**TITLE:**

# Acute L-Glutamine Supplementation does not improve Gastrointestinal Permeability, Injury or Microbial Translocation in Response to Exhaustive High Intensity Exertional-Heat Stress


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## ABSTRACT

**Purpose:** Exertional-heat stress adversely distrupts (GI) barrier integrity and, through subsequent microbial translocation (MT), can result in potentially fatal exertional-heat stroke. Acute glutamine (GLN) supplementation is a potential nutritional countermeasure, although the practical value of current supplementation regimens is questionable. **Method:** Ten males completed two high-intensity exertional-heat stress tests (EHST) involving running in the heat (40°C and 40% relative humidity) at lactate threshold to volitional exhaustion. Participants ingested GLN (0.3 g·kg·FFM$^{-1}$) or a non-calorific placebo (PLA) one hour prior to the EHST. Venous blood was drawn pre-, post- and one-hour post-EHST. GI permeability was assessed using a serum dual-sugar absorption test (DSAT) and small intestinal epithelial injury using plasma Intestinal Fatty-Acid Binding Protein (I-FABP). MT was assessed using the *Bacteroides*/total 16S DNA ratio. **Results:** Volitional exhaustion occurred after 22:19 ± 2:22 (minutes: seconds) in both conditions, during which whole-body physiological responses and GI symptoms were not different ($p$ > 0.05). GI permeability (serum DSAT) was greater following GLN (0.043 ± 0.020) than PLA (0.034 ± 0.019) ($p$ = 0.02; $d$ = 0.47), but small intestine epithelial injury (I-FABP) increased comparably ($p$ = 0.22; $\eta^2 p = 0.16$) following the EHST in both trials (GLN Δ = 1.25 ± 0.63 ng·ml$^{-1}$; PLA Δ= 0.92 ± 0.44 ng·ml$^{-1}$). GI MT (*Bacteroides*/total 16S DNA ratio) was unchanged in either condition following the EHST ($p$ = 0.43). **Conclusion:** Acute low-dose (0.3 g·kg$^{-1}$ fat free mass) GLN supplementation ingested one hour before high-intesity exertional-heat stress worsened GI permeability, but did not influence either small intestinal epithilial injury or microbial translocation.

**Highlights:**

- The pathophysiology of exertional-heat stroke is widely hypothesised to be at least in part attributable to a systemic inflammatory response caused by the leak of gastrointestinal microbes into the circulating blood.
- Acute high-dose (0.9 g·kg·FFM$^{-1}$) L-glutamine supplementation is widely promoted as a practical strategy to protect gastrointestinal barrier integrity during exertional-heat stress. However, previously validated doses are often poorly tolerated and cannot be recommended for widespread implementation.
- This study examined the efficacy of low-dose (0.30 g·kg·FFM$^{-1}$; ~20 grams) acute L-glutamine supplementation on small intestinal injury, permeability, and microbial translocation in response a high-intensity exertional-heat stress test to exhaustion (20 – 30 minutes). This type of exercise accounts for the majority of exertional-heat stroke cases in the military.
- Despite being universally well-tolerated across all participants, acute low-dose L-glutamine supplementation worsened gastrointestinal permeability, without influencing either small intestinal injury or microbial translocation. These findings do not support the application of low-dose L-glutamine supplementation to help prevent exertional-heat stroke.

## ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| CV | Coefficient of Variation |
| DSAT | Dual Sugar Absorption Test |
| EDTA | Ethylenediaminetetraacetic acid |
| EHST | Exertional Heat Stress Test |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FFM | Fat Free Mass |
| GI | Gastrointestinal |
| GFR | Glomerular Filtration Rate |
| GLN | Glutamine |
| HPLC | High Performance Liquid Chromatography |
| HR | Heart Rate |
| I-FABP | Intestinal Fatty-Acid Binding Protein |
| ISAK | International Society for the Advancement of Anthropometric Kinanthropometry |
| L/R | Lactulose-to-Rhamnose |
| LT | Lactate Threshold |
| MT | Microbial Translocation |
| mVAS | Modified Visual Analogue Scale |
| PBS | Phosphate-Buffered Saline |
| PLA | Placebo |
| 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 |

$\dot{V}O_{2max}$ Maximal Oxygen Uptake

## INTRODUCTION

Exertional Heat Stroke (EHS) is the most severe disorder along a continuum of heat-related illnesses (Leon and Bouchama, 2011). It is a condition that sporadically affects individuals engaged in arduous physical activity, including labourers, athletes, military personnel, and emergency first responders (Epstein and Yanovich, 2019). Whilst direct mortality from EHS is uncommon where rapid (< 1 hour) whole-body cooling is provided (Leon and Bouchama, 2011), many casualties experience chronic health complications because of residual organ damage (Wallace et al., 2007). The pathophysiology of EHS is believed to be at least in part attributable to a systemic inflammatory response caused by gastrointestinal (GI) microbial translocation (MT) into the systemic circulation (Lim, 2018). Consequently, contemporary research has focussed on evaluating the efficacy of nutritional countermeasures to support GI barrier integrity in response to exertional-heat stress (Ogden et al., 2020a).

