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TITLE:

Influence of Aerobic Fitness on Gastrointestinal Barrier Integrity and Microbial Translocation Following a Fixed-Intensity Military Exertional Heat Stress Test

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RUNNING HEAD:

Low Aerobic Fitness is Associated with increased Gastrointestinal Injury and Microbial Translocation Following Exertional Heat Stress

ABSTRACT

Purpose: Exertional-heat stress adversely disrupts gastrointestinal (GI) barrier integrity, whereby subsequent microbial translocation (MT) can result in potentially serious health consequences. To date, the influence of aerobic fitness on GI barrier integrity and MT following exertional-heat stress is poorly characterised. **Method:** Ten untrained (UT; $VO_{2max} = 45 \pm 3 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) and ten highly trained (HT; $VO_{2max} = 64 \pm 4 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) males completed an ecologically valid (military) 80-minute fixed-intensity exertional-heat stress test (EHST). Venous blood was drawn immediately pre- and post-EHST. GI barrier integrity was assessed using the serum dual-sugar absorption test (DSAT) and plasma Intestinal Fatty-Acid Binding Protein (I-FABP). MT was assessed using plasma *Bacteroides*/total 16S DNA. **Results:** UT experienced greater thermoregulatory, cardiovascular and perceptual strain ($p < 0.05$) than HT during the EHST. Serum DSAT responses were similar between the two groups ($p = 0.59$), although Δ I-FABP was greater ($p = 0.04$) in the UT ($1.14 \pm 1.36 \text{ ng}\cdot\text{ml}^{-1}$) versus HT ($0.20 \pm 0.29 \text{ ng}\cdot\text{ml}^{-1}$) group. *Bacteroides*/Total 16S DNA ratio was unchanged (Δ ; -0.04 ± 0.18) following the EHST in the HT group, but increased (Δ ; 0.19 ± 0.25) in the UT group ($p = 0.05$). Weekly aerobic training hours had a weak, negative correlation with Δ I-FABP and *Bacteroides*/Total 16S DNA responses. **Conclusion:** When exercising at the same absolute workload, UT individuals are more susceptible to small intestinal epithelial injury and MT than HT individuals. These responses appear partially attributable to greater thermoregulatory, cardiovascular, and perceptual strain.

KEYWORDS:

Exercise, I-FABP, Endotoxemia, Exertional Heat Stroke, Gut Health

ABBREVIATIONS:

ANOVA	Analysis of variance
CV	Coefficient of Variation
DSAT	Dual Sugar Absorption Test
EDTA	Ethylenediaminetetraacetic acid
EHS	Exertional Heat Stroke
EHST	Exertional Heat Stress Test
ELISA	Enzyme Linked Immunosorbent Assay
GI	Gastrointestinal
HPLC	High Performance Liquid Chromatography
HR	Heart Rate
HT	Highly Trained
I-FABP	Intestinal Fatty-Acid Binding Protein
I-HSP	Intracellular Heat Shock Protein
ISAK	International Society for the Advancement of Anthropometric Kinanthropometry
LPS	Lipopolysaccharide
L/R	Lactulose-to-Rhamnose
MT	Microbial Translocation
qPCR	Quantitative Polymerase Chain Reaction
RH	Relative Humidity
RPE	Rate of Perceived Exertion
SD	Standard Deviation
SEM	Sensor Electronics Module
T _{core}	Core Body Temperature
T _{body}	Mean Body Temperature
T _{skin}	Mean Skin Temperature
TS	Thermal Sensation
UT	Untrained
$\dot{V}O_{2max}$	Maximal Oxygen Uptake

INTRODUCTION

The gastrointestinal (GI) microbiota is a complex ecosystem formed of up to 100 trillion micro-organisms, which have co-evolved inside humans and perform multiple symbiotic functions (Cani, 2018). To prevent systemic immune activation, the microbiota is contained inside the GI lumen, a function that is tightly regulated by the multi-layered GI barrier (Wells et al., 2017). Exertional heat stress negatively disrupts the integrity of the GI barrier (Costa et al., 2017), and in a manner broadly associated with the severity of thermal strain (Pires et al., 2017; Ogden et al., 2020a). Though poorly characterised, the mechanisms driving this response are widely believed to be attributable to the combined influence of localised ischemic injury following hypoperfusion, and paracellular tight junction breakdown following hyperthermia-mediated cytotoxicity (Dokladny et al., 2016). In cases of severe GI barrier integrity loss, subsequent systemic microbial translocation (MT) can trigger a sequela of pro-inflammatory responses (Deitch, 2012). These responses may underpin several potentially serious health conditions that affect physically active populations (e.g. military personnel, firefighters, athletes), including: exercise-induced anaphylaxis (Christensen et al., 2019), central-fatigue (Vargas and Marino, 2016) and exertional heatstroke (Lim, 2018).

Exertional Heat Stroke (EHS) is the most severe condition along a continuum of heat-related illnesses (Leon and Bouchama, 2011). The most widely accepted medical definition of EHS includes: a pathological rise in core body temperature (T_{core} ; $>40^{\circ}\text{C}$); central nervous system dysfunction (e.g. delirium, coma); and multiple organ failure (Bouchama and Knochel, 2002). In military settings, EHS poses a significant threat to operational performance and can have long-term career/health implications for incapacitated personnel (Epstein et al., 2012). The incidence of EHS in armed forces is estimated to be *circa* 0.5/1000 cases per person-year in both the United Kingdom (Stacey et al., 2016) and the United States (Army Forces Health Surveillance Centre, 2020). This prevalence is primarily attributable to the widespread exposure of highly-motivated individuals to strenuous physical activity, often whilst wearing encapsulating clothing and/or when deployed to hot ambient environments (Epstein et al., 2012). Given these issues, various policies have been published that provide guidance on EHS management (Belval et al., 2018; Military Headquarters of the Surgeon General, 2019). However, until recently, little consideration had been given to the relevance of GI-MT within the pathophysiology of EHS (Lim, 2018; Ogden et al., 2020a).

Various intrinsic (e.g. age) and extrinsic (e.g. clothing) risk factors have been consistently advocated to predispose military personnel to EHS (Westwood et al., 2020). Aerobic fitness is one well-characterised intrinsic risk factor, whereby failure of a recent mandatory fitness test, has been associated with a 2-8 fold increased odds-ratio of EHS in comparison to a successful test (Wallace et al., 2006; Moore et al., 2016; Nelson et al., 2018). Improved cardiovascular stability (e.g. plasma volume) and cellular thermotolerance (e.g. intracellular heat shock protein [I-HSP] expression) are key mechanisms that likely explain the benefits afforded by improved aerobic fitness (Selkirk and McLellan, 2001; Kazman et al., 2013). During self-paced physical activity, modification of thermoregulatory behaviour (e.g. slower pacing) helps to mitigate the enhanced risk of EHS experienced by less trained personnel (Selkirk and McLellan, 2001). However, these behavioural modifications are often unattainable during group-paced physical activities, which are a frequent setting where military EHS hospitalizations have been reported to arise (Epstein et al., 1999; Stacey et al., 2015). Despite this knowledge, the influence of aerobic fitness on GI barrier integrity, MT and subsequent EHS have not been adequately characterised (Ogden et al., 2020a).

