocytes, allowing I-FABP to enter circulation via paracellular diffusion (Van Wijck et al., 2012b) and be subsequently detected in the urine or plasma. Despite the half-life of I-FABP being only eleven minutes, this protein has become a popular marker of enterocyte damage and intestinal injury in clinical studies (Derikx et al., 2010; van Wijck et al., 2011a; Dokladny, Zuhl and Moseley, 2016; Pugh et al., 2017b; Wells et al., 2017). The rapid clearance of I-FABP into the plasma or urine permits a quick diagnosis of clinical intestinal pathology, since its appearance can occur before any visual indicators (Khadaroo et al., 2014)

Circulating levels of I-FABP increase following exercise (van Wijck et al., 2011a; March et al., 2017), trauma (Timmermans et al., 2015), and in cases of heat-stroke patients where core temperature exceeds 40º C (Zhang et al., 2015). Research to measure intestinal permeability and injury on passively heat-stressed humans is limited. Data regarding the intestinal responses to heat-stress has thus been collated from clinical studies of heat-stroke patients, or exercise studies. As such, in the author's best knowledge, this is the first study to purposefully quantify I-FABP concentrations in response to passive heat stress i.e., induced by hot-water immersion and not exercise with, or without, additional environmental stress. Our resting I-FABP values were similar to those observed by other researchers (van Wijck et al., 2011a; Pugh et al., 2017b; Jonvik et al., 2018), however we report higher post-test values. Specifically, Pugh et al. (2017b) reported a 72 % increase in I-FABP from baseline in response to treadmill running at 70% of maximal oxygen uptake in 30°C heat, whilst van Wijck et al. (2011a) report a 99 % increase in response to 60 minutes of cycling at 70% maximum workload capacity.

In the present study an increase of 204 and 118 % in I-FABP was observed during the glutamine and placebo trials, respectively. Aside from hyperthermia, the mechanisms underlying such a large increase in I-FABP remain unclear. It has previously been suggested that hemodynamic parameters such as low blood pressure (< 70mmHg) and haemoglobin levels (<80 % of the lower normal value limit), in response to abdominal trauma may contribute to intestinal injury (Timmermans et al., 2015). However, the present study reports no correlation between I-FABP and arterial blood pressure (r = 0.221), and a minor, negatively correlated relationship between Hb and I-FABP (r = -0.247). The results of the present study therefore indicate, for the first time, that in-vivo heat stress alone may be sufficient to elicit enterocyte intestinal injury. It should be remembered that in present study, along with the majority of literature (van Wijck et al., 2011a; Van Wijck et al., 2012b; Barberio et al., 2015; Lis et al., 2015; Karhu et al., 2017; March et al., 2017), single point measures of I-FABP have been utilised and may fail to reflect the total injury load expressed; I-FABP has a relatively fast elimination profile. Future research should consider reporting data as area under the curve, as originally proposed to estimate release profiles (van Wijck et al. (2011a).

GI permeability has previously been increased in response to exercise (Costa et al., 2017b), and *in vitro* by subjecting Caco-2 cell culture models to physiologically relevant increases in heat-stress (Dokladny, Moseley and Ma, 2006; Davison et al., 2016). Since exercise results in an increase in core temperature, it has been suggested that hyperthermia is a major contributing factor towards compromised gastrointestinal integrity, potentially in a dose-dependent manor (Pires et al., 2017). Following a twenty-four hour exposure to elevated temperatures (37 to 41°C), Caco-2 cells demonstrated a linear inverse relationship between transepithelial resistance (TER) and paracellular permeability (Dokladny, Moseley and Ma, 2006). Davison et al. (2016) similarly observed an increase in permeability of HT29 and Caco02 cells in response to an increase in temperature of 2°C, from 37°C to 39°C.

Similarly, core temperatures in excess of 39° C have also been correlated to augmented intestinal permeability (Pires et al., 2017). In the present study, mean peak core temperature was 39.2 °C, with no difference between conditions (P= 0.855). These temperatures are similar to those seen in participants exercising for sixty minutes during at 70% of $\dot{V}O_{2\,max}$ (Zuhl et al., 2015) where an increase in intestinal permeability was observed. However, intestinal hypoperfusion, caused by the redistribution of blood flow away from the gut during exercise, has also been considered a major contributor to the underlying mechanisms augmenting intestinal permeability. Blood flow to the gut can be reduced by as much as 40-50% within 20 minutes of the onset of exercise (Rehrer et al., 2001), therefore reducing the amount of oxygen available for the perfusion of the intestinal organs. Unfortunately, one limitation of the present study is that we were unable to measure mesenteric blood flow, thus the blood flow response to passive heat stress cannot be determined in the present model. Despite this limitation, data again indicates, that short term hyperthermia alone does not result in an increase in intestinal permeability in passively heat-stressed humans. Together, these results indicate no attenuating effect on core

temperature by glutamine supplementation and that a combination of hyperthermia and intestinal ischemia mediated via mesenteric hypoperfusion may be necessary to augment enterocyte permeability in this population.

L-glutamine is the most abundant amino acid in human muscle and plasma, with normal plasma ranges between normal range of plasma glutamine is 500- 750μmol/L. Maintenance of this range is coordinated by the net balance between glutamine release and uptake, with the gut being responsible for a high proportion of uptake (Felig and Wahren, 1971). Under conditions of catabolic stress, such as infection, trauma, burns and prolonged exercise, the endogenous glutamine pool is depleted (Rao and Samak, 2012; Timmermans et al., 2015). It has previously been suggested that a reduction in intestinal glutamine is associated with an increase in intestinal permeability and that glutamine supplementation can repair intestinal membrane integrity (Camilleri et al., 2012). Whilst glutamine supplementation has ameliorated intestinal permeability and injury when compared to placebo (Hond et al., 1999; Pugh et al., 2017c), the present study demonstrates no difference in GI permeability or injury between conditions. Two-hour post HWI L/R ratio values were similar to those reported in resting conditions in this thesis (Chapter 5) and previous studies (Pugh et al., 2017b), and also following 60 minutes of treadmill running (Chapter 4). Furthermore, despite similar conditions, the values from the present study are higher than those previously reported from passive heat stress by HWI (Chapter 6). A limitation exists in the present study as L/R values are obtained from one time point, taken two hours post-ingestion of the dual-sugar probe solution. As such, it cannot be concluded whether intestinal permeability changed in these participants.

The occurrence of GI symptoms is common during exercise (de Oliveira, Burini and Jeukendrup, 2014), with several contributing factors being proposed including heat-stress (Snipe et al., 2017), intestinal ischemia (ter Steege et al., 2012) and psychological stress (Wilson, 2017). We have previously demonstrated that exercise intensity could affect the appearance of GI symptoms in a stepwise manner (see Chapter 3). Passive heat-stress did not increase the appearance of subjective GI symptoms, yet our participants reported a high occurrence of nausea, a common symptom of heat-illness (Howe and Boden, 2007). It was therefore decided, for the present study, to quantify the appearance of heat-stress related symptoms, rather than gastrointestinal symptoms. It has previously been shown that the appearance of GI symptoms does not correlate with intestinal injury or permeability (Karhu et al., 2017; Pugh et al., 2017c). Whilst increased levels of endotoxin release cannot be ruled out in the present study, in absence of augmented intestinal permeability, endotoxemia would have been unlikely, which can cause similar subjective symptoms (Lambert, 2008); therefore, the appearance of heat-stress symptoms would suggest alternative underlying mechanism.