$_{L}$-glutamine (GLN) is a conditionally essential amino acid and the preferential energy source of intestinal enterocytes (Lacey and Wilmore, 1990). During severe catabolism where intracellular GLN concentrations become depleted, exogenous GLN supplementation appears to protect GI barrier integrity (Wischmeyer, 2006). In relation to EHS, high-dose GLN supplementation (0.90 $g \cdot kg^{-1}$ fat free body mass [FFM]) increases plasma GLN availability and protects GI barrier integrity when consumed 2 hours before ~60 minutes of moderate intensity (70% $VO_{2max}$) running in the heat (Zuhl et al., 2015; Pugh et al., 2017a). Despite appearing highly efficacious, this GLN bolus may lack ecological validity given reports of adverse GI symptoms (e.g. nausea, bloating) in some individuals that can persist for several hours (Ogden et al., 2020d). In consideration of this issue, one potential solution is to reduce the GLN dose to *circa* 0.2 – 0.3 $g \cdot kg \cdot$body $mass^{-1}$, where tolerance is markedly improved (Ward et al., 2003; Ogden et al., 2020d). Furthermore, given that *in vitro* evidence shows key intracellular pathways (e.g. heat shock protein 70) are upregulated within 1-hour of GLN supplementation (Wischmeyer et al., 1997), it might be advantageous for supplementation to occur more proximal to exercise-onset when considering the short notice of some occupational deployments involving high intensity exertional-heat stress (e.g. firefighting, military first responders).

Short-duration, high-intensity exercise (e.g. 5-10 km runs) is a common risk factor for EHS, especially in occupational settings (Westwood et al., 2020). For example, in French soldiers, retrospective analysis of 182 EHS cases, concluded that 84% of cases occurred during an 8-km time race in battle clothes (Abriat et al., 2014). Likewise, in Israeli soldiers, retrospective analysis of 150 EHS casualties, concluded 43% of incidents occurred within the first 1-hour of exercise onset and 57% of incidents that occurred specifically during running were within the first 5-km (Epstein et al., 1999). Whilst the combined influence of exercise intensity and duration on GI barrier integrity has never been directly examined, studies applying short-duration (< 30 mins) high-intensity (>75% $VO_{2max}$) protocols typically report a greater relative (%) increase in GI permeability (Marchbank et al., 2011; Davison et al., 2016) and MT (Shing et al., 2013; Barberio et al., 2015) than long-duration (1-3 hours) low-intensity (≤ 70% $VO_{2max}$) exercise. Despite this evidence, virtually all nutritional countermeasures previously recommended to support GI barrier integrity in response to exertional-heat stress have only ever been examined in response to 1-2 hours moderate intensity cycling or running (Ogden et al., 2020a). Thus, it is important to consider whether acute low-dose GLN supplementation can protect GI barrier integrity and prevent downstream MT during exhaustive high intensity exertional heat stress where the severity of GI ischemia is more pronounced (Otte et al., 2001).

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

## METHODS

### Participants and Ethical Approval

Ten healthy males volunteered to participate in the present study (age = 29 ± 7 years; height = 1.78 ± 0.10 cm; body mass = 81.8 ± 9.3 kg; body fat = 16.5 ± 5.0%; $\dot{V}O_{2max}$ = 48 ± 6 $ml \cdot kg^{-1} \cdot min^{-1}$). All participants were non-smokers, habitually active (>4 $h \cdot week^{-1}$), non-endurance trained (≤ 55 $ml \cdot kg \cdot min^{-1}$ $VO_{2max}$) and unacclimated to hot environments. No participant self-reported taking pharmacological medications or having suffered from an acute illness within 14 days prior to data collection. Informed consent was obtained for each participant following a full written and oral explanation of the experimental procedures. The study protocol was approved by Plymouth MARJON University Research Ethics Committee (Approval Code: EP082) and was conducted in accordance with the principles outlined in the *Declaration of Helsinki*.

### Experimental Overview

Participants visited the laboratory on three occasions. Baseline anthropometrics, maximal oxygen uptake ($\dot{V}O_{2max}$) and lactate threshold (LT) were assessed during the first visit. The two subsequent visits were main experimental trials, where participants were supplemented with either glutamine (GLN) or placebo (PLA) in a randomised, counterbalanced, double-blind, cross-over design. Trial order was determined by a computer-generated random number generator (*www.randomizer.org*). Study trials were separated by 7-14 days (Ogden et al., 2020b).

During both main experimental trials, participants completed an exertional-heat stress test (EHST), consisting of a 30-minute fixed-intensity treadmill run in the heat (40°C and 40% relative humidity) on a fixed 1% gradient at the speed previously calculated to represent the participants normothermic anaerobic LT. Data collection coincided with non-summer months in the United Kingdom. A schematic illustration of the protocol is shown in Figure 1.

[Figure 1 – Insert Here]

### Dietary and Lifestyle Controls

Dietary supplementation and prolonged thermal exposures were prohibited from 14

days before and until the end of data collection (Ogden et al., 2020a). Abstinence from alcohol, caffeine, strenuous physical activity and non-steroidal anti-inflammatory drugs (e.g. ibuprofen) was self-attested for 48 hours before main experimental visits. Participants adhered to a ≥ 10 hour overnight fast and consumed 500 ml of plain water two hours prior. Participants remained fasted throughout all main experimental trials and were provided a 12 ml·kg$^{-1}$ bolus of ambient temperature water (28-30°C) to drink over 20 minutes following the EHST.

**$_{L}$-Glutamine Supplementation**

GLN supplementation consisted of 0.3 g·kg $^{-1}$ fat free body mass of GLN crystalline powder ($_{L}$-glutamine Elite, Myprotein, Northwich, UK), which was freshly suspended in 500 ml of water/lemon (4:1 ratio) flavour sugar-free cordial (10kcal [aspartame, saccharine]. Robinsons, UK). Participants ingested the entire fluid bolus within 5-10 minutes, finishing one hour before commencing the EHST. Placebo supplements comprised of the same water/lemon flavour sugar-free cordial; and were matched for taste and consistency. Both supplements were administered from an opaque bottle to match visual appearance.

**Anthropometric Measurements**

Barefoot height was measured on a stadiometer (HM-200, Marsden, Rotherham, UK) and nude body mass on an electronic scale (MC 180 MA, Tanita, Tokyo, Japan). Skinfold thicknesses were taken in duplicate (coefficient of variation [CV] = 1.8%) at the bicep, tricep, subscapular and suprailliac using skinfold callipers (Harpenden, Holtain Ltd, Crymych, UK). Body density was calculated in line with Durnin and Womersley (1974).