In a pioneering study, untrained individuals (VO_{2max} 37-44 ml·kg·min⁻¹) experienced a *circa* 100% increase in GI MT (plasma lipopolysaccharide [LPS]) when assessed at fixed 0.5°C T_{core} increments above 38.5°C during a low-intensity (4.5 km·h⁻¹, 2% incline) EHST in a 40°C ambient environment (Selkirk et al., 2008). In comparison, GI MT was unchanged from rest throughout this protocol in highly trained individuals (VO_{2max} 54-73 ml·kg·min⁻¹), despite this group presenting an increase in both exercise and thermal capacity. The notion that increased aerobic fitness protects GI barrier integrity during exertional-heat stress was not supported in a follow-up study by Morrison et al. (2014), who conversely demonstrated trained individuals (VO_{2max} 64 ± 4 ml·kg·min⁻¹) to have greater intestinal injury (plasma intestinal fatty-acid binding protein [I-FABP]) than untrained individuals (VO_{2max} 46 ± 4 ml·kg·min⁻¹) during a 90-minute self-paced EHST. In this study, mean and peak thermoregulatory strain were comparable between the two fitness groups. Together, these findings suggest that elevated aerobic fitness causes more pronounced GI barrier integrity loss during relative intensity exercise, potentially caused via greater splanchnic hypoperfusion, whilst GI MT is conversely blunted, potentially caused via an improved capacity for systemic microbial neutralisation (Lim et al., 2019).

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

METHODS

Participants and Ethical Approval

Twenty healthy males volunteered to participate in the present study (Table 1). All participants were classified as untrained (UT; $n= 10$; $\leq 50 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $\leq 3 \text{ h}\cdot\text{week}^{-1}$) or highly trained (HT; $n= 10$; $\geq 60 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $\geq 6 \text{ h}\cdot\text{week}^{-1}$) based upon *a priori* criteria for maximal oxygen uptake and weekly involvement in cardiovascular training (Morrison et al., 2014). Two further participants ($n= 2$) whose training status fell between the classification criteria following preliminary aerobic fitness assessment were excluded. There were no participant withdrawals following assignment. The primary activities of the UT group were recreational level sports ($n= 4$ football, $n= 1$ rugby, $n= 1$ judo, $n= 1$ downhill skiing, $n= 3$ weight lifting), whilst the primary activities of the HT group were regional level aerobic sports ($n= 8$ running, $n= 1$ cycling, $n= 1$ triathlon). This demographic (age, body composition, $\text{VO}_{2\text{max}}$) is considered too broadly represent the heterogeneity of individuals enlisted in military ground combat roles worldwide (Fallowfield et al., 2019). Females were excluded as thermoregulation (Shechter and Boivin, 2010) and GI barrier function (Farage et al., 2009) are both influenced by menstruation. A general medical questionnaire was used to screen for a previous history of GI, cardiorespiratory and metabolic illness. No participant self-reported taking pharmacological medications or having suffered from an acute illness within 14 days prior to data collection. Written informed consent was obtained from each participant after they had been provided with a full written and verbal explanation of the experimental procedures. The study protocol was approved by MARJON University Research Ethics Committee (Approval Code: EP096) and was conducted in accordance with the principles outlined in the *Declaration of Helsinki (2013)*.

[Table 1 – Insert Here]

Experimental Overview

This study applied a two-way independent groups design. Participants visited the laboratory on two occasions. Baseline anthropometrics and maximal oxygen uptake ($\dot{V}O_{2max}$) were assessed during the first visit. The second visit comprised an intermittent exercise-heat stress test (EHST). The EHST consisted of two bouts of 40 minutes fixed-intensity treadmill walking ($6 \text{ km}\cdot\text{h}^{-1}$ and 7% gradient) in the heat (35°C and 30% relative humidity; RH). The exercise bouts were separated by 20 minutes seated recovery, including 4 minutes of forearm cold water immersion (de Groot et al., 2013). This protocol was designed in line with military work/rest schedule guidance, consistent across different militaries worldwide (Spitz et al., 2012), and has previously been shown to demonstrate acceptable reliability for whole-body physiological and GI barrier integrity assessment (Ogden et al., 2020b). Data collection was undertaken in Plymouth, United Kingdom, where mean daily ambient temperature at a local meteorological station (Camborne, United Kingdom; latitude: 50.218° N) remained below 20°C (Met Office, 2019). A schematic representation of the experimental protocol is provided in Figure 1.

[Figure 1 – Insert Here]

Dietary and Lifestyle Controls

Dietary supplementation (e.g. glutamine, probiotics, bovine colostrum) and prolonged thermal exposures (e.g. saunas, sunbeds) were prohibited from 14 days before until the end of data collection (Ogden et al., 2020a). Alcohol, caffeine, strenuous physical activity and non-steroidal anti-inflammatory drugs (e.g. ibuprofen) were all abstained for 48 hours before main experimental visits (van Wijck et al., 2012). Participants adhered to a ≥ 10 hour overnight fast and consumed 500 ml of plain water two hours prior to the experimental visits. Conformity with all pre-trial controls was assessed in writing upon laboratory arrival using a pre-trial control questionnaire. Participants remained fasted throughout main experimental trials (Edinburgh et al., 2018), but were permitted a $12 \text{ ml}\cdot\text{kg}^{-1}$ bolus of ambient temperature water ($28\text{-}30^{\circ}\text{C}$) in the 20 minutes following both 40-minute exercise bouts. This volume of fluid is in line with worldwide military guidance (Spitz et al., 2012), whilst the temperature has

ecological validity for military field operations conducted in hot ambient environments.

Anthropometric Measurements

Participants' height, body mass and body fat were measured following the International Society for the Advancement of Kinanthropometry (ISAK) guidelines (Marfell-Jones et al. 2006). Height was measured barefoot using a stadiometer to the nearest 0.1 cm (HM-200, Marsden, Rotherham, UK), body mass was measured on an electronic scale to the nearest 0.05 kg (MC 180 MA, Tanita, Tokyo, Japan). Skinfold thicknesses were taken in duplicate (coefficient of variation [CV] = 2.1%) by the same researcher at the bicep, tricep, subscapular and suprailiac using skinfold callipers to the nearest 0.1 cm (Harpenden, Holtain Ltd, Crymych, UK). Predictions of body density were calculated using age- and sex-relevant equations (Durnin and Womersley, 1974).