In the present study, we observed a high incidence of subjective symptoms of heat illness, with no effect from glutamine supplementation. The exact mechanisms underpinning the pathophysiology of heat-illness symptoms are not clear, and are likely due to a combination of factors. Peripheral vasodilation occurs during heat stress as a mechanism to dissipate heat, however, heat dissipation was

likely impaired by hot-water immersion, compromising the loss of heat via radiation into the external environment. An increase in heart rate was observed, a mechanism utilized to maintain cardiac output and prevent an attenuation in blood pressure (Costrini et al., 1979). This impairment in vascular control, combined with the un-compensable nature of hot-water immersion likely caused heat load to exceed heat dissipation capacity, leading to an accumulation of body heat. Together, these physiological responses may have affected cerebral blood flow and increased the temperature of blood supply to the brain. Subsequently, brain temperature (Romanovsky, 2007; Walter and Carraretto, 2016) may have increased, leading to a cascade of physiological mechanisms, including syncope (Seto, Way and O'Connor, 2005), which have been associated with the appearance of symptoms of heat exhaustion, including nausea, lightheadedness and confusion (Aggarwal et al., 2008; Cheshire, 2016; Walter and Carraretto, 2016).

During short periods (30 – 45 minutes) of intestinal hypoperfusion, the upper and more mature epithelial cells found at the villus tips, whereby I-FABP is exclusively expressed, become compromised. This results in a retraction of the basal membrane, creating a sub-epithelial space. Subsequently, the less mature enterocytes are pulled together by the contraction of the basal membrane, forming a new villus tip, and the upper damaged cells detach, shedding into the intestinal lumen, increasing circulating levels of I-FABP (Grootjans et al., 2016). As such, the epithelial lining is quickly restored, with the paracellular tight junctions thus maintaining a barrier to the translocation of luminal microbiota or larger particles such as lactulose (Derikx et al., 2008a; Grootjans et al., 2016). Theoretically, this would explain the mechanism behind our observation of intestinal injury without an increase in the L/R ratio. However, this physiological mechanism is underpinned by intestinal ischemia, resulting from a sustained reduction in blood flow to the gut.

Passive heat stress induced by a water perfusion suit exposure has previously reduced hepatic blood flow by up to 60% (Rowell et al., 1968; Rowell et al., 1970; Rowell, 1974), similar to those values observed in response to exercise ranging from 50 to 70% maximal oxygen uptake (Rehrer et al., 2001; Rehrer et al., 2005; Thijssen, Steendijk and Hopman, 2009). Because of ethical and logistical restrictions, these measures were not performed in the present study. The data suggests, however, that cardiovascular adjustments occurred in the participants, namely an increase in cardiac output indicated by an augmented heart rate. These mechanisms serve to promote thermoregulation and dissipation of heat away from the core to the environment (Crandall and Wilson, 2015). Together with the work by (Rowell, 1974), it cannot be ruled out that some degree of reduced splanchnic blood flow occurred in the participants of the present study. However this notional hypoperfusion appears not to be of sufficient magnitude and duration to induce an increase in intestinal permeability.

The results of the present study indicate, for the first time, that passive heat-stress results in intestinal injury, indicated by an increase in plasma I-FABP. Whilst encouraging, the post-HWI sample reflects the I-FABP concentration of only the previous 11 minutes (given the half-life of I-FABP). As such it is unclear whether these values represent the highest concentrations of I-FABP. Further samples

taken during and further post-HWI could indicate a timeline of I-FABP dynamics in response to this passive hyperthermia stimulus. The present study was also able to demonstrate that passive heat stress induced symptoms of heat-illness, particularly nausea and that nausea is positively correlated to core temperature. This data may provide valuable information towards nutritional interventions for athletes who are prone to symptoms of nausea during exercise or competition, as measures could be implemented to offset the increase in core temperature. The data from the present study also suggests that 60 minutes of passive heat stress may not be sufficient to elevate intestinal permeability. These data also demonstrate that intestinal injury and subsequent shedding of the villus tips, thus releasing I-FABP into circulation, may precede tight-junction dysfunction; providing evidence that the mechanisms outline by Grootjans et al. (2016) may also be reflected in humans. No attenuating effect from acute glutamine supplementation was observed on any of our measures including intestinal permeability, injury, core temperature and symptoms. Since we observed no increase in serum L/R ratio, the results of the current study suggest that hyperthermia alone may not be sufficient to induce increased intestinal permeability.

Chapter 8: Synthesis of Findings

8.1 Realisation of Aims

The primary aim of this thesis was to investigate the relationship between exercise intensity and the role of hyperthermia in eliciting increased GI dysfunction, and subjective GI symptoms. In addition, a secondary aim was to examine potential nutritional intervention strategies that could offset any observed disturbances to the intestinal barrier. Specifically, the potential of glutamine to reduce GI permeability and injury will also be examined. To achieve these aims, the following objectives were addressed:

8.1.1 Objective 1. Compare the reported magnitudes in intestinal permeability when the timing of the dual-sugar probe solution is changed in response to exercise performance.

This objective was addressed in Chapter 4 (Study 1). The principal findings indicate that GI permeability as represented by the surrogate measure of L/R ratio indicate no significant difference was when the dual-sugar probe solution was ingested either pre-, during or post-exercise. Furthermore, treadmill running at approximately ~80% $\dot{V}O_{2\,max}$ for a duration of 60 minutes was insufficient to induce the appearance of subjective gastrointestinal symptoms.

8.1.2 Objective 2: Investigate the effects of exercise intensity on GI permeability, damage and symptoms

This objective was addressed in Chapter 5 (Study 2). Two-hour serum sample analysis showed a significant increase in intestinal permeability with increasing exercise intensity, in a stepwise manner. The findings from Chapter 5 indicate that exercise intensities above Critical Power (CP) are sufficient to compromise gastrointestinal barrier integrity permitting the translocation of the sugar probe markers. These results also indicated that shorter periods of exercise, when performed at an intensity above the heavy domain, are also sufficient to induce an increase in intestinal permeability similar to those observed in longer duration exercise occurring at lower intensities noted in literature. Furthermore, the running exercise intensity threshold associated with augmented intestinal permeability in this population is suggested to occur at or above 90% CP. A trend was also observed between core temperature and intestinal permeability. Significantly higher serum L/R ratios were returned in the heavy and severe exercise conditions, in which rectal temperature rose above threshold 1 (38.5 °C) and threshold 2 (38.9 °C), respectively. However, this data only represents three participants so does not

allow for strong conclusions to be made regarding the robustness of this association and the causal mechanistic associations between permeability and elevation in temperature.

The data from Chapter 5 which showed a clear disassociation between exercise intensity and GI symptom expression which putatively suggests no observable causal link between exercise intensity and the appearance of gastrointestinal symptoms in this short duration laboratory protocol. In this study, the same two participants accounted for the appearance of GI symptoms in both the *heavy* and *severe* exercise conditions. Furthermore, Chapter 5 also indicated that D-lactate may not be a suitable biomarker of intestinal damage in an exercise model. However, this assertion must be bracketed with the caveat that future investigations move toward a more sensitive assay protocol with lower threshold of detection that presently utilised.