**Lactate Threshold and Maximal Oxygen Uptake**

The anaerobic lactate threshold (LT) was determined using a discontinuous incremental treadmill test (Desmo HP, Woodway GmbH, Weil am Rhein, Germany) undertaken in normothermic laboratory conditions (18-22°C, 40-60% RH) utilising the method of Chalmers et al. (2015). The test began at a speed corresponding to a rating of perceived exertion of 11 on a fixed 1% inclination. Belt speed was increased by 1 km·h$^{-1}$ every 4-minutes, until rating of perceived exertion ≥ 18. Capillary fingertip blood samples were collected between stages and we analysed in duplicate for $_{L}$-lactate concentration (CV = 0.4%) using an

automated biochemical analyser (Biosen C-Line, EKF Diagnostics GmbH, Magdeburg, Germany). Anaerobic LT was classified using the modified D-max method by plotting a third order polynomial regression curve between incremental lactate measurements and determining the maximal perpendicular distance on a straight line formed by the workload one bout preceding a ≥ 0.40 mmol·l$^{-1}$ rise in blood lactate above baseline and the final lactate point (Bishop et al., 1998).

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 in normothermic laboratory conditions (18-22°C, 40-60% RH).Following a five-minute warm-up at 6 km·h$^{-1}$, the test began at a speed of 10 km·h$_{-1}$ on a 1% inclination. The treadmill speed was then increased at 1 km·h$^{-1}$ increments every three minutes until reaching 13 km·h$^{-1}$, when inclination was then increased by 2% every two minutes. The test was terminated when the participant reached volitional exhaustion. The criteria used to establish a true $VO_{2max}$ included three from: (1) a plateau in $VO_2$ (an increase ≤ 2 ml·kg·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) a rating of perceived exertion of 20 (Winter et al. 2007). The highest 30 second average $\dot{V}O_2$ was taken to be $\dot{V}O_{2max}$.

**Exertional-Heat Stress Test**

The EHST commenced in the morning (08:30 ± 1 hour). Upon laboratory arrival, participants provided a mid-flow urine sample to assess hydration status via urine osmolality (Osmomat 3000, Gonotec, Berlin, Germany; CV = 1.3%) and urine specific gravity (3741 Pen-Urine S.G, Atago Co. Ltd, Tokyo, Japan; CV = < 0.1%). Participants measured their own 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). Participant 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 ~40 °C (GLN: 40.5 ± 0.3 °C; PLA: 40.0 ± 0.5 °C; *p*= 0.44) and ~40% RH (GLN: 38 ± 1%; PLA: 38 ± 1%; *p*= 0.59). On entry to the chamber, skin thermistors (EUS-UU-VL3-O, Grant Instruments, Cambridge, UK) were affixed to the participant's skin using a single layer of cotton tape (KT Tape®, KT Health, UT, USA). Mean skin temperature ($T_{skin}$)

(Ramanathan, 1964) and mean body temperature (Tbody) (Jay and Kenny, 2007) were calculated using standard equations.

Participants then undertook the pre-defined EHST, consisting of a 30-minute fixed-intensity treadmill run on a fixed 1% gradient at speed equating to normothermic anaerobic LT. Termination of the EHST was 30 minutes of running or volitional exhaustion, whichever came first. If the first trial was terminated early, EHST duration was successfully matched in the second trial. An a *priori* minimum duration threshold of 20-minutes was selected as exercise of this intensity and duration has previously been shown to cause a marked increase (~200%) in GI permeability measured using the DSAT (Marchbank et al., 2011; Davison et al., 2016). 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). All data, including RPE, thermal sensation (TS; Toner et al., 1986) and GI symptoms (Gaskell et al., 2019) were reported at 10-minute intervals. GI symptoms are presented as the incidence (%) and accumulated severity of symptoms, grouped following previous guidance (Gaskell et al., 2019). Upon EHST termination, participants were removed from the environmental chamber and their post-EHST nude body mass was recorded. Absolute sweat loss was calculated from the change in dry nude body mass from pre-to-post EHST.

**Blood Collection and Analysis**

Forearm venous blood samples (12 ml) were drawn immediately pre, post and one-hour post EHST. Participants stood upright for 20 minutes before collection to allow capillary filtration pressure to stabilise. Samples were collected into serum-separator (SST II) and $K_2$ EDTA tubes (Becton Dickinson and Company, Plymouth, UK). Samples were centrifuged at 1300*g* for 15 minutes at 4°C to separate serum and plasma. Aliquots were frozen at -80°C until analyses. All blood handling was performed with sterile (pyrogen, DNA free) pipette tips and microtubes.

**Haematology**

Haemoglobin was measured using a portable photometric analyser (Hemocue® Hb 201+, EFK Diagnostics, Madeburg, Germany; duplicate CV = 0.6%) and haematocrit using the

microcapillary technique (Haematospin 1400, Hawksley and Sons Ltd, Lancing, England; duplicate CV = 0.3%). 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 trials and the low molecular weights of quantified analytes.

**Dual-Sugar Absorption Test**

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 at the start of the EHST. Probe concentrations were determined in duplicate serum samples collected 90 minutes following probe ingestion (i.e. 60-70 minutes post-EHST) using high performance liquid chromatography (HPLC) (Ogden et al., 2020b). The 90 minute serum DSAT was utilised as an alternative to the traditional 5-hour urine DSAT, based on recent exercise gastroenterology research reporting serum/plasma concentrations post probe ingestion to be more responsive than urine for detecting small transient losses in GI barrier integrity (van Wijck et al., 2011; Pugh et al., 2017b). The recovery of both sugars was determined per litre serum ($mg \cdot l^{-1}$), where the lactulose/$_{L}$-rhamnose (L/R) ratio was then corrected relative (%) to the concentration of sugar consumed. The combined L/R coefficient of variation was 6.5%.

