TITLE:

No benefit of low dose acute L-glutamine supplementation on small intestinal permeability, epithelial injury or bacterial translocation biomarkers in response to subclinical exertional-heat stress: A randomised cross-over trial

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This study aimed to assess the influence of acute low-dose glutamine supplementation on small intestinal permeability, injury and bacterial translocation biomarkers in response to exertional-heat stress. Whilst the glutamine supplement was well-tolerated, gastrointestinal injury, permeability or bacterial translocation was not improved, in comparison to a non-calorific placebo.

ABSTRACT

INTRODUCTION: Exertional-heat stress disrupts gastrointestinal permeability, and through subsequent bacterial translocation, can result in potentially fatal exertional-heat stroke. Glutamine supplementation is a potential countermeasure, although previously validated doses are not universally well-tolerated. **METHODS:** Ten males completed two 80-minute subclinical exertional-heat stress tests (EHST) following either glutamine (0.3 g·kg·FFM^{-1}) or placebo supplementation. Small intestinal permeability was assessed using the lactulose/rhamnose dual-sugar absorption test and small intestinal epithelial injury using Intestinal Fatty-Acid Binding Protein (I-FABP). Bacterial translocation was assessed using total 16S bacterial DNA and *Bacteroides*/total 16S DNA ratio. **RESULTS:** The glutamine bolus was well tolerated, with no participants reporting symptoms of gastrointestinal intolerance. Small intestinal permeability was not influenced by glutamine supplementation (p = 0.06), though a medium effect size favouring the placebo trial was observed (d = 0.73). Both small intestinal epithelial injury (p < 0.01) and *Bacteroides*/total 16S DNA (p = 0.04) increased following exertional-heat stress, but were uninfluenced by glutamine supplementation. **CONCLUSION:** Acute low-dose oral glutamine supplementation does not protect gastrointestinal injury, permeability, or bacterial translocation in response to subclinical exertional-heat stress.

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Ethics Approval: The study protocol was approved by Plymouth MARJON University Research Ethics Committee (Approval Code: EP097) and was conducted in accordance with the principles outlined in the 1964 Declaration of Helsinki and its later amendments

Consent to Participate: Informed consent was obtained from all individual participants included in the study.

Consent for Publications: Patients signed informed consent regarding publishing their anonymised data.

ABBREVIATIONS

ANOVA	Analysis of variance
CV	Coefficient of Variation
EDTA	Ethylenediaminetetraacetic acid
EHST	Exertional Heat Stress Test
ELISA	Enzyme Linked Immunosorbent Assay
FFM	Fat Free Mass
HPLC	High Performance Liquid Chromatography
I-FABP	Intestinal Fatty-Acid Binding Protein
L/R	Lactulose-to-Rhamnose
mVAS	Modified Visual Analogue Scale
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
$\dot{V}O_{2max}$	Maximal Oxygen Uptake

INTRODUCTION

Exertional heat stroke is the most severe disorder along the continuum of heat-related illnesses (Leon and Bouchama, 2011). It is a condition that sporadically affects young individuals engaged in arduous physical activity, including athletes, military personnel, and labourers. Whilst direct mortality from exertional heat stroke is largely preventable with provision of rapid (< 1 hour) whole-body cooling (DeMartini et al., 2015), many casualties experience lifelong health complications because of residual organ damage (Wallace et al., 2007). The pathophysiology of EHS has been hypothesised to be at least partially attributable to a systemic inflammatory response triggered by the translocation of pathogenic microbes from the gastrointestinal lumen into the systemic circulation (Lim, 2018; Fung et al., 2021; Walter et al., 2021). Consequently, contemporary research has focussed on evaluating the efficacy of nutritional countermeasures to support intestinal permeability, epithelial injury, and bacterial translocation in response to exertional-heat stress (Costa et al., 2020; Ogden et al., 2020a).

$_{L}$-glutamine is the most abundant free amino acid in the human body (Newsholme et al., 2003). It is classified as a conditionally essential nutrient, given it is the preferential energy source of rapidly proliferating cells (e.g. leukocytes), but becomes depleted during severe catabolism (Lacey and Wilmore, 1990). In addition to being an important energy substrate, $_{L}$-glutamine performs various other essential physiological roles, including: nitrogen transportation; gluconeogenesis; acid-base regulation; and the biosynthesis of glutathione, nitric oxide and heat shock proteins (Gleeson, 2008). Based on these functions, $_{L}$-glutamine supplementation has previously been demonstrated to protect small intestinal permeability and epithelial injury in response to exertional-heat stress (Zuhl et al., 2014, 2015; Pugh et al.,

2017). For example, Zuhl et al. (2014) found one week of daily $_{L}$-glutamine supplementation (0.9 g·kg·fat free mass^{-1} [FFM]) attenuated the ~3-fold rise in small intestinal permeability following one-hour of running at 70% VO_{2max} in the heat (30°C). These findings were subsequently replicated with a single acute glutamine bolus (0.9 g·kg·FFM^{-1}) ingested two hours prior to exertional-heat stress (Zuhl et al., 2015; Pugh et al., 2017).