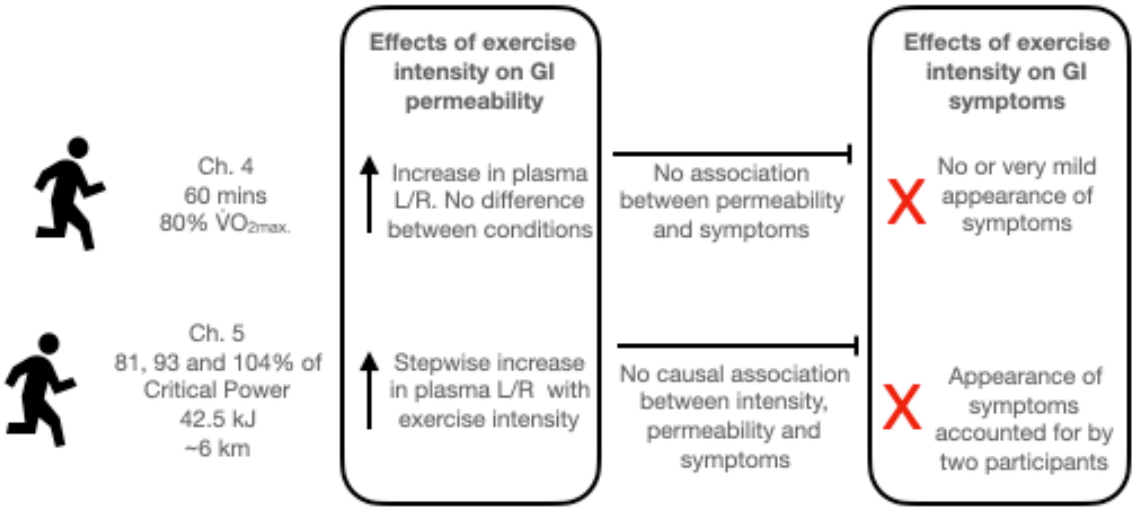

Figure 23: Schematic representation of the main findings from this thesis in regard to the effects of exercise intensity on GI permeability and GI symptom expression.

8.1.3 Objective 3: Explore the responses in GI permeability, damage and symptoms in response to passive hyperthermia

This objective was addressed in Chapters 6 (Study 3) and 7 (Study 4). In Chapter 6, despite elevating core temperatures to thresholds 1 (38.5 °C) and 2 (38.9 °C), no significant increases in intestinal permeability were observed. In Chapter 7, despite mean core temperature being elevated above 39 °C in both trial conditions, intestinal permeability was not augmented. The core temperatures observed across both chapters are approximately similar to those observed in participants exercising for sixty minutes at 70% of $\dot{V}O_{2\,max}$ (Zuhl et al., 2015) which evidenced increased intestinal permeability. An increase in heart rate was observed, which is indicative of a redirection of blood flow away from the

splanchnic area towards the periphery to promote heat dissipation. However, the hypothesised reduction in splanchnic blood flow may not have been of sufficient magnitude to perturb dissipation of heat load away from the GI walls, impair oxygen delivery to the villi that would result in an increase in intestinal permeability. Moreover, no meaningful expression of a broad range of gastrointestinal symptoms was noted with heat exposure, apart from nausea was frequently reported and showed a strong correlation with elevated core temperature.

8.1.4 Objective 4: Investigate the efficacy of glutamine to reduce GI permeability and damage in response to passive hyperthermia

In Chapter 7 (Study 4), participants received an acute dose of glutamine or placebo prior to hot water immersion, whereby core temperature was elevated above 39 °C. The results indicated no effect of acute glutamine supplementation on intestinal permeability, yet an increase in intestinal injury was observed, indicated by an elevation in plasma I-FABP concentrations. Together, these results suggest that passive hyperthermia induced by 60 minutes of hot-water immersion is sufficient to result in apical structural damage to the intestinal villus, but the epithelial lining of the GI tract may recover before an increase in intestinal permeability manifests (Grootjans et al., 2016). Furthermore, passive hyperthermia resulted in the appearance of symptoms of heat-illness, particularly those associated with the head, such as *nausea*, *tiredness*, *dizziness*, *thirst* and *feeling lightheaded, and confusion.* However, acute supplementation with glutamine at 0.5 $g.kg^{-1}$ body mass failed to impact any of the measures in the study including L/R ratio, I-FABP concentrations, and core temperature or heat-illness symptoms.

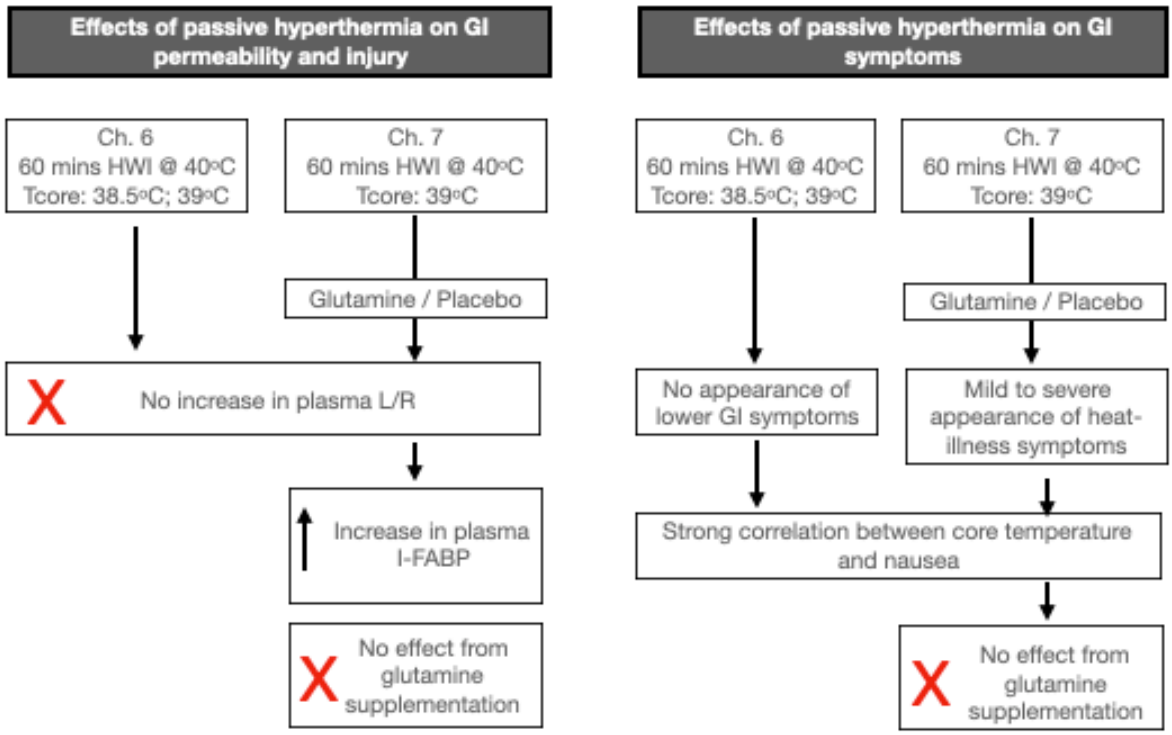

Figure 24: Schematic representation of the main findings from this thesis in regard to the effects of passive hyperthermia, with or without glutamine supplementation on GI permeability and injury, and symptoms.