Maximal Oxygen Uptake

Maximal oxygen uptake ($\dot{V}O_{2max}$) was determined using an incremental treadmill test (Desmo HP, Woodway GmbH, Weil am Rhein, Germany) to volitional exhaustion. The test was undertaken in normothermic laboratory conditions (18-22°C, 40-60% RH). The starting speed of the treadmill was 10 km·h⁻¹ on a fixed 1% inclination. Treadmill speed was increased by 1 km·h⁻¹ increments every three minutes until reaching 13 km·h⁻¹, when inclination was then increased by 2% every two minutes (Ogden et al., 2020b). Expired metabolic gases were measured continuously using a breath-by-breath metabolic cart (Metalyser 3B, Cortex, Leipzig, Germany). Heart rate (HR; Polar FT1, Polar Electro OY, Kempele, Finland) and rating of perceived exertion (RPE; Borg, 1970) was measured during the final ten seconds of each stage. The highest 30 second average $\dot{V}O_2$ was taken to be $\dot{V}O_{2max}$. The criteria used to define a true $\dot{V}O_{2max}$ included three from: (1) a plateau in $\dot{V}O_2$ (an increase ≤ 2 ml·kg⁻¹·min⁻¹) despite increasing exercise intensity; (2) a respiratory exchange ratio ≥ 1.15 ; (3) a heart rate ≤ 10 b·min⁻¹ of the age-predicted maximum (220-age); and (4) an RPE of 20 (Winter et al. 2007).

Exercise-Heat Stress Test

The EHST commenced in the morning (08:30 \pm 1 hour) to standardise for the influence of circadian variation (Waterhouse et al., 2005). Upon laboratory arrival, participants provided a mid-flow urine sample to assess hydration status. Duplicate urine osmolality

measurements were undertaken via freeze-point depression (Osmomat 3000, Gonotec, Berlin, Germany; CV = 0.4%) and urine specific gravity via a digital refractometer (3741 Pen-Urine S.G, Atago Co. Ltd, Tokyo, Japan; CV = <0.1%). Each participant also provided a capillary blood sample into a K₂EDTA microtube (Microvette®, Sarstedt, Numbrecht, Germany) for duplicate assessment of plasma osmolality using freeze-point depression (CV = 0.2%). Pre-defined criteria for euhydration was: (1) a urine osmolality ≤ 700 mOsm.kg⁻¹; and (2) urine specific gravity ≤ 1.020 AU (Casa et al., 2005). This control was met by all participants without need for additional fluid provision. Participants measured their nude body mass (180 MA, Tanita MC, Tokyo, Japan), before self-inserting a single-use rectal thermistor (T_{core}; Phillips 21090A, Guildford, UK) 12 cm beyond the anal sphincter. A HR monitor was positioned around participants' chest (EQ02, Equivital™, Cambridge UK). Dress-state was standardised using summer military clothing (i.e. jacket [neck zipped, sleeves extended], trousers, boxer briefs, socks, trainers). The environmental chamber was regulated at $\sim 35^{\circ}\text{C}$ (UT: $35.0 \pm 0.2^{\circ}\text{C}$; HT: $35.1 \pm 0.3^{\circ}\text{C}$; $p = 0.54$) and $\sim 30\%$ RH (UT: $32 \pm 5\%$; HT: $30 \pm 3\%$; $p = 0.27$). On entry to the chamber, skin thermistors (EUS-UU-VL3-O, Grant Instruments, Cambridge, UK) were affixed to the participant's chest, arm, thigh and calf using one layer (5 x 5 cm) of cotton tape (KT Tape®, KT Health, UT, USA). Mean skin temperature (T_{skin}) was calculated using standard equations (Ramanathan, 1964).

Participants then undertook the EHST (i.e. 40-minute walking bout; 20-minute seated rest; 40-minute walking bout). Throughout the EHST, T_{core} and T_{skin} were recorded using a temperature logger (Squirrel SQ2010, Grant Instruments, Cambridge, UK) and HR was recorded using a Sensor Electronics Module (SEM) unit (EQ02, Equivital™, Cambridge UK). Mean whole-body temperature (T_{body}) was calculated from simultaneous T_{core} and T_{skin} measurements (Jay and Kenny, 2007). All data, including RPE and thermal sensation (TS; Toner et al., 1986), were reported at 20-minute intervals. The standardisation of instructions for perceptual measures included ensuring participants had a clear understanding of anchoring the top and bottom ratings to previous experiences of: (1) no exertion (RPE = 6) to maximum exertion (RPE = 20) and; (2) unbearably cold (TS = 0) to unbearably hot (TS = 8). Between the two walking bouts, participants immersed their forearms in a $\sim 15^{\circ}\text{C}$ (UT: $15.6 \pm 1.3^{\circ}\text{C}$, HT: $16.3 \pm 0.7^{\circ}\text{C}$; $p = 0.17$) cold-water bath to help relieve physiological strain (de Groot et al., 2013). Upon EHST termination, participants were removed from the environmental

chamber, towel-dried and their post-EHST nude body mass recorded. Absolute sweat losses were calculated from the change in nude body mass from pre-to-post EHSTs after correction for fluid intake, blood withdrawal and urine output.

Blood Collection and Analysis

Venous blood samples (12 ml) were drawn immediately prior to and immediately following the EHST (< 2 minutes). At rest, participants stood upright for a minimum of 20 minutes before blood withdrawal to allow capillary filtration pressure to stabilise (Shirreffs and Maughan, 1994). The forearm was sterilised with an 80% isopropyl alcohol wipe and blood was immediately drawn from an antecubital vein under minimal stasis (<30 seconds). Samples were collected directly into a K₂ EDTA vacutainer (Becton Dickinson and Company, Plymouth, UK). A 0.5 ml aliquot of blood was immediately removed for haematological analysis and samples then centrifuged at 1300g for 15 minutes at 4°C to separate plasma. Aliquots were frozen at -80°C until analyses within 2 years. All blood handling was performed with sterile (pyrogen, DNA free) pipette tips and microtubes.

Haematology

Haemoglobin was measured using a portable photometric analyser (Hemocue® Hb 201+, EFK Diagnostics, Madeburg, Germany; duplicate CV = 0.5%) and haematocrit using the microcapillary technique following centrifugation at 14,000g for 4 minutes at room temperature (Haematospin 1400, Hawksley and Sons Ltd, Lancing, England); duplicate CV = 0.4%). Plasma volume was estimated using standard equations (Dill and Costill, 1974). Post-exercise analyte concentrations were left uncorrected for acute plasma volume shifts, given the similarity of responses between groups and the low molecular weights of quantified analytes.