**Intestinal Fatty-Acid Binding Protein**

I-FABP (1:4 plasma dilution) was measured in duplicate plasma samples pre and immediately post EHST using a solid-phase sandwich ELISA (DY3078, DuoSet, R&D systems, Minneapolis, USA) following manufacturer instructions. This time-point was selected to target the peak response previously reported following a similar intensity/duration EHST (Barberio et al., 2015). The intra-assay coefficient of variation was 5.5%.

**Bacterial DNA**

Bacterial DNA was measured in duplicate plasma samples collected pre and immediately post EHST using a quantitative real-time polymerase chain reaction assay (qPCR) (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. Total 16S bacterial DNA was quantified according to Ogden et al. (2020b). *Bacteroides* species DNA were quantified using a double-dye probe/primer kit (Path-Bacteroides-spp, Genesig, Primerdesign Ltd, Chandler's Ford, UK). Ratio data are presented as Bacteroides/total bacterial DNA. This time-point was selected to target the peak MT response previously reported following a similar intensity/duration EHST (Barberio et al., 2015).The intra-assay coefficients of variation were 6.4% (total 16S) and 27.7% (*Bacteroides*).

**Statistics**

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 and sphericity using Mauchly's Test. Error outliers were classified *a priori* based on recommended cut-offs of ± 2.24 standard deviations for normally distributed data and 4 standard deviations for non-normally distributed data (Aguinis et al., 2013). For this analysis, one participants Bacteroides data was removed as an outlier. To identify between-trial differences over time, a two-way analysis of variance (ANOVA) with repeated measures was applied, which is robust to minor violations of normal distribution (Maxwell, 1990). For aspherical data, Greenhouse-Geiser corrections were applied for epsilon < 0.75, whilst the Huynh-Feldt correction was applied for epsilon > 0.75. Where significant interaction effects were identified, post-hoc Holm-Bonferroni stepwise corrected t-tests were used to determine the location of variance. When there was only a single comparison, a paired t-test was used to determine between-trial differences. Effect sizes were calculated using partial eta squared ($\eta^2p$) for ANOVA comparisons and Cohens D ($d = [PLA_{mean} - GLN_{mean}] / PLA_{SD}$) for paired t-test (Lakens, 2013). The magnitude of effect was classified as small ($\eta^2p$ = 0.01; d = 0.2), medium ($\eta^2p$ = 0.06; d = 0.5) and large ($\eta^2p$ = 0.14; d = 0.8) based on standard criteria (Lakens, 2013). Statistical significance was accepted at the alpha level of $p \leq 0.05$. Data are presented as mean ± standard deviation (SD). GI symptom severity is presented as accumulated mean ± range of reported symptoms $\geq 1$ (Gaskell et al., 2019).

**Power Analysis**

A sample size estimation was calculated *a priori* (G*Power 3.1, Kiel, Germany). Anticipated effect sizes were derived from previous studies (Zuhl et al., 2015) comparing the influence of acute glutamine (0.9 g·kg·$FFM^{-1}$) on DSAT responses following exertional-heat

379 stress. In total, 6 participants were considered necessary to detect a significant interaction
380 effect using a two-way ANOVA with standard alpha (0.05) and beta (0.8) values.

381

## RESULTS

### EHST Duration

Treadmill speed at lactate threshold was 11.7 ± 1.4 km·h$^{-1}$. All participants were able to replicate EHST duration (22:19 ± 2:22 minutes: seconds) across the two trials (range = 20:00 – 26:12 minutes: seconds).

### Thermoregulation

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

### Cardiovascular

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

[Figure 2 – Insert Here]

### Perception

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

**Gastrointestinal Barrier Integrity**

The DSAT (lactulose/$_L$-rhamnose ratio) was 26% greater ($p$ = 0.02; d = 0.47) following the GLN (0.043 ± 0.020), in comparison to the PLA (0.034 ± 0.019) trial (Figure 3A). There was no difference in I-FABP responses between the two trials (Figure 3B; time x trial interaction; $p$ = 0.20; $\eta^2p = 0.16$). In both conditions, I-FABP concentration increased from pre- (GLN: 1.21 ± 0.67 ng·ml$^{-1}$; PLA: 1.62 ± 0.82 ng·ml$^{-1}$) to post-EHST (GLN: 2.46 ± 1.17 ng·ml$^{-1}$; PLA: 2.54 ± 1.07 ng·ml$^{-1}$). There was no difference in the Δ I-FABP response between the GLN (1.25 ± 0.63 ng·ml$^{-1}$ [117 ± 63%]) and PLA (0.92 ± 0.44 ng·ml$^{-1}$ [63 ± 29%]) trials ($p$ = 0.22; d = 0.73).

[Figure 3 – Insert Here]

**Microbial Translocation**

Total 16S DNA concentrations responded comparably across both trials (Figure 4A; time x trial interaction; $p$ = 0.84; $\eta^2p < 0.01$), albeit total 16S DNA concentrations were 13% lower throughout the GLN trial (trial; $p$ = 0.04; $\eta^2p = 0.34$). The EHST had no influence on total 16S DNA concentration (pre: GLN = 3.56 ± 0.74 µg·ml$^{-1}$; PLA = 4.00 ± 1.05 pg·µl$^{-1}$; post: GLN = 3.38 ± 0.40 pg·ml$^{-1}$; PLA = 3.86 ± 0.43 pg·ml$^{-1}$). There was no difference in the Δ total 16S DNA response between the GLN (-0.18 ± 0.78 µg·ml$^{-1}$) and PLA (-0.14 ± 1.33 µg·ml$^{-1}$) trials ($p$ = 0.95; d = 0.03). *Bacteroides*/total 16S DNA ratio was unchanged ($p$ = 0.34) from pre- (GLN = 0.07 ± 0.07; PLA = 0.02 ± 0.05) to post-EHST (GLN = 0.05 ± 0.09; PLA = 0.05 ± 0.05) in both trials (Figure 4B; time x trial interaction; $p$ = 0.37; $\eta^2p = 0.08$). The Δ *Bacteroides*/total 16S

438 DNA ratio was comparable between the GLN (-0.04 ± 0.11) and PLA (0.03 ± 0.08) trials ($p$ =
439 0.45; d = 0.52). *Bacteroides* concentrations were below the limit of detection in 24/40 samples
440 (ratio data presented as zero).