8.2 General Discussion

8.2.1 The timing of ingestion of sugar-probe test solutions for the determination of intestinal permeability

A large body of literature exists investigating the effects of exercise with, or without, additional environmental or nutritional stress such as heat, fluid restriction or NSAID ingestion on gastrointestinal symptomology and function (Table 1). Whilst these authors have all aimed to investigate intestinal permeability, diversity in the methods used between studies makes comparison between studies difficult. For the determination of intestinal permeability, previous research has employed the standard sugar absorption test procedure, utilising a sugar-probe test solution containing a large non-metabolisable molecule (lactulose) with a smaller molecule which is absorbed by paracellular transport through the enterocytes (L-rhamnose). However, a wide variation exists in the composition (concentration) of the test solutions and the timing of ingestion. For example, researchers have utilised different quantities of lactulose and L-rhamnose, or provided the test solution before (Lambert et al., 2008), during (Pals et al., 1997) or post-exercise (Smetanka et al., 1999). Furthermore, various methods

of physiological, environmental or nutritional challenges have been employed, including running (Zuhl et al., 2015) or cycling (van Wijck et al., 2011a), heat (Snipe et al., 2017), and the ingestion of NSAIDs (Lambert et al., 2007). These variations make comparisons between studies difficult due to factors that may affect the absorption and pharmacokinetics of the sugar-probes. Individualistic factors such as gastric emptying rate, intestinal transit or habitual diet may affect the transport and absorption of sugar probes through the digestive system into the blood stream (Travis and Menzies, 1992; Costa et al., 2017a; Jeukendrup, 2017). Indeed, different concentrations of sugar probes used in these test express differential kinetic and absorptive responses in the small intestine and as such may impact interpretation of data (van Nieuwenhoven et al., 1999). To the author's knowledge, this was the first study to compare the reported magnitudes of L/R ratio when ingestion of the sugar-probe test solution was provided at different times in relation to the exercise bout. This study demonstrated that in response to 60 minutes of treadmill running at approximately 80% $\dot{V}O_{2\,max}$, the timing of sugar-probe ingestion does not significantly affect the reported L/R ratio. However, the data demonstrates consistently lower ratios of L/R when the test solution is ingested post-exercise. These results could be explained by previous literature on mouse models, when the sugar-probes are ingested post-exercise, that restorative mechanisms have initiated the repair of enterocyte tight-junctions proteins and activation of the actin-myosin protein complexes between enterocytes sufficiently enough to prevent transport of the sugar-probes through the intestinal tract before they could reach the small intestine (Grootjans et al., 2016). However, other studies have detected large increases in permeability using the 5-hr urinary sample method, following post-exercise ingestion of the L/R test solution, in response to a relatively short bout of exercise (20 minutes treadmill running in temperate conditions at 80% $\dot{V}O_{2\,max}$) (Marchbank et al., 2011; Davison et al., 2016). Previous research by Marchbank et al. (2011) demonstrated an approximate 2.5 fold increase between baseline and post-exercise L/R ratio values in response to 20 minutes treadmill running in temperate conditions at 80% $\dot{V}O_{2\,max.}$ Interestingly, these researchers reported baseline values of 0.222 which are higher than the 2-hr post test-solution ingestion values of the present study (0.015), which were collected after 1 hour of exercise at 80% $\dot{V}O_{2\,max}$. Considering the participants of the current study completed a three-fold duration of similar exercise, in similar conditions, at a similar intensity, it is difficult to explain the discrepancies between these results.

Since, to date, only one study has provided the sugar-probe solution post-exercise (Smetanka et al., 1999), future research should continue to provide test solutions either prior to, or during, exercise.

8.2.3 The effects of exercise intensity on gastrointestinal permeability and symptoms on triathletes

It has previously been suggested that intestinal permeability increases in a stepwise manner with exercise intensity (Pals et al., 1997), and that a critical threshold of exercise intensity of 70% $\dot{V}O_{2\,max}$ is

required to induce an increase in intestinal permeability (Costa et al., 2017b). The results from Chapter 4 support this hypothesis, whereby exercise at 80% $\dot{V}O_{2\,max}$ was able to induce an increase in intestinal permeability in all participants. Furthermore, in Chapter 5, a large increase in permeability was observed between the Moderate and Heavy intensity domains, representing approximately 70 and 80% % $\dot{V}O_{2\,max,}$ respectively. However, inconsistencies in the data presented by Pals et al. (1997) showed a lower % recovery of lactulose (an indicator of small intestinal permeability) following exercise at 60% $\dot{V}O_{2\,max}$ than 40% $\dot{V}O_{2\,max,}$ followed by a sharp increase following exercise at 80% $\dot{V}O_{2\,max.}$ Although the results showed a stepwise increase in the L/R ratio, the lactulose recovery data would suggest that the intestinal barrier may notionally recover integrity at 60% $\dot{V}O_{2\,max.,}$ thus reducing estimates of GI permeability. Furthermore, their data representing the % recovery of rhamnose showed a modest stepwise decrease with exercise intensity, showing exercise at 40% $\dot{V}O_{2\,max}$ returning higher values than 80% $\dot{V}O_{2\,max}$, suggesting that transcellular transport may decrease or remain unchanged with increasing exercise intensity. The results are a contrast to those obtained within this thesis and all other studies, which showed a stepwise increase in the recovery of both lactulose and rhamnose (data not presented), as well as an increasing ratio of the sugar-probes in accordance with exercise intensity. The data from this thesis would suggest that intestinal permeability, expressed through L/R ratio, increases in a stepwise manner with exercise intensity. Together with the results from Chapter 4, it would appear that an exercise intensity of 80%% $\dot{V}O_{2\,max}$ elicits a large increase in permeability. Indeed, even short duration exercise of 20 minutes at 80%% $\dot{V}O_{2\,max}$ has been shown to increase intestinal permeability (Marchbank et al., 2011).

The appearance of GI symptoms during training or competition is common amongst athletes (de Oliveira, Burini and Jeukendrup, 2014). Some factors such as diet (Lis et al., 2016), hot environments (Costa et al., 2017b) and life stress (Wilson, 2017) can lead to an increased development of symptoms, but the role of exercise intensity has thus far been unclear. If taken collectively, the results from this study appear to demonstrate a link between increasing exercise intensity and the appearance of GI symptoms. However, further interpretation of the data shows that the same two participants accounted for the appearance of symptoms throughout the study, both of which were female. Furthermore, acute high-intensity interval running failed to elicit an increase in GI symptoms despite participants exercising at 120% $\dot{V}O_{2\,max}$ (Pugh et al., 2017b), whilst in Chapter 7, 60 minutes of running at 80% $\dot{V}O_{2\,max}$ failed to elevate the expression of GI symptoms. These data suggest that the appearance of GI symptoms during exercise may be highly individualistic with a possible difference in sexes, yet a clear link between GI symptoms and exercise intensity cannot be concluded based upon present data.

8.2.4 The effects of passive hyperthermia on intestinal permeability, injury and GI symptoms

Exercise can cause an increase in intestinal permeability with hypoperfusion and heat being identified as two key mediators of exercise-induced GI dysfunction (Costa et al., 2017b; Pires et al., 2017). The