Dual-Sugar Absorption Test (DSAT)

Participants orally ingested a standard sugar probe solution containing 5 g Lactulose (Lactulose Oral Solution, Sandoz, Holzkirchen, Germany) and 2 g L-Rhamnose (L-rhamnose FG, 99% pure, Sigma Aldrich, Missouri, USA) dissolved within 50 ml of plain water (osmolality = ~750 mOsm·kg⁻¹) ten minutes into the EHST. Probe concentrations were determined in duplicate from serum samples collected 90 minutes post probe ingestion (i.e. immediately

post-EHST) following a previously described high performance liquid chromatography (HPLC) protocol (Fleming et al., 1996). The recovery of both sugars was determined per litre serum ($\text{mg}\cdot\text{l}^{-1}$), where the lactulose/L-rhamnose (L/R) ratio was then corrected relative (%) to the concentration of sugar consumed. The limit of detection was $0.1 \text{ mg}\cdot\text{l}^{-1}$. The combined L/R ratio CV was 8.8%.

Intestinal Fatty-Acid Binding Protein

I-FABP (1:4 plasma dilution) was measured in duplicate plasma samples immediately pre and post EHST using a solid-phase sandwich ELISA (DY3078, DuoSet, R&D systems, Minneapolis, USA) following manufacturer instructions for optimising the assay for plasma samples. Briefly, this involved supplementing the standard reagent diluent (from the DuoSet ELISA) with 10% heat-inactivated normal goat serum (DY005, Reagent Additive 1, R&D systems, Minneapolis, USA). This diluent was used for diluting assay standards and samples. Standard reagent diluent without additional normal goat serum was used to reconstitute the detection antibody and dilute Streptavidin-HRP to the working concentration. The detection antibody was diluted to the working concentration in reagent diluent with 2% normal goat serum. The capture antibody was reconstituted and diluted to the working concentration in PBS without carrier protein. The intra-assay CV was 4.0%.

Bacterial DNA

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

bacterial DNA. The intra-assay CV was 9.8% for total 16S DNA and 18.8% for *Bacteroides* DNA.

Statistical Analyses

All statistical analyses were performed using Prism Graphpad software (Prism V.8, La Jolla, California, USA). Comparisons were made after first establishing normal distribution using a Shapiro-Wilk test ($p > 0.05$). A two-way analysis of variance (ANOVA) was used to identify between group differences over time (time x group). If Mauchly's test for sphericity was violated, Greenhouse Geiser corrections were applied for $\epsilon < 0.75$, while the Huynh-Feldt correction was used for less severe asphericity. Where significant interaction effects were identified, post-hoc Holm-Bonferroni step-wise corrected t-tests were used to determine the location of variance. When there was only a single comparison, an unpaired t-test or non-parametric Mann-Whitney test (physical activity, body mass, DSAT, peak/ Δ *Bacteroides*/total 16S DNA, peak/ Δ I-FABP, HR mean) was used to determine between-group differences. Relationships were assessed using a non-parametric Spearman's rank correlation coefficient. Correlations were classified as small (≤ 0.69), moderate (0.70-0.89) and large (≥ 0.90) (Vincent and Weir, 1995). Outliers were defined as ± 2.4 SD units (normally distributed data) or ± 4.0 SD units (non-normally distributed data) outside of the mean and were removed from subsequent analysis (Aguinis et al., 2013). Statistical significance was accepted at the alpha level of $p \leq 0.05$. Data are presented as mean \pm standard deviation (SD) unless otherwise stated.

Power Analysis

A sample size estimation was calculated *a priori* using specialist statistical power software (G*Power 3.1, Kiel, Germany). Anticipated effect sizes were derived from previous studies comparing I-FABP (Morrison et al., 2014) and endotoxin (Selkirk et al., 2008) responses between individuals of low and high aerobic fitness following exertional-heat stress. In total, ≥ 9 (I-FABP) and ≥ 4 (endotoxin) participants per group were calculated necessary to detect a significant interaction effect using a two-way ANOVA with standard alpha (0.05) and beta (0.8) values.

RESULTS

Thermoregulatory

T_{core} increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group compared with the HT group (Figure 2A; time x group interaction; $p < 0.01$). Peak (UT: $38.88 \pm 0.32^{\circ}\text{C}$, HT: $38.21 \pm 0.30^{\circ}\text{C}$; $p < 0.01$), mean (UT: $37.99 \pm 0.29^{\circ}\text{C}$, HT: $37.63 \pm 0.19^{\circ}\text{C}$; $p < 0.01$) and Δ (UT: $1.97 \pm 0.31^{\circ}\text{C}$, HT: $1.35 \pm 0.38^{\circ}\text{C}$; $p < 0.01$) T_{core} were all greater in the UT group. T_{skin} was increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group (Figure 2B; time x group interaction; $p < 0.01$). Peak (UT: $36.23 \pm 0.53^{\circ}\text{C}$, HT: $35.56 \pm 0.59^{\circ}\text{C}$; $p = 0.02$) and Δ (UT: $1.84 \pm 0.61^{\circ}\text{C}$, HT: $0.81 \pm 0.52^{\circ}\text{C}$; $p < 0.01$) T_{skin} were greater in the UT group, but mean (UT: $35.55 \pm 0.36^{\circ}\text{C}$, HT: $35.56 \pm 0.33^{\circ}\text{C}$; $p = 0.26$) T_{skin} was similar. T_{body} was increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group (Figure 2C; time x group interaction; $p < 0.01$). Peak (UT: $38.75 \pm 0.28^{\circ}\text{C}$, HT: $38.08 \pm 0.32^{\circ}\text{C}$; $p < 0.01$), mean (UT: $37.98 \pm 0.29^{\circ}\text{C}$, HT: $37.63 \pm 0.19^{\circ}\text{C}$; $p < 0.01$) and Δ (UT: $1.95 \pm 0.32^{\circ}\text{C}$, HT: $1.24 \pm 0.34^{\circ}\text{C}$; $p < 0.01$) T_{body} were greater in the UT group. Mean sweat rate (UT: $1.52 \pm 0.23 \text{ l}\cdot\text{h}^{-1}$; HT: $1.30 \pm 0.25 \text{ l}\cdot\text{h}^{-1}$; $p = 0.07$) and % body mass loss (UT: $1.23 \pm 0.26\%$; HT: $1.13 \pm 0.32\%$; $p = 0.39$) were similar between groups.