Unfortunately, high-dose oral glutamine ingestion (≥ 0.75 g·kg·FFM^{-1}) appears to have limited ecological validity, given reports of gastrointestinal symptoms such as bloating, nausea and vomiting in some study participants (Ward et al., 2003; Pugh et al., 2017; Ogden et al., 2020d). One solution to overcome this issue is to reduce the glutamine dose to *circa* 0.2-0.3 g·kg^{-1} where adverse gastrointestinal symptoms become negligible (Ziegler et al., 1990; Ogden et al., 2020d). Whilst the importance of regular macronutrient ingestion (e.g. 30 – 60 g·hour^{-1} of liquid carbohydrate) has been widely-advocated as a nutritional countermeasure to protect small intestinal permeability during exertional-heat stress (Lambert et al., 2001; Snipe et al., 2017; Flood et al., 2020; Jonvik et al., 2020), it is not a universally effective strategy (Lambert et al., 2001; Sessions et al., 2016; Pugh et al., 2020), whilst in favourable studies the protection afforded on the gastrointestinal barrier had little influence on downstream bacterial translocation (Snipe et al., 2017; Jonvik et al., 2020). Furthermore, during short-notice occupational deployments where exertional-heat stroke is commonplace, such as military operations, wildland fire fighting and emergency first responses, consideration must be given to the logistical constraints associated with any proposed nutritional intervention. This includes the ability to carry the required food/drink on person, acceptable gastrointestinal tolerance and affordability (Ogden et al., 2020a). Based on these considerations, low acute dose glutamine supplementation (0.2-0.3 g·kg^{-1})

would appear to have better practicality than other previously researched nutritional countermeasures (e.g. chronic bovine colostrum or probiotic supplementation). The efficacy of low dose glutamine supplementation (12 grams) has previously been demonstrated to protect small intestinal permeability when ingested 30 minutes prior to gastrointestinal disturbance induced by a non-steroidal anti-inflammatory pharmaceutical (Hond et al., 1999).

The aim of the present study was to assess the influence of acute low-dose (0.3 $g \cdot kg \cdot FFM^{-1}$) oral glutamine supplementation on small intestinal permeability, epithelial injury and bacterial translocation in response to exertional-heat stress. The primary hypothesis was that glutamine supplementation would protect small intestinal permeability, epithelial injury and reduce bacterial translocation in response to exertional-heat stress.

METHODS

Twelve healthy males volunteered to participate in the study. Two participants withdrew before study completion due to injury independent of the study; therefore, data is reported for the remaining ten completing participants (Table 1). All participants were non-smokers, who habitually exercised (>4 $h \cdot week^{-1}$), were non-endurance trained ($VO_{2max} \leq 55$ $ml \cdot kg \cdot min^{-1}$) and unacclimated to hot environments. This demographic could be considered broadly representative of UK military personnel in ground combat roles (Fallowfield et al., 2019). A general medical questionnaire was used to screen for previous histories of gastrointestinal, cardiorespiratory and metabolic illnesses. No participant self-reported taking pharmacological medications or having suffered from an acute respiratory or gastrointestinal 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

184 (Approval Code: EP097) and was conducted in accordance with the principles outlined in the
185 1964 Declaration of Helsinki and its later amendments.

186 [Table 1 – Insert Here]

187 Participants visited the laboratory on three occasions. Baseline anthropometrics and
188 maximal oxygen uptake ($\dot{V}O_{2max}$) were assessed during the first visit. The two subsequent
189 visits were main experimental trials, where participants were supplemented with either
190 glutamine or placebo in a randomised, counterbalanced, double-blind cross-over design. Trial
191 order was determined by a computer-generated random number generator
192 (*www.randomizer.org*) and participants assigned *a priori* by an individual independent of
193 primary data collection. Study trials were separated by 7-14 days to minimise the influence of
194 prior exertional-heat stress on thermoregulatory (Barnett and Maughan, 1993) and
195 gastrointestinal biomarker (Ogden et al., 2020b) responses.