redirection of blood away from the splanchnic region toward the periphery, to aid thermoregulation and nutrient delivery, leading to hypoperfusion and reduced oxygen availability to the enterocyte cells of the intestinal tract (Otte et al., 2001; Rehrer et al., 2001). However, the specific contributions of hypoperfusion and hyperthermia towards GI dysfunction are unclear, as such we do not yet understand which may be the primary causal factor. Previous literature has demonstrated that heat-stress can augment intestinal permeability in both exercise and *in vitro* studies (Dokladny, Moseley and Ma, 2006; Dokladny, Zuhl and Moseley, 2016; Snipe et al., 2018a), by disrupting the integrity of the tight-junction protein complexes which bridge the enterocytes (Ogden et al., 2020). Recently, Pires et al. (2017) identified two key thresholds of core temperature from exercise-related studies whereby intestinal permeability is augmented. However, their review fails to distinguish the synergistic impact of hypoperfusion on intestinal permeability. This study aimed to investigate the effects of passive hyperthermia on intestinal permeability, which theoretically acted to minimise the effects of exercise-induced hypoperfusion. Previous research has demonstrated that some decline (20-40%) in intestinal blood flow can occur with passive hyperthermia (Rowell et al., 1970; Rowell, 1974), however these measures could not be completed in this study due to ethical and logistical restrictions. An interesting observation of the present study was the increase in serum L/R ratio in the control condition, possibly indicating the 'normal' translocation/transport rate of the sugar probes across the intestinal barrier. Furthermore, no difference was observed in the serum L/R ratio between conditions. In Chapter 5, it was demonstrated that exercise in the Heavy domain, equating to approximately 80% $\dot{V}O_{2\ max}$ was sufficient to elicit an increase in intestinal permeability, despite the duration of exercise only being approximately 32 minutes which is consistent with others (Marchbank et al., 2011). Mean core temperature within this condition (37.2 °C) was just below the 38.5°C lower threshold outlined by (Pires et al., 2017), whilst rectal temperature was equal to the threshold. However, the present study failed to observe an increase in intestinal permeability when core temperature was elevated to the lower 38.5°C threshold. Together, this data suggests that an exercise stimulus, or indeed a combination of physiological challenges in addition to core temperature rise, may be required to elicit an increase in intestinal permeability. Whilst no appearance of GI symptoms was reported, passive hyperthermia resulted in an increase in nausea, a symptom of heat illness (Howe and Boden, 2007). The core temperatures reached in this study are similar to those reported from exercise studies where intestinal permeability was increased (Pals et al., 1997; Morrison, Cheung and Cotter, 2014; Snipe et al., 2018b). Furthermore, participants received water at regular periods during this study, and displayed no indications of dehydration which can also augment intestinal permeability (Lambert et al., 2008). Collectively, these results would suggest that hypoperfusion may be the key underlying mechanism towards augmented intestinal permeability during exercise.

8.2.5 The effects of glutamine supplementation on gastrointestinal permeability, injury and symptoms in response to heat-stress

Previous research has demonstrated that supplementation with glutamine can attenuate intestinal permeability and injury, and the rise in core temperature in response to heat stress (Mondello et al., 2010; Rao and Samak, 2012; Soares et al., 2014; Pugh et al., 2017c). In response to running at 70 % $\dot{V}O_{2\,max}$ in 30°C heat, Pugh et al. (2017c) reported a protective effect of glutamine supplementation at 0.25, 0.5 and 0.9 $g.kg^{-1}$ on intestinal permeability in a stepwise manner. Whilst no effect on core temperature was observed, glutamine also attenuated intestinal injury indicated by a reduction in I-FABP. These results are in contrast to those of Soares et al. (2014) who observed an attenuating effect of glutamine on the core temperature of rats in response to heat stress. In Chapter 6, passive hyperthermia induced a rise in core temperature but no further increase in intestinal permeability when compared to control, however I-FABP was not quantified in this study. Furthermore, the effects of glutamine on core temperature dynamics in response to heat stress had not been investigated. In chapter 7 it was demonstrated that passive hyperthermia through HWI can induce intestinal injury and permeability, as represented by an increase in I-FABP and serum L/R ratio, respectively. Additionally, glutamine supplementation showed no effect on I-FABP, intestinal permeability or core temperature. Moreover, despite similar conditions, final core temperature (compared to the HOT condition) and pre-experimental protocols, intestinal permeability was elevated approximately two-fold in this study, when compared than in Chapter 7. Previous literature has demonstrated that the apical cells of the villus are shed under conditions of ischemia, but a zipper-like mechanism acts to recover the intestinal lining (Grootjans et al., 2016). This process may precede the breakdown of the tight junction complexes which permit the transport of otherwise impassable molecules, such as lactulose, through the intestinal barrier into the portal blood. As such, an increase in I-FABP would be observed without an accompanying increase in the serum L/R ratio. Since glutamine has previously demonstrated an attenuating effect on intestinal permeability in response to exercise in the heat, these effects may be more pronounced in the presence of exercise-induced hypoperfusion. Similarly to Chapter 6, logistical and ethical restrictions prevented the quantification of intestinal blood flow during the study, yet previous literature suggests intestinal blood flow may have been reduced by some degree in the current study (Rowell et al., 1968; Rowell et al., 1970). This study therefore supports the hypothesis that exercise-induced hypoperfusion, when combined with an adequate increase in core temperature, underlies the appearance of increased intestinal permeability.

In this thesis, exercise at 70% $\dot{V}O_{2\,max}$ and above, as well as passive hyperthermia has failed to induce a rise in GI symptoms often reported by athletes during training or competition (de Oliveira, Burini and Jeukendrup, 2014). However in Chapter 6, participants frequently reported the appearance of symptoms related to heat-illness such as nausea or dizziness (Coris, Ramirez and Van Durme, 2004). Furthermore, this study demonstrated a strong relationship between core temperature and nausea. Exercise in the heat

has seen an elevation in reported symptoms, including nausea, when compared to temperate conditions (Snipe et al., 2017). Future studies may aim to differentiate symptoms into those relating to the gut and those relating to heat illness. Together, the underlying mechanisms governing the appearance of GI symptoms in response to exercise remain unclear and warrant further investigation.

8.3 Thesis Limitations

8.3.1 Chapter 4

A limitation in Chapter 4 was the small sample size. Whilst mean data is presented in the results of this thesis, the wide deviations demonstrate a large difference in the individual response in intestinal permeability. Examination of the results indicated that one participant displayed large variances in intestinal permeability during one experimental visit when compared to their other trials. Despite confirming adherence to the pre-experimental conditions it is the belief of the present author that these conditions may not have been followed. Another limitation to this study was the participant cohort did not include any females. Indeed, there is a distinct lack of research investigating the response in intestinal permeability in response to exercise in females. Some research suggests that the prevalence of GI symptoms is higher amongst female athletes (de Oliveira, Burini and Jeukendrup, 2014), however this is contradictory to other reports (Snipe and Costa, 2018a). Indeed, in Chapter 5, the two participants who accounted for the increase in GI symptoms were both females, suggesting more research is warranted investigating the appearance of GI symptoms in female athletes. It is worthy of note that female participants were invited to participate in the study, but no volunteers were received.

8.3.2 Chapter 5

Previous literature has demonstrated core temperature to be an important influencing factor of exercise-induced GI dysfunction (Pires et al., 2017). However, during the study, persistent problems with the rectal probe equipment resulted in this data only being recorded from three participants; the remaining participants' core temperature was quantified using tympanic infrared thermometer. The validity of aural temperature for measurement of core temperature in indoor conditions has been questioned, with readings being -0.67 °C when compared to rectal temperature (Ganio et al., 2009). As such, the lack of validity limits the confidence of the author to make conclusions on the core temperature data. Again, the cohort of this study presented a limited number of female athletes.

8.3.3 Chapter 6

A limitation of the study is that mesenteric blood flow was not measured; therefore it is difficult to delineate the relative contribution of hypoperfusion to the observed results. Previous research has

demonstrated that passive hyperthermia can reduce mesenteric blood blow (Rowell et al., 1968; Rowell et al., 1970), yet this was the first study to investigate the response in intestinal permeability to passive heat stress. Despite elevating core temperatures to thresholds previously identified as associated with augmented intestinal permeability (Pires et al., 2017), ethical restrictions meant hot water immersion was limited to a total of 60 minutes. As such, core temperature did not reach these thresholds for a further 15 minutes. This amount of time may not have been sufficient to elicit disruption to the tight-junction complexes (Grootjans et al., 2016). Future research should aim to quantify the response in intestinal permeability to increased durations of passive hyperthermia, however, the hot-water immersion model proved an efficient method for elevating and maintaining core temperature. A further limitation to this study was that I-FABP was not measured. These measures would have added to the limited body of literature examining the effects of passive hyperthermia on intestinal damage.