Hydration and Cardiovascular

Basal urine osmolality (UT: $273 \pm 109 \text{ mOsmol}\cdot\text{kg}^{-1}$, HT: $261 \pm 164 \text{ mOsmol}\cdot\text{kg}^{-1}$; $p = 0.87$), urine specific gravity (UT: $1.007 \pm 0.005 \text{ AU}$, HT: $1.005 \pm 0.006 \text{ AU}$; $p = 0.56$) and plasma osmolality (UT: $296 \pm 5 \text{ mOsmol}\cdot\text{kg}^{-1}$, HT: $295 \pm 3 \text{ mOsmol}\cdot\text{kg}^{-1}$; $p = 0.65$) were similar between groups. The Δ plasma volume following the EHST were similar (UT: $0.22 \pm 2.59\%$, HT: $0.89 \pm 2.49\%$; $p = 0.59$). HR was increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group (Figure 2D; time x group interaction; $p < 0.01$). Peak (UT: $173 \pm 9 \text{ bpm}$; HT: $133 \pm 11 \text{ bpm}$; $p < 0.01$), mean (UT: $156 \pm 10 \text{ bpm}$; HT: $119 \pm 6 \text{ bpm}$; $p < 0.01$) and Δ (UT: $105 \pm 17 \text{ bpm}$; HT: $78 \pm 13 \text{ bpm}$; $p < 0.01$) HR were all greater in the UT group.

Perception

RPE was increased throughout the EHST (time; $p < 0.01$) and to a greater extent in the UT group (Figure 2E; time x group interaction $p < 0.01$). Peak (UT: $17 \pm 2 \text{ AU}$; HT: $11 \pm 2 \text{ AU}$; $p < 0.01$), mean (UT: $14 \pm 1 \text{ AU}$; HT: $10 \pm 1 \text{ AU}$; $p < 0.01$) and Δ (UT: $6 \pm 3 \text{ AU}$; HT: $2 \pm 2 \text{ AU}$; $p < 0.01$) RPE were all higher in the UT group. TS was increased throughout the EHST (time; $p <$

0.01) and to a greater extent in the UT group (Figure 2F; time x group interaction; $p < 0.01$). Peak (UT: 7.0 ± 0.5 AU; HT: 6.0 ± 1.0 AU; $p < 0.01$), mean (UT: 6.0 ± 0.5 AU; HT: 5.5 ± 0.5 AU; $p < 0.01$) and Δ (UT: 2.5 ± 1.0 AU; HT: 1.0 ± 1.0 AU; $p < 0.01$) TS were all higher in the UT group.

[Figure 2 – Insert Here]

Gastrointestinal Barrier Integrity

The DSAT (lactulose/L-rhamnose ratio) was similar between the UT (0.039 ± 0.030) and HT (0.027 ± 0.011) groups (Figure 3A; $p = 0.59$). I-FABP concentration increased (time; $p = 0.01$) from pre- (UT: 1.17 ± 0.35 ng·ml⁻¹; HT: 1.81 ± 1.10 ng·ml⁻¹) to post-EHST (UT: 2.31 ± 1.34 ng·ml⁻¹; HT: 2.01 ± 1.03 ng·ml⁻¹), and to a greater extent in the UT group (Figure 3B; time x group interaction; $p = 0.05$). This interaction effect was not visible at either time point following post-hoc correction. The Δ I-FABP response was however greater in the UT (1.14 ± 1.35 ng·ml⁻¹ [119 ± 77%]) versus the HT (0.20 ± 0.29 ng·ml⁻¹ [16 ± 27%]) group ($p = 0.02$).

[Figure 3 – Insert Here]

Microbial Translocation

Total 16S DNA was unchanged (time; $p = 0.34$) from pre- (UT = 5.50 ± 1.38 µg·ml⁻¹; HT = 5.54 ± 0.74 µg·ml⁻¹) to post-EHST (UT = 5.60 ± 0.82 pg·µl⁻¹; HT = 5.94 ± 0.94 pg·µl⁻¹) in both groups (Figure 4A; time x group interaction; $p = 0.56$). There was no difference in the Δ total 16S DNA response between the UT (0.10 ± 1.16 µg·ml⁻¹) and HT (0.40 ± 1.13 µg·ml⁻¹) groups ($p = 0.56$). *Bacteroides*/total 16S DNA ratio displayed a significant time x group interaction (Figure 4B; $p = 0.04$). However, there was no significant difference in *Bacteroides*/total 16S DNA ratio between groups at either pre- (UT = 0.14 ± 0.10 ; HT = 0.20 ± 0.21 ; $p = 0.44$) or post- (UT = 0.32 ± 0.26 ; HT = 0.16 ± 0.08 ; $p = 0.13$) the EHST following *post hoc* adjustment. The Δ *Bacteroides*/total 16S DNA ratio was greater in the UT (0.18 ± 0.25) versus HT (-0.04 ± 0.18) group ($p < 0.01$). Unfortunately, *Bacteroides* concentrations were below the limit of detection in 7/40 samples (in these cases ratio data are presented as zero).

[Figure 4 – Insert Here]

Associations

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

DISCUSSION

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

nutritional supplementation) and/or attenuate thermal strain (e.g. reduced load carriage, cooling) during group-paced occupational activities.

The DSAT is the gold-standard *in vivo* technique to assess GI permeability (Bischoff et al., 2014). The traditional endpoint of the DSAT is the 5-hour urinary recovery ratio of pre-ingested lactulose-to-L-rhamnose (L/R), though serum assessment offers a valid alternative over a reduced (1-2 hour) time course (Fleming et al., 1996). Previous work from our laboratory has demonstrated the serum DSAT to increase ~2-fold above resting levels ([rest] = 0.014 ± 0.006 , [post-EHST] = 0.028 ± 0.005 ; $p = 0.02$) in a mixed-cohort of recreationally trained ($VO_{2max} = 40-55 \text{ ml}\cdot\text{kg}\cdot\text{min}^{-1}$) males utilising an identical EHST to herein (Ogden et al., 2020b). Contrary, to the *a priori* hypothesis, GI permeability was comparable between the UT and HT individuals following the EHST in the present study, whilst absolute responses were in line with our previous research (Ogden et al., 2020b). This is the first study to assess the influence of aerobic fitness on GI permeability using the DSAT in response to either exercise or heat stress. Given the difficulty of obtaining intestinal biopsies in healthy humans, and the absence of available evidence from animal models, it is difficult to speculate whether aerobic fitness influences localised pathways (e.g. I-HSP) concentration, GI tight junction structure) that strengthen GI barrier integrity (Ogden et al., 2020a). Indirect mechanistic evidence is equally inconsistent. For example, pro-inflammatory cytokine (Landers-Ramos et al., 2014; Morrison et al., 2014) and stress hormone (Wright et al., 2010; Reihmane et al., 2012) concentrations, which dysregulate GI tight junctions structure (Dokladny et al., 2016), do not appear to be influenced by aerobic fitness in response to exertional-heat stress. In comparison, the expression and activity of I-HSP's in blood leukocytes are more pronounced in HT individuals following exertional-heat stress, though whether a comparable response is initiated in GI tissue is unknown (Fehrenbach et al., 2001).