441 [Figure 4 – Insert Here]

442

## DISCUSSION

The aim of this study was to determine the influence of low-dose (0.3 g·kg·FFM$^{-1}$) acute oral GLN supplementation on GI barrier integrity and MT biomarkers in response to a high-intensity exhaustive EHST. This protocol has ecological relevance for situations where EHS arises in occupational and sport settings, whilst the nutritional intervention has fewer practical limitations than previously recommended protocols (Zuhl et al., 2014, 2015, Pugh et al., 2017a). The main findings were that acute low-dose oral GLN ingestion worsened GI permeability measured using the serum DSAT compared to PLA, but that small intestinal epithelial injury measuring using plasma I-FABP was similar between the two conditions. There was no evidence of increased GI MT (*Bacteroides*/total 16S DNA ratio) following the EHST in either the GLN or PLA trial. The GLN bolus was well-tolerated, with few adverse subjective GI symptoms and comparable whole-body physiological (e.g. $T_{core}$, heart rate) responses reported across both trials. Thus, these data suggest no benefit of low-dose (0.3 g·kg·FFM$^{-1}$) acute oral GLN supplementation, or even detrimental effects, on GI barrier integrity responses to exertional-heat stress.

I-FABP is a high-sensitivity biomarker of small intestinal epithelial injury, whereas the DSAT assesses functional GI permeability. In the present study, overall mean Δ I-FABP (0.88 ng·ml$^{-1}$ [57%]) and absolute DSAT (L/R = 0.034 ± 0.019) responses were very comparable in the PLA trial to previous research from our laboratory (Ogden et al., 2020b, 2020c) in response to a low-intensity (6 km·h$^{-1}$; 7% incline) 80-minute EHST in a 35°C (25% RH) environment (I-FABP = Δ 0.83 ng·ml$^{-1}$ [53%]; DSAT = 0.028 ± 0.012). The participant demographic (e.g. $VO_{2max}$ = ~50 ml·kg·min$^{-1}$), analytical methodology, and severity of physiological strain (e.g. peak $T_{core}$ = ~38.5-39.0°C) were all comparable between each study. Taken together, these data suggest that the exercise intensity and durations used in these studies all have a comparable influence on GI barrier integrity, when tightly controlling for confounding variables. Whilst the small increase in I-FABP in the present study is comparable to other high-intensity, short-duration (20-40 minute) exercise protocols (e.g. Barberio et al., 2015 [Δ 0.30 ng·ml$^{-1}$; 46%]) a greater increase in GI permeability *circa* 200% was anticipated with this form of exercise (e.g. Marchbank et al., 2011; Davison et al., 2016 [DSAT = ~0.09 - 0.11]). Despite widespread suggestions that prolonged duration (≥2 hours), moderate-intensity (~60% $VO_{2max}$) exertional-heat stress initiates the greatest disruption of GI barrier integrity (Costa et al.,

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

Acute oral GLN supplementation at doses *circa* 0.9 g·kg$^{-1}$ fat free body mass is a proposed nutritional countermeasure to support GI barrier integrity during exertional-heat stress (Zuhl et al., 2015; Pugh et al., 2017a). Despite previous favourable evidence applying this intervention, notably a ~40-50% reduction in GI permeability and ~25% reduction in epithelial injury following one hour of moderate intensity (70% $VO_{2max}$) exertional-heat stress (Zuhl et al., 2015; Pugh et al., 2017a), this dosage of GLN has potential practical limitations, most notably causing nausea, bloating and vomiting in some individuals (Ward et al., 2003; Ogden et al., 2020d). In comparison, GLN doses *circa* 0.3 g·kg$^{-1}$ fat free body mass are largely well tolerated without inducing GI symptoms and have been shown to improve GI barrier integrity in clinical care settings (Shu et al., 2016) and moderate intensity exertional-heat stress (Pugh et al., 2017a). However, contrary to the *a priori* hypothesis, the present study found an acute 0.3 g·kg$^{-1}$ fat free body mass bolus of GLN ingested one hour before running at LT pace to exhaustion in the heat (40°C/40% RH), increased GI permeability (serum DSAT) by 26% and had no influence on small intestinal epithelial injury (I-FABP), when compared to a non-calorific PLA supplement. The size of effect of GLN in worsening GI permeability (serum DSAT) was considered small (d = 0.47), however, is of comparable magnitude to the beneficial effects reported by Pugh et al. (2017a) when GLN was ingested in doses of 0.25 (d = 0.60) and 0.5 (d = 0.50) g·kg$^{-1}$ fat free body mass two hours prior to exertional-heat stress. Likewise, Pugh et al. (2017a) reported no effect (d = 0.02) of 0.25 g·kg$^{-1}$ fat free body mass GLN supplementation on post exertional-heat stress I-FABP concentrations and a small effect with 0.5 g·kg$^{-1}$ fat free body mass (d = 0.46). Whilst Pugh et al. (2017a) concluded that acute GLN supplementation protects GI barrier integrity in a dose dependant manner, we would challenge this statement, instead concluding that low-dose acute GLN supplementation (0.25 – 0.3 g·kg$^{-1}$ fat free body mass) has a negligible effect on GI barrier integrity in response to exertional-heat stress.