8.3.4 Chapter 7

Similarly to Chapter 6, in this study, mesenteric blood flow was not measured. Again, the cohort of participants included no females. Furthermore, serum L/R data was obtained from only one time point; whilst the results demonstrated no effect of glutamine supplementation, it is difficult to conclude whether an increase in intestinal permeability occurred.

9 Conclusions and implications

The methodological approaches used to the investigate intestinal permeability changes as a result of exercise across a range of exercise modalities, intensities and environmental conditions have noted wide variances in GI permeability biomarkers . Data presented in this thesis demonstrate that the timing of L/R sugar-probe ingestion do not significantly affect the reported serum L/R ratio. However, it is recommended that sugar-probe solutions are provided either pre- or during (up to 30 min), rather than at the end of the experimental protocol.

This thesis has also demonstrated that intestinal permeability increases in a stepwise manner with exercise intensity. A similar stepwise relationship was observed with core temperature. The relationship between exercise intensity, GI symptoms and, furthermore, biological sex requires further attention since two female participants accounted for the appearance of the majority of symptoms in the study. Exercise studies across this thesis demonstrate weak relationships between intestinal permeability and the appearance of GI symptoms. Indeed, research has demonstrated that intestinal permeability can be increased in response to exercise, but there has been no relationship established with intestinal permeability and the appearance of symptoms or indeed a decline in exercise performance. Furthermore, the results of this thesis have failed to support a link between an increase in core

temperature and augmented intestinal permeability, at least when in absence of exercise-induced hypoperfusion. The studies presented in this thesis, and previous research (Table 1) may demonstrate that augmented intestinal permeability occurs, however, comparisons between the specific magnitudes of ratios of lactulose/ rhamnose cannot be made.

Hyperthermia and hypoperfusion represent two key mechanisms underlying the increase in intestinal permeability associated with exercise (Costa et al., 2017b; Pires et al., 2017). Using a passive heat stress model, this thesis demonstrates that hyperthermia may be secondary to hypoperfusion in eliciting the greatest increase in intestinal permeability. Minor increases in intestinal permeability were observed in hyperthermia, but no difference was present between control conditions in a thermoneutral environment and following heat stress. It remains unclear why intestinal permeability increased in the control condition, as such this research requires further investigation into daily and hourly variance in intestinal permeability. The use of I-FABP as a marker of intestinal damage in response to exercise has recently gained attention. Yet, a review of the literature identifies a large range in the responses to exercise, no nominal data exists outlining the day-to-day variations in circulating I-FABP, no population specific values exist and in relation to trained individuals no consideration of I-FABP adaptive responses to training exists. Chapter 7 was the first study in humans to report an increase in I-FABP in response to passive hyperthermia. Yet, this increase was not ameliorated by acute glutamine supplementation. Furthermore, acute glutamine supplementation returned no effect on intestinal permeability or core temperature.

The results of this thesis suggest that hypoperfusion, rather than hyperthermia, may be a predominant casual factor in the appearance of increased intestinal permeability during exercise. The studies undertaken in this thesis demonstrate an increase in intestinal permeability in response to exercise, but this effect is not observed when exercise stress is removed, despite a similar increase in core temperature achieved. Furthermore, the results in this thesis suggest no protective (attenuation) effect of acute glutamine supplementation on core temperature elevation, intestinal permeability or injury, or heat-illness symptoms in response to passive hyperthermia.

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Zuhl, M.N., Lanphere, K.R., Kravitz, L., Mermier, C.M., Schneider, S., Dokladny, K. and Moseley, P.L. (2014b) Effects of oral glutamine supplementation on exercise-induced gastrointestinal permeability and tight junction protein expression. *J Appl Physiol (1985)*, 116 (2), 183-191.

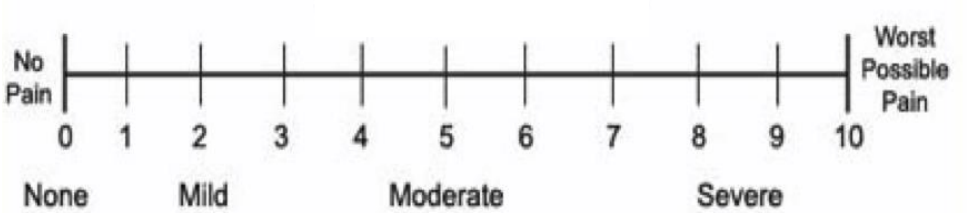
Appendix 1

1.1 Adapted GI symptom questionnaire

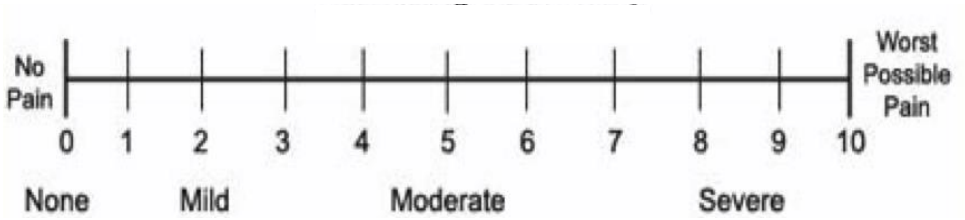
Gastrointestinal Screening Questionnaire

Below are a series of scales 0-10 regarding symptoms of gastrointestinal upsets. Please indicate on each scale whether you have any symptom of the stated condition during or after training over the last 6 weeks. No symptoms is 0 and 10 is extreme symptoms.