I-FABP is the prominent biomarker of small intestinal epithelial injury and is tightly associated with localised splanchnic perfusion (van Wijck et al., 2011; Bischoff et al., 2014). In the present study, the increase (Δ) in I-FABP following exertional-heat stress was comparable to previous research utilising similar intensity (60-70% VO_{2max}) and duration (60-90 minute) EHSTs (e.g. Szymanski et al., 2017 [87%, $\Delta 0.800 \text{ ng}\cdot\text{ml}^{-1}$]; Ogden et al., 2020b [56%, $\Delta 0.834 \text{ ng}\cdot\text{ml}^{-1}$]). In comparison, larger Δ I-FABP responses have been reported following longer

EHSTs (≥ 120 minutes) of similar intensity (e.g. Snipe et al., 2018 [432%, $\Delta 1.230 \text{ ng}\cdot\text{ml}^{-1}$]; Gaskell et al., 2019 [710%; $\Delta 1.805 \text{ ng}\cdot\text{ml}^{-1}$]). In comparison to GI permeability responses, Δ I-FABP concentrations were more pronounced in the UT group than in the HT group following the EHST in the present study. This finding is in direct opposition to previous evidence by Morrison et al. (2014) who found HT individuals to experience more pronounced small intestinal injury than UT individuals during a 90-minute relative-intensity EHST. Whilst Morrison et al. (2014) proposed that HT individuals may redistribute a greater proportion of cardiac output away from the splanchnic organs than UT individuals during exertional-heat stress to support thermoregulation, they also acknowledge a limitation of their research was that their UT group had a lower overall thermal impulse given that 5/8 participants were unable to complete the EHST. The present finding that small intestinal epithelial injury is reduced in HT individuals in response to exertional-heat stress is supported by research in livestock, which demonstrate aerobically trained animals to better sustain splanchnic perfusion during passive heat stress in comparison to untrained animals (Sakurada and Hales, 1998). In humans, splanchnic cardiovascular stability has not been directly examined in response exertional-heat stress, however, given aerobic training characteristically increases blood plasma volume (Sawka et al., 2011) and splanchnic arterial luminal area (Gabriel and Kindermann, 1996), comparable responses might be anticipated.

Bacterial DNA is an emerging biomarker of GI MT through high-sensitivity conserved 16S gene sequencing (Paise et al., 2016). In comparison to traditional GI MT biomarkers (e.g. endotoxin), bacterial DNA assessment appears less susceptible to issues surrounding exogenous contamination given that ability to target microbial phyla/species (e.g. *Bacteroides*) with high GI specificity (Ogden et al., 2020a). The assessment of total 16S DNA is to control for co-variables that influence *Bacteroides* DNA concentration independent of GI MT, such as the efficiency of DNA extraction, immune function, and DNase concentrations (March et al., 2019). In the present study, stable and comparable total 16S DNA concentrations were evident across both groups, however, only the UT group experienced a significant increase in the *Bacteroides*/total 16S DNA ratio following the EHST. Previous studies have reported similar basal *Bacteroides*/total 16S DNA ratios as herein ($\sim 0 - 1.0$), with a tendency to increase following 60-80 minutes exertional-heat stress, though large inter- and intra-individual variability in responses were apparent (March et al., 2019; Ogden et al.,

2020b). In both previous studies, participants' aerobic fitness was a potential co-variate, with VO_{2max} ranging between 40-60 ml·kg⁻¹·min⁻¹. In support of the present findings, UT individuals were shown to experience significant systemic endotoxemia during exhaustive walking in the heat, however, this response was absent in HT individuals despite exhibiting a longer exercise capacity (Selkirk et al., 2008). The findings of Selkirk et al. (2008), where blood was collected at fixed 0.5°C T_{core} increments, suggest that the protective benefits of aerobic fitness largely occur independent of absolute thermoregulatory strain. Correspondingly, given a lack of strong association between any GI barrier integrity and MT biomarker in the present study, this might infer that HT individuals might also acquire an improved capacity for systemic microbial neutralisation. Though speculative, indirect evidence has previously shown higher levels of aerobic fitness (e.g. VO_{2max} , sports performance) to increase certain anti-microbial defences, including: immunoglobulin G and M concentration (Bosenberg et al., 1988; Camus et al., 1997); high density lipoprotein concentration (Lippi et al., 2006); CD14⁺CD14 monocyte profile (Selkirk et al., 2009); and hepatic reticuloendothelial (Kupffer cell) endotoxin phagocytosis (Komine et al., 2017).

Whole-body physiological responses – including thermoregulatory, cardiovascular, and perceptual strain – were all more pronounced in the UT versus HT individuals throughout the EHST. These results were highly anticipated, given that aerobic training is well-characterised to induce a plethora of physiological adaptations that both support thermoregulation (e.g. increased evaporative heat loss) and lower the relative metabolic cost (e.g. increased cardiac output) of fixed-absolute intensity exercise (Havenith et al., 1995; Cheung and McLellan, 1998). This reduction in whole-body physiological strain might have contributed towards blunting the Δ I-FABP and *Bacteroides*/total 16S DNA response in the HTt participants within the present study. Relevantly, a recent systematic review outlined an exercise-induced T_{core} threshold of 38.6°C for GI barrier integrity loss (DSAT, I-FABP and endotoxin) to be commonplace (>50% incidence) and of 39.0°C for GI barrier integrity loss to be universal (100% incidence; Pires et al., 2017). In the present study, 9/10 UT participants had a peak T_{core} that exceeded 38.6°C, including 4 participants whose T_{core} exceeded 39.0°C, whilst only 2/10 HT participants surpassed the 38.6°C T_{core} threshold and none the 39.0°C threshold. Likewise, small associations were evident between peak T_{core} with both Δ I-FABP and *Bacteroides*/total 16S DNA when data for the entire participant cohort ($n= 20$) was

accumulated. Mechanistically these responses appear logical, given that hyperthermia disrupts GI barrier integrity in a broadly dose dependant manner (Dokladny et al., 2016). In the present study, small positive associations were also found between: (1) I-FABP and mean RPE and; (2) *Bacteroides*/total 16S DNA with peak T_{body} , mean HR, and mean RPE. The independent effect of these whole-body physiological responses on GI barrier integrity/MT have never been directly assessed and warrant future investigation utilising more valid methodologies (e.g. clamped T_{core}).