There are several potential explanations why 0.3 GLN g·kg$^{-1}$ fat free body mass GLN supplementation did not improve GI barrier integrity in response to exertional-heat stress. First, the supplementation regime might have been insufficient to upregulate key mechanistic pathways, including intracellular heat shock protein (I-HSP) expression; epithelial cell proliferation; and glutathione biosynthesis (Singleton and Wischmeyer, 2006). Second, the serum DSAT undertaken at a single timepoint has potential to be confounded by the influence of prior GLN ingestion on gastric emptying rate (Du et al., 2018). Potentially GLN ingestion delayed the delivery of Lactulose/$_{L}$-rhamnose to the small intestine, thus altering the time-course of peak responses (Sequeira et al., 2014). Third, previous research on this topic was conducted following an overnight fast, where the PLA was non-calorific (Zuhl et al., 2014, 2015, Pugh et al., 2017a; Osborne et al., 2019). Given macronutrient ingestion improves intestinal perfusion during exertional-heat stress (Snipe et al., 2017), favourable responses with GLN supplementation might simply relate to the dose-dependent effects of energy provision *per se*. Finally, the intensity of both exercise (anaerobic LT intensity; mean HR = 171 b·min$^{-1}$) and heat stress (40°C, 40% RH) that participants were exposed to in the present study was markedly greater than previous research reporting favourable benefits of GLN supplementation on GI barrier integrity in response to 1-hour of running in the heat at an intensity corresponding to 70% $VO_{2max}$ (Zuhl et al., 2015 [35°C, 2-20% RH]; Pugh et al., 2017a; [30°C, 40% RH). In accordance with Osborne et al. (2019) who recently reported minimal effect of supplementing with a 0.9 g·kg$^{-1}$ fat free body mass dose of GLN 1-hour prior to a 20-km cycling time trial in the heat (35°C; 50% RH) on post exercise I-FABP concentrations, we propose that GLN supplementation has limited effect on protecting GI barrier integrity during high-intensity exertional-heat stress where intestinal ischemia is more pronounced. As proposed by Costa et al. (2020), this finding suggests that the benefits of GLN supplementation during low-intensity exertional-heat stress might be largely attributable to improved microvascular hyperemia, through production of localised metabolic vasodilators (i.e. nitric oxide) or simply the presence of nutrients within the small intestine, mechanisms that potentially become overwhelmed as exercise intensity increases.

Bacterial DNA is a novel biomarker to examine GI MT through high-sensitivity 16S gene sequencing (Paisse et al., 2016). In comparison to traditional GI MT biomarkers (e.g. endotoxin), bacterial DNA assessment is 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 controls against the effect of co-variates that influence *Bacteroides* DNA concentrations independent of GI MT, such as the efficiency of DNA extraction and DNase concentrations (March et al., 2019). In the present study, *Bacteroides*/total 16s DNA concentrations were stable over time across both trials, although the majority of samples ($n$ = 24/40) were below the sensitivity of analysis (hence the high CV between duplicates. Previous research demonstrated comparable basal *Bacteroides*/total 16S DNA ratios as presently reported (~0 – 1.0), though the proportion of samples with *Bacteroides* DNA concentrations below the assay sensitivity was less than presently reported (March et al., 2019; Ogden et al., 2020b, 2020c). In response to exertional-heat stress, March et al. (2019) found the *Bacteroides*/total 16S DNA ratio tended to increase ($p$ = 0.07) by ~65% (placebo arm) directly following 60 minutes moderate intensity (70% $VO_{2max}$) running in the heat (30°C/60% RH). Similarly, previous research from our laboratory found *Bacteroides*/total 16S DNA ratio increased by 129% following an 80-minute fixed-intensity EHST in untrained individuals of comparable demographic to the present study (Ogden et al., 2020c). Based on our reported findings to date, we promote future development of improved bacterial DNA methodologies for MT assessment. Considerations include: (1) assessment in whole blood samples, whereby GI microbial DNA concentrations are several magnitudes greater than plasma; and (2) simultaneous assessment of other major GI microbial phyla (Paisse et al., 2016).

Though previous research demonstrated acute oral GLN supplementation to support GI barrier integrity during exertional-heat stress, there is no evidence that these benefits translate to blunted GI MT. A primary aim of this research was therefore to address this gap in the literature, hence the selection of a high-intensity EHST previously proven to induce GI MT (Shing et al., 2013; Barberio et al., 2015). However, inconsistent with the *a priori* hypothesis, the *Bacteroides*/total 16S DNA ratio was similar between the GLN and PLA trials. Concordant with the present findings, earlier studies of GLN and GI barrier responses following exertional-heat stress, were also unable to induce measurable GI MT, measured by plasma endotoxin concentration, despite evoking a ~200% increase in GI permeability (Zuhl et al., 2015) and an ~83% increase in small intestinal injury (Osborne et al., 2019). In the present study, total 16S DNA concentrations exhibited a significant overall trial effect, where

concentrations were 13% lower after GLN supplementation. This response was unexpected given that GLN simultaneously increased GI permeability and had no influence on the *Bacteroides*/total 16S DNA ratio. Whilst total 16S DNA concentrations have previously been suggested as a method to assess GI MT in clinical settings (Fukui, 2016), this analysis is potentially confounded by variables independent of GI MT (e.g. DNase concentrations) when considered in response to exercise (March et al., 2019). Therefore, despite previous evidence suggesting GLN supplementation to improve systemic microbial neutralisation capacity in clinical care patients (Shu et al., 2016), these benefits were not reproducible following high-intensity exertional heat stress.