196 During both main experimental trials, participants completed an intermittent
197 exertional-heat stress test (EHST), consisting of two bouts of 40 minutes fixed-intensity
198 treadmill walking (6 $km \cdot h^{-1}$ and 7% gradient) in the heat (35°C and 30% relative humidity; RH).
199 The exercise bouts were separated by 20-minutes seated recovery. This protocol was
200 designed in line with military work/rest schedule guidance, consistent across different
201 militaries worldwide (Spitz et al., 2012). In comparison with previous research from our
202 laboratory that applied this study design (Ogden et al., 2020b, 2020c), forearm cold water
203 immersion and fluid replacement interventions were removed, with the aim to enhance
204 gastrointestinal barrier insult (Costa et al., 2019). Data collection coincided with non-summer
205 months in Plymouth, United Kingdom, where daily mean ambient temperature at a local

meteorological station (Camborne, United Kingdom; latitude: 50.218 ° N) remained below 20°C (Met Office, 2020). A schematic illustration of the protocol is shown in Figure 1.

[Figure 1 – Insert Here]

Dietary supplements (e.g. probiotics) and prolonged thermal exposures (e.g. saunas) were prohibited from 14 days before until the end of data collection. Alcohol, caffeine, strenuous physical activity, non-steroidal anti-inflammatory drugs (e.g. ibuprofen) and spicy foods were all abstained for 48 hours before main experimental visits. Dietary macronutrient composition in the days prior to trial visits was not tightly controlled to minimise participant burden, although participants were requested to match their habitual diet as close as possible between repeated visits. Participants adhered to a ≥ 10 hour overnight fast and consumed 500 ml of plain water two hours prior to main experimental visits. Conformity with all pre-trial controls was self-attested in writing upon laboratory arrival using a questionnaire. Participants remained fasted throughout main experimental trials (Edinburgh et al., 2018).

Glutamine supplementation consisted of 0.3 $g \cdot kg^{-1}$ fat free mass of glutamine crystalline powder ($_{L}$-glutamine Elite, Myprotein, Northwich, UK; Batch Number: W920126073), which was freshly suspended in 500 ml of water/lemon flavour cordial (4:1 ratio; Fruit Squash – no added sugar, Robinsons, UK). Participants ingested the entire fluid bolus within a 5-10 minute period, finishing one hour before commencing the EHST. Placebo supplements were taste and consistency matched, comprised of the identical water/lemon flavour sugar-free cordial alone. Both supplements were administered in an opaque bottle to match visual appearance. Supplements were prepared by an individual independent of the study.

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] = 2.2%) 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 ($\dot{V}O_{2max}$) was determined using an incremental treadmill test (Desmo HP, Woodway GmbH, Weil am Rhein, Germany) to volitional exhaustion described in detail elsewhere (Ogden et al., 2020b).

The EHST commenced in the morning (08:30 ± 1 hour) to standardise the influence of circadian variation (Gaskell et al., 2020b). 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 = 0.5%) and urine specific gravity via a digital refractometer (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 heart rate monitor was positioned around participants' chest (EQ02, Equivital™, Cambridge UK) and was measured using a Sensor Electronics Module (SEM) unit (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 ~35°C (glutamine: 35.3 ± 0.3°C; placebo: 35.3 ± 0.2°C; p= 0.15) and ~30% RH (glutamine: 31 ± 1%; placebo: 30 ± 1%; p= 0.44). On entry to the chamber, skin thermistors (EUS-UU-VL3-O, Grant Instruments, Cambridge, UK) were affixed to the participant using one layer (5 x 5 cm) of cotton tape (KT Tape®, KT Health, UT, USA); and mean skin temperature (T_{skin}) was calculated using standard equations (Ramanathan, 1964).

Throughout the EHST, T_{core} and T_{skin} were continuously recorded using a temperature logger (Squirrel SQ2010, Grant Instruments, Cambridge, UK) and heat rate 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 rating of perceived exertion (RPE; Borg et al., 1970), thermal sensation (Toner et al., 1986) and gastrointestinal symptoms (Gaskell et al., 2019) were reported at 20-minute intervals. For each perceptual measure, participants were given standardised instructions to ensure understanding of anchoring the top and bottom ratings to previous perceptions or experiences during exertional-heat stress. Gastrointestinal symptoms are presented as the incidence (%) and accumulated severity of symptoms, grouped following previous guidance (Gaskell et al., 2019). Absolute sweat losses were calculated from the change in dry nude body mass from pre-to-post EHST.