LIMITATIONS

Despite the execution of a tightly controlled methodological design, the present results were not without some limitations. First, the EHST only evoked moderate disturbance of GI barrier integrity and MT, potentially limiting the practical application of these findings in more severe situation's indicative of EHS. However, the present EHST was selected as it had strong ecological validity in representing group-based military field activities (Spitz et al., 2012; Military Headquarters of the Surgeon General, 2019). Second, *Bacteroides* DNA analysis had poor analytical reliability (CV = 18.8%). This is largely attributable to a proportion of samples being close or below the assays minimum level of detection ($1 \text{ copy} \cdot \mu\text{l}^{-1}$). Despite this limitation, our group has previously characterised the absolute test-retest reliability of *Bacteroides*/total 16S using the present EHST, whereby it is noted that the statistically significant Δ response between the UT and HT group reported herein exceeds the typical error of measurement (ratio = 0.077) and 95% limits of agreement (ratio = 0.213) previously reported (Ogden et al., 2020b). To further improve analytical reliability, future analysis might consider the assessment of whole-blood samples, given that bacterial 16S DNA concentrations in the buffy coat and red blood cells far exceed that of plasma (Paisse et al., 2016). Third, a basal DSAT was not performed to minimise the burden placed on participants with the aim of improving overall adherence. This lack of basal DSAT correction likely contributes to the lack of association between this biomarker with both Δ I-FABP and *Bacteroides*/total 16S DNA responses. Fourth, females were excluded from participation due to unavailability of menstruation hormone testing. Previous evidence has shown no influence of sex on GI barrier integrity responses to exertional-heat stress (Snipe and Costa, 2018). Finally, the HT group were statistically older and had a lower body fat percentage than the UT group. The difference in chronological age between the two groups is unlikely to have clinical

relevance, given basal GI permeability appears relatively stable across the lifespan (Saweirs et al., 1985; Saltzman et al., 1995). In comparison, a small positive association was found between % body fat and the Δ *Bacteroides*/total 16S DNA ratio (but not DSAT or I-FABP responses), which is a limitation concordant with previous research in this field (Selkirk et al., 2008; Morrison et al., 2014).

CONCLUSION

This is the first study to extensively assess the influence of aerobic fitness on GI barrier integrity and MT biomarkers in response to exertional-heat stress. There was no difference in GI permeability (serum DSAT) between the two groups, but there was more pronounced small intestinal epithelial injury (I-FABP) following the EHST in the UT group. These findings suggest that the GI barrier is more resistant to perturbation in HT individuals, though not to the extent where GI permeability is measurably altered. Likewise, GI MT (*Bacteroides*/total 16S DNA) only increased following the EHST in the UT group. Given that GI permeability was not different between the two groups, this suggests that GI MT neutralization might also be upregulated with aerobic fitness. These data broadly support conclusions drawn from studies assessing the impact of exertional heat stress on either GI barrier integrity or MT in isolation. It should be noted that peak thermoregulatory responses (e.g. $T_{\text{core}} = 38\text{-}39.5^{\circ}\text{C}$) were sub-clinical when compared with situations where exertional-heat stroke predominately arise (e.g. $T_{\text{core}} > 40.0^{\circ}\text{C}$). These findings should help inform occupational EHS doctrine, in relation to the management of UT individuals to support GI barrier integrity (e.g. reduced load carriage, cooling) during group-paced physical activity in the heat.

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Consent for Publication: Signed

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Authors Contributions:

1) made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work. HO, JF, RC, GD, SF, SD, AM, CW, JL

2) drafted the work or revised it critically for important intellectual content. HO, JF, RC, GD, SD, JL

3) approved the version to be published. HO, JF, RC, GD, SF, SD, AM, CW, JL

4) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. HO, JF, RC, GD, SF, SD, AM, CW, JL

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Table 1. Participant demographic characteristics