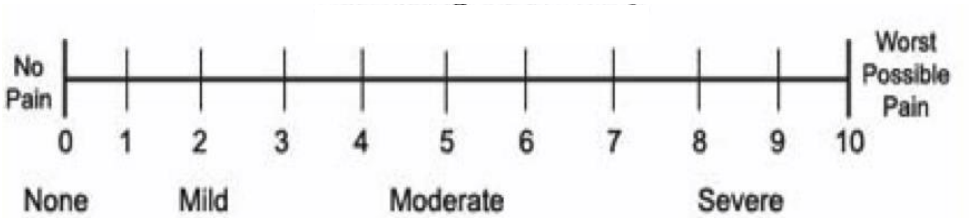
Side Stitch



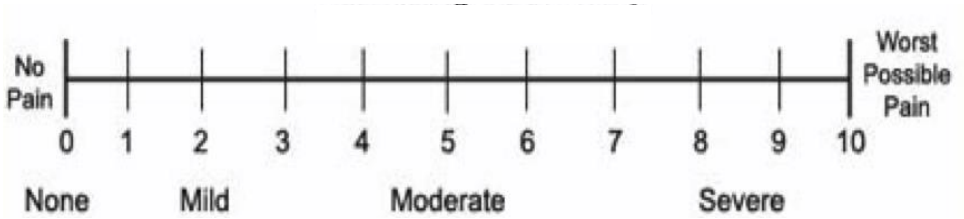
Nausea



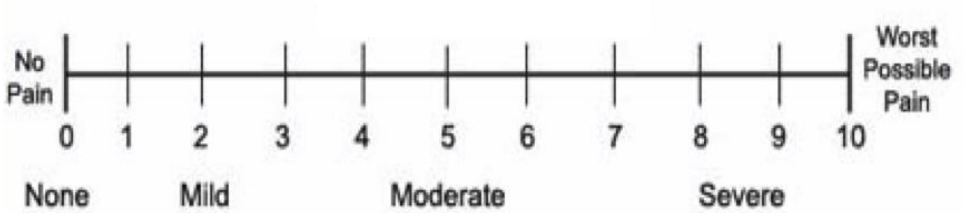
Bloating



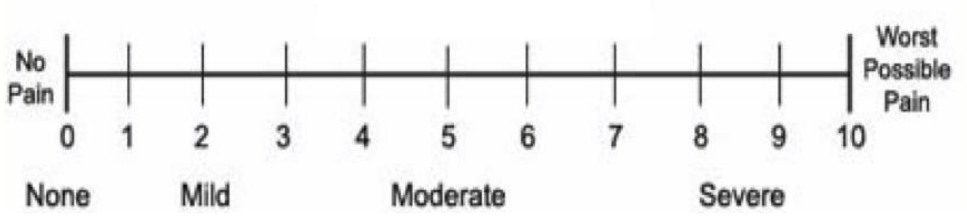
Urge to burp



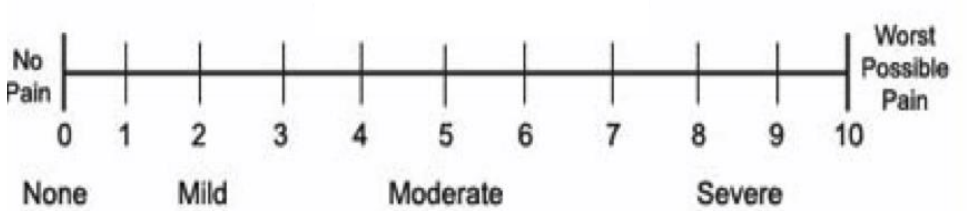
Urge to vomit



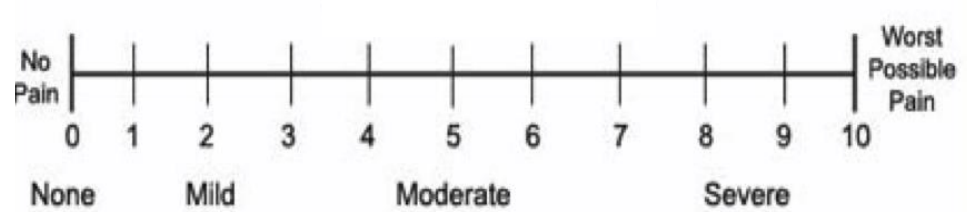
Urge to defecate



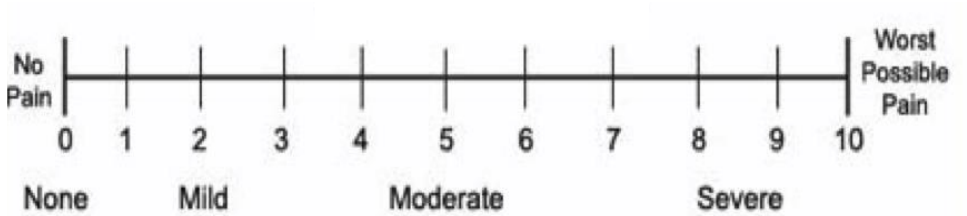
Diarrhoea



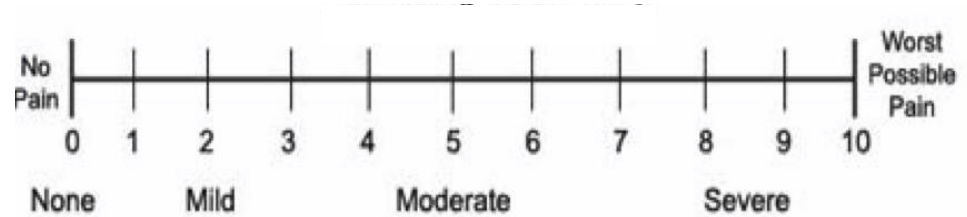
Flatuation



Stomach Cramps



Stomach Upsets



1.2 State-Trait Anxiety Inventory questionnaire

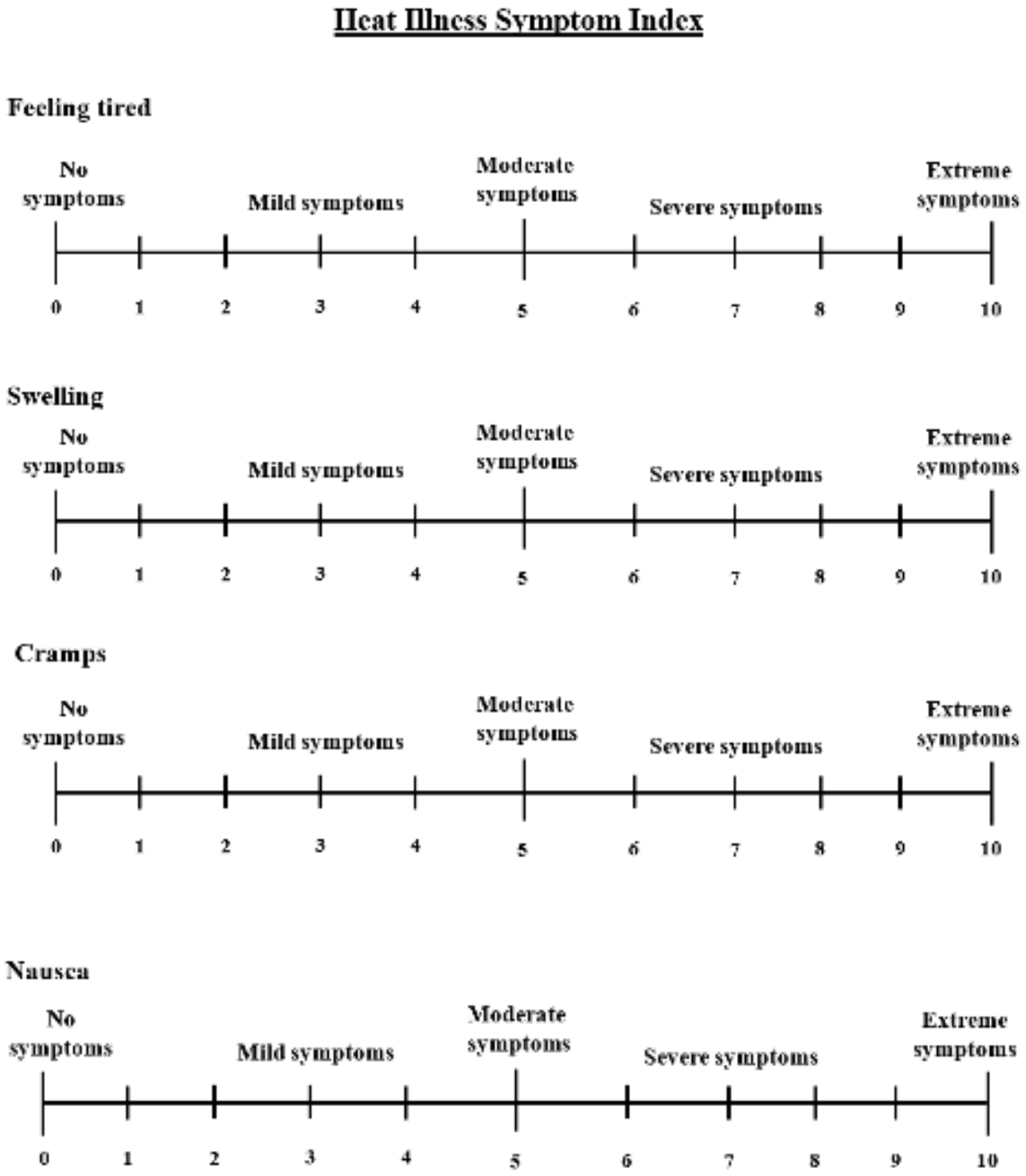
Appendix– A
State-Trait Anxiety Inventory
STAI Form Y-1