Venous blood samples (12 ml) were drawn immediately pre and post the EHST. At rest, participants stood upright for a minimum of 20 minutes before collection to allow capillary filtration pressure to stabilise (Shirreffs and Maughan, 1994). Blood was drawn from a forearm antecubital vein under minimal stasis (<30 seconds) and following sterilisation with an 80% isopropyl alcohol wipe. Samples were collected proportionally into serum-separator (SST II) and K_2 EDTA tubes (Becton Dickinson and Company, Plymouth, UK). The SST II tube

was allowed to clot for 30-40 minutes at room temperature. A 0.5ml aliquot of K_2EDTA blood was removed for immediate haematological analysis. Samples were centrifuged at 1300*g* for 15 minutes at 4°C to separate serum and plasma. Aliquots were transferred into microtubes without disrupting cells and then frozen at -80°C until analyses. All blood handling was performed with manufacturer certified sterile (pyrogen, DNA free) pipette tips and microtubes.

Haemoglobin was measured using a portable photometric analyser (Hemocue® Hb 201+, EFK Diagnostics, Madeburg, Germany; Duplicate; CV = 0.4%) and haematocrit using the microcapillary technique following centrifugation at 14,000*g* for 4 minutes at room temperature (Haematospin 1400, Hawksley and Sons Ltd, Lancing, England; CV = 0.6%). Changes in 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. Plasma osmolality was examined in duplicate (CV = <0.1%) using freeze-point depression (Osmomat 3000, Gonotec, Berlin, Germany).

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 serum samples collected 90 minutes following probe ingestion (i.e. 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 (mg·l^{-1}), where the lactulose/$_L$-rhamnose (L/R) was then corrected relative (%) to the concentration of

296 sugar consumed. The limit of detection was 0.1 $mg \cdot l^{-1}$. The combined L/R coefficient of
297 variation was 8.7%.

298 I-FABP (1:4 plasma dilution) was measured in duplicate plasma samples pre and
299 immediately post EHST using a solid-phase sandwich ELISA (DY3078, DuoSet, R&D systems,
300 Minneapolis, USA) following manufacturer instructions. The intra-assay coefficient of
301 variation was 2.0%.

302 Bacterial DNA was measured in duplicate plasma samples collected pre and
303 immediately post EHST using a quantitative real-time polymerase chain reaction assay (qPCR)
304 on a LightCycler 96 instrument (LightCycler 96, Roche, Basel, Switzerland). DNA was isolated
305 from plasma using a Quick-DNA Mini Prep Plus kit (D4068, Zymo Research, Irvine, CA, USA)
306 following manufacturer's instructions. The elution buffer was heated to 65°C before use. Total
307 16S bacterial DNA was quantified according to March et al. (2019) using a universal library
308 probe (Roche, Basel, Switzerland), with standards (E2006-2, Zymo Research, Irvine, CA, USA)
309 and primers (Eurogentec, Liège, Belgium) specific to a 16S region (limit of detection 0.1
310 $pg \cdot \mu l^{-1}$). *Bacteroides* species DNA were quantified using a commercial double-dye
311 probe/primer kit using 5 μl^{-1} of DNA template following manufacturer's instructions (Path-
312 Bacteroides-spp, Genesig, Primerdesign Ltd, Chandler's Ford, UK). Where Bacteroides
313 concentrations were < 0.2 $copy \cdot \mu l^{-1}$ (i.e. less than 1 copy of DNA in 5 μl^{-1} DNA template), these
314 were considered as artefact and subsequently reported as zero. Negative controls (PCR grade
315 water) for the entire extraction process were below the limit of detection for both measures.
316 Ratio data are presented as *Bacteroides*/total bacterial DNA (*Bact./*16S). The intra-assay
317 coefficients of variation were 6.4% (total 16S) and 24.5% (*Bacteroides*).

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. A two-way analysis of variance (ANOVA) with repeated measures (time x trial) was used to identify differences over time. 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 corrected t-tests were used to determine the location of variance. When there was only a single comparison (lactulose/rhamnose), a paired t-test was used to determine between-trial differences. Statistical significance was accepted at the alpha level of $p \leq 0.05$. Effect sizes were calculated based on Cohens D with Hedges small sample size correction (hedges *g*), with the magnitude of effect classified as small (*d* = 0.2), medium (*d* = 0.5) and large (*d* = 0.8) based on standard criteria (Lakens, 2013). Data are presented as mean ± standard deviation (SD). Gastrointestinal symptom severity is presented as accumulated mean ± range of reported symptoms ≥ 1 (Gaskell et al., 2019).