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

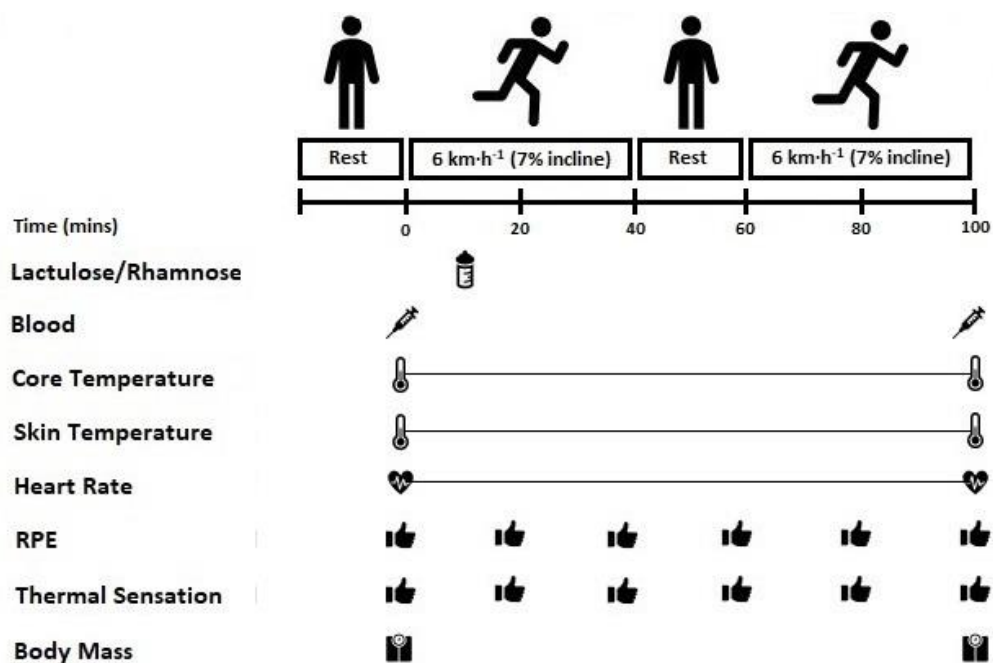


Figure 1. Schematic illustration of the experimental measurement timings

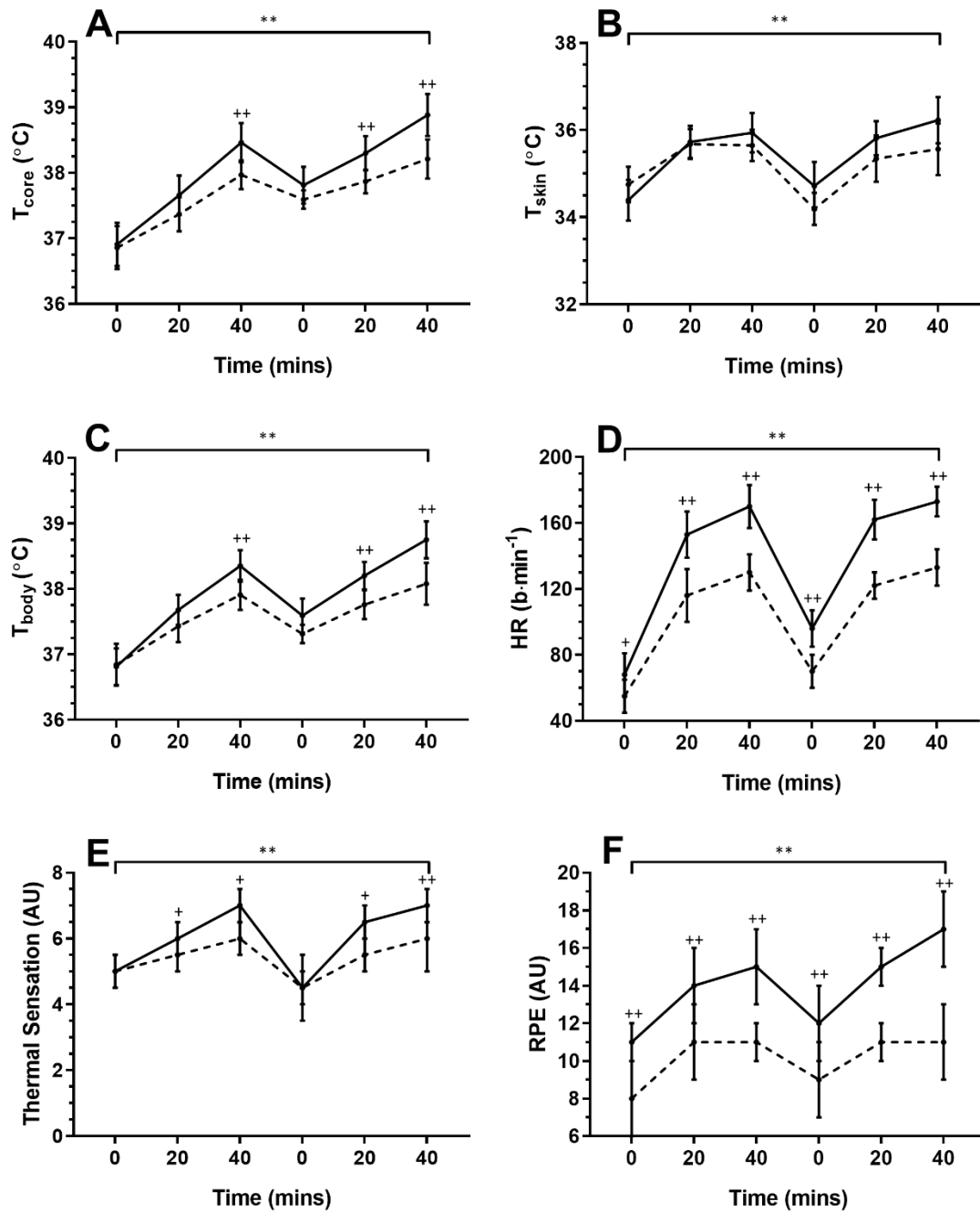


Figure 2. Whole-body physiological responses to EHSTs: (A) = core temperature; (B) = mean skin temperature; (C) = mean body temperature; (D) = heart rate; (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = UT, broken line = HT. Significant overall effect of time (* $p \leq 0.05$; ** $p \leq 0.01$). Significant group \times time interaction (+ $p \leq 0.05$; ++ $p \leq 0.01$).

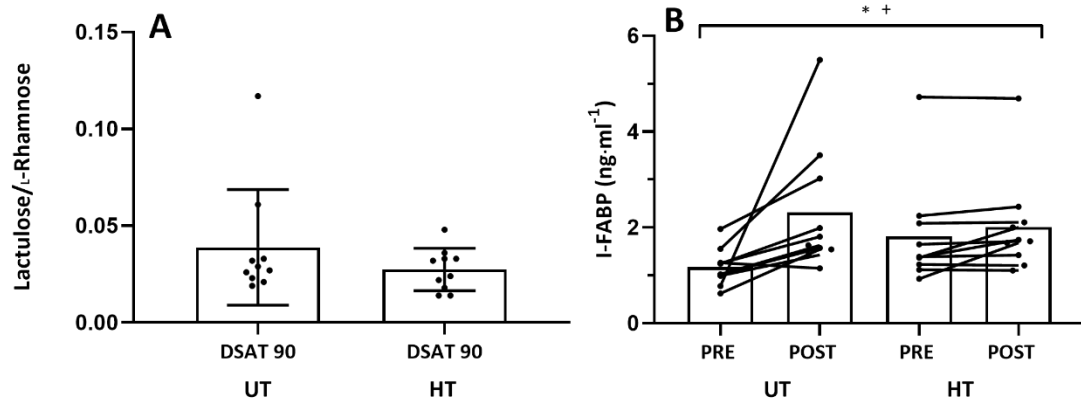


Figure 3. Gastrointestinal barrier integrity responses pre- and immediately post- the exertional-heat stress tests: (A) = L/R ratio (DSAT) at 90 minutes; (B) I-FABP. UT = untrained group, HT = highly trained group. Significant overall effect of time (* $p \leq 0.05$; ** $p \leq 0.01$). Significant group * time interaction (+ $p \leq 0.05$; ++ $p \leq 0.01$).

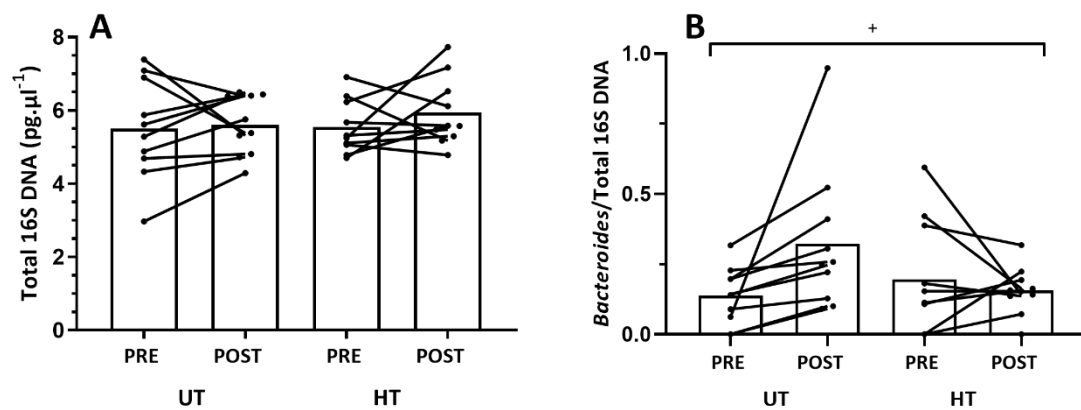


Figure 4. Gastrointestinal microbial translocation responses pre- and immediately post- the exertional-heat stress tests: (A) = total 16S DNA; (B) *Bacteroides*/total 16S DNA. UT = untrained group, HT = highly trained group. Significant group * time interaction (+ $p \leq 0.05$; ++ $p \leq 0.01$).