**LIMITATIONS**

Despite execution of a tightly controlled methodological design, the present results were not without some limitations. First, the EHST only evoked moderate disturbance of GI barrier integrity and MT, likely attributable to only a moderate rise in $T_{core}$ given all participants failed to complete the 30-minute EHST. Second, a basal DSAT was not performed to minimise participants' time burden, with the overall aim of improving compliance. Consequently, this prevented direct assessment of the EHST on GI permeability, whereby absolute responses were markedly lower than previous research using comparable intensity/duration exercise protocols (Marchbank et al., 2011; Davison et al., 2016). Third, implementation of an isocaloric PLA would have reduced concerns regarding the external influence of macronutrient provision on GI barrier integrity. The decision to utilise a non-calorific PLA was selected to ensure consistency with previous studies on this topic (Zuhl et al., 2014, 2015; Pugh et al., 2017a; Osborne et al., 2019). Finally, although trials were undertaken following a >10 hour overnight fast, diet was not standardised in the days prior to testing. The impact of this lack of standardisation is unclear, however, previous research report no effect of manipulating either carbohydrate (Moncada-Jimenez et al., 2009) or gluten (Lis et al., 2015) availability on GI barrier responses to exercise when trials were conducted in the fasted state.

## CONCLUSION

These findings do not support the application of acute low-dose oral $_{L}$-glutamine supplementation to help prevent exertional-heat stroke in occupational and athletic settings by reducing gastrointestinal permeability, small intestinal injury, and microbial translocation.

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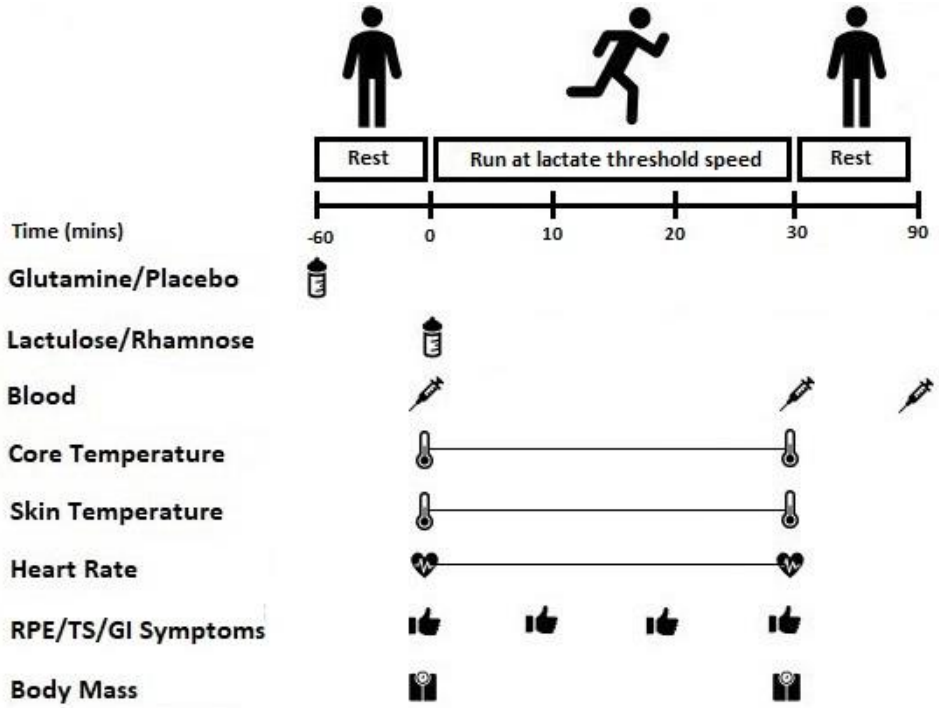


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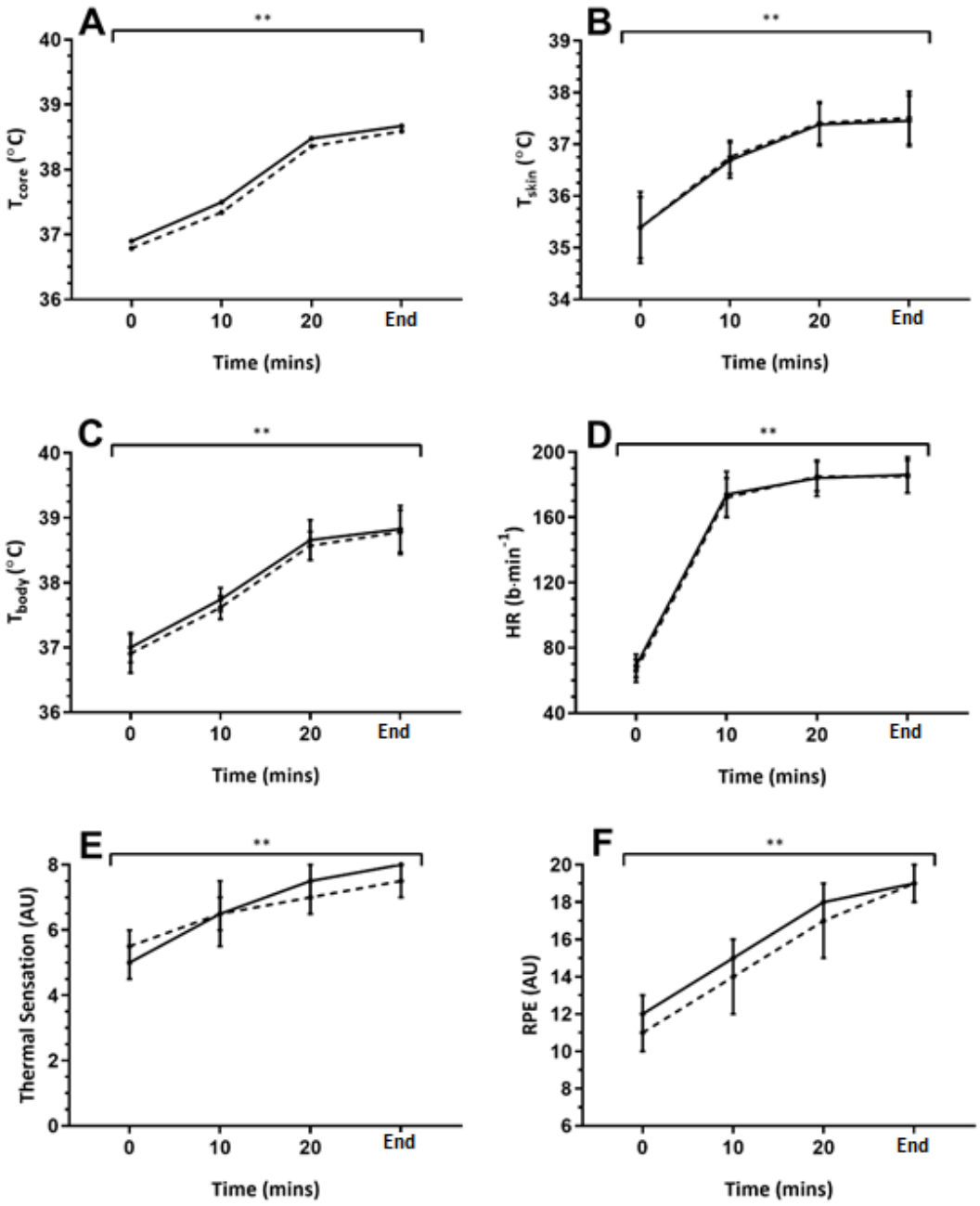
763 **Figure 1**. Schematic illustration of the experimental measurement timings