Name……Date……Age..... Sex: Male

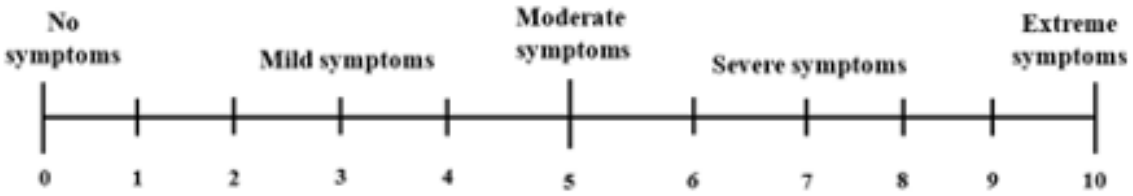
DIRECTIONS: A number of statements which people have used to describe themselves are given below. Read each statement and then write the number in the blank at the end of the statement that indicates how you feel right now, that is, at this moment. There is no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe your present feelings best

S. No.		Not at all	Some What	Moderately so	Very much so
1.	I feel calm	1	2	3	4
2.	I feel secure	1	2	3	4
3.	I am tense	1	2	3	4
4.	I feel Strained	1	2	3	4
5.	I feel at ease	1	2	3	4
6.	I feel upset	1	2	3	4
7.	I am presently worrying over possible misfortunes	1	2	3	4
8.	I feel satisfied	1	2	3	4
9.	I feel frightened	1	2	3	4
10.	I feel comfortable	1	2	3	4
11	I feel self confident	1	2	3	4
12.	I feel nervous	1	2	3	4
13.	I am Jittery	1	2	3	4
14.	I feel indecisive	1	2	3	4
15.	I am relaxed	1	2	3	4
16.	I feel content	1	2	3	4
17.	I am worried	1	2	3	4
18.	I feel confused	1	2	3	4
19.	I feel steady	1	2	3	4
20.	I feel pleasant	1	2	3	4

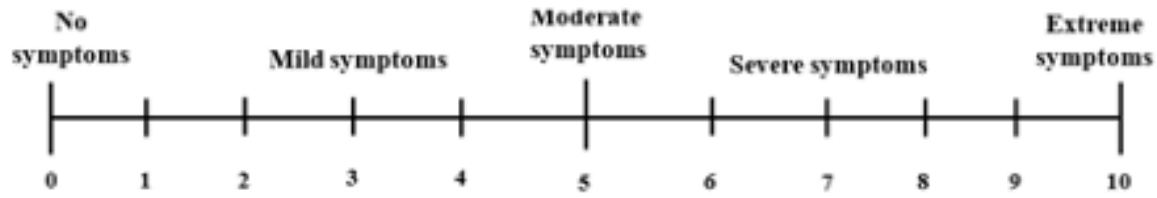
1.3 Adapted heat-illness symptoms questionnaire



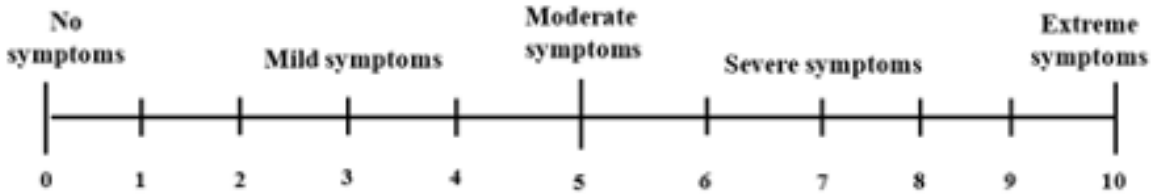
Dizziness



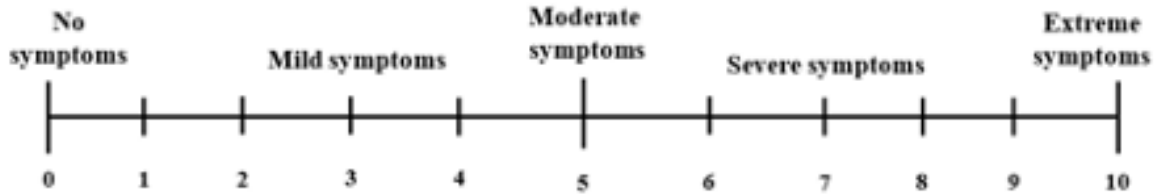
Thirst



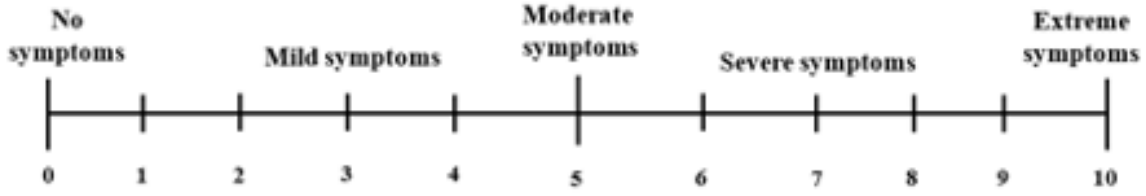
Vomiting



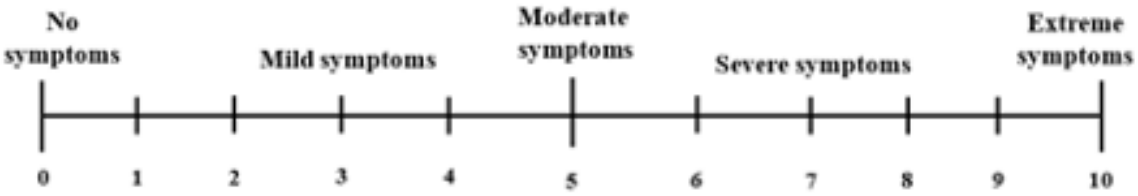
Confusion



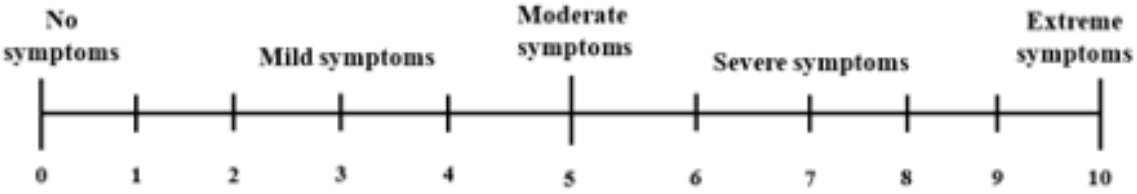
Muscle Weakness



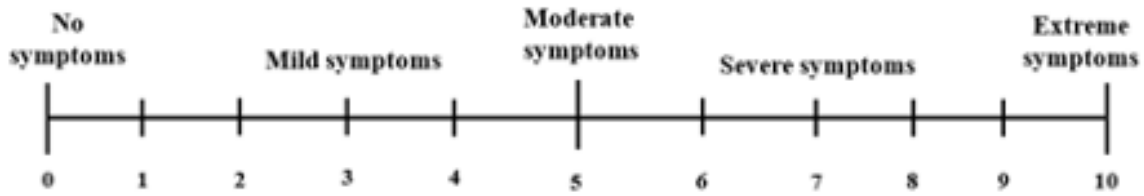
Heat sensation on the head or neck



Chills



Stopping sweating



Feeling lightheaded