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765 **Figure 2.** Whole-body physiological responses to a high-intensity exertional heat stress test:
766 (A) = core temperature; (B) = mean skin temperature; (C) = mean body temperature; (D) =
767 heart rate; (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = GLN,
768 broken line = PLA. Significant overall effect of time (*$p \leq 0.05$; ** $p \leq 0.01$).

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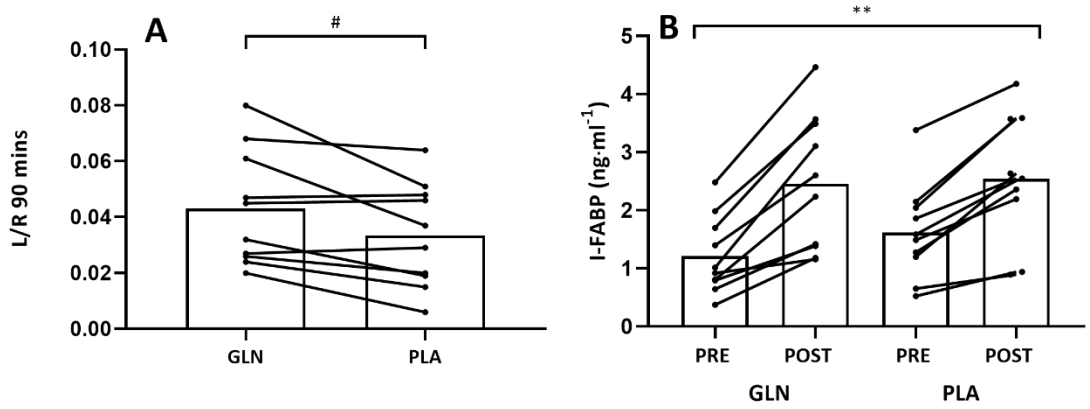


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773 **Figure 3.** GI barrier integrity responses to a high-intensity exertional heat stress test: (A) =
774 L/R ratio (DSAT) at 90 minutes; (B) I-FABP. Significant overall effect of time (*$p \leq 0.05$; ** $p \leq$
775 0.01). Significant effect of trial (# $p \leq 0.05$).

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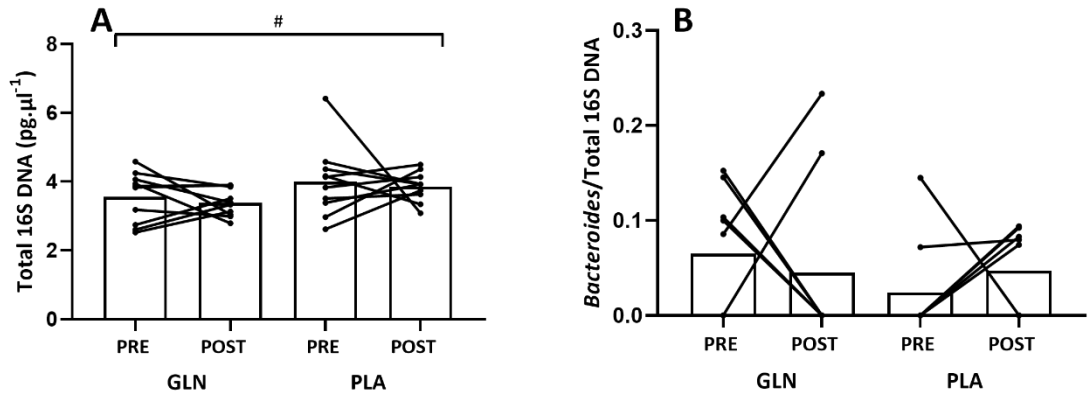


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778 **Figure 4.** GI MT responses to a high-intensity exertional heat stress test: (A) = total 16S DNA;
779 (B) *Bacteroides*/total 16S DNA ($n = 9$). Significant overall effect of trial (# $p \leq 0.05$).

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