A sample size estimation was calculated *a priori* using specialist statistical power software (G*Power 3.1, Kiel, Germany). Anticipated effect size was derived from a previous study comparing the influence of acute glutamine (0.9 $g \cdot kg \cdot FFM^{-1}$) on small intestinal permeability responses (Zuhl et al., 2015) following exertional-heat stress. In total, ≥ 6 participants were considered necessary to detect a significant interaction effect using a two-way ANOVA with standard alpha (0.05) and beta (0.8) values.

RESULTS

Basal urine osmolality (glutamine: 383 ± 256 mOsmol·kg^{-1}; placebo: 336 ± 217 mOsmol·kg^{-1}; p = 0.57), urine specific gravity (glutamine: 1.010 ± 0.008 AU; placebo: 1.009 ± 0.006 AU; p = 0.53) and plasma osmolality (glutamine: 299 ± 3 mOsmol·kg^{-1}; placebo: 298 ± 3 mOsmol·kg^{-1}; p = 0.15) were similar between conditions. The Δ plasma volume following the EHST was comparable in both the glutamine (-3.26 ± 1.81%) and placebo (-3.46 ± 1.59%) trials (p = 0.62).

Small intestinal permeability (lactulose/rhamnose ratio) was not different between the glutamine (0.030 ± 0.012) and placebo (0.023 ± 0.006) trials (p = 0.06; Figure 2A), though with a medium effect size favouring placebo supplementation (g = 0.73). I-FABP concentration increased from pre (glutamine: 2.16 ± 0.98 ng·ml^{-1}; placebo: 2.60 ± 1.04 ng·ml^{-1}) to post-EHST (glutamine: 4.70 ± 1.31 ng·ml^{-1}; placebo: 3.98 ± 1.70 ng·ml^{-1}) in both trials (time; p < 0.01). There was an overall time x trial interaction effect (p = 0.03) for I-FABP (Figure 2B), however, after post hoc correction this effect was not significant either pre- or post-EHST (p > 0.05). There was a small effect size for post-EHST I-FABP concentration favouring placebo supplementation (g = 0.47). No trial order effects were identified (p > 0.05).

[Figure 2 – Insert Here]

Total 16S DNA was unchanged from pre- (glutamine = 6.08 ± 0.98 µg·ml^{-1}; placebo = 6.55 ± 1.75 pg·µl^{-1}) to post-EHST (glutamine = 5.97 ± 0.98 pg·ml^{-1}; placebo = 6.87 ± 0.91 pg·ml^{-1}) in either trial (Figure 2C; time x trial interaction; p = 0.49). The effect size for post-EHST total 16S DNA was large (g = 0.95) in favour of the glutamine trial. *Bacteroides*/total 16S DNA ratio increased (time; p = 0.04) from pre (glutamine = 0.05 ± 0.05; placebo = 0.09 ± 0.08)

to post-EHST (glutamine = 0.12 ± 0.11; placebo = 0.26 ± 0.41) in both trials (Figure 2D; time x trial interaction; p = 0.21). The effect size for post-EHST total 16S DNA was small (d = 0.60) in favour of the glutamine trial. In 11/40 samples, *Bacteroides* concentrations were below the analytical limit of detection (0.2 copy·μl^{-1}) suggestive of no *Bacteroides* DNA present in the sample (ratio data presented as zero). No trial order effects were identified (p > 0.05).

There was no difference in T_{core} (Figure 3A; time x trial; p = 0.98), mean T_{skin} (Figure 3B; time x trial; p = 0.49) or T_{body} (Figure 3C; time x trial; p = 0.93) between the glutamine and placebo trials. Peak T_{core} was 38.6 ± 0.4°C in the glutamine trial and 38.6 ± 0.5°C in the placebo trial, respectively (p > 0.05). Mean whole body sweat (glutamine: 1.8 ± 0.3 l·h^{-1}; placebo: 1.8 ± 0.3 l·h^{-1}; p = 0.43) and % body mass loss (glutamine: 2.0 ± 0.3%; placebo: 2.0 ± 0.5%; p = 0.48) were similar between conditions. Heart rate increased to a similar extent throughout the EHST between the two trials (Figure 3D; time x trial; p = 0.96). Peak heart rate was 161 ± 13 b·min^{-1} in the glutamine and 161 ± 19 b·min^{-1} in the placebo trial, respectively.

[Figure 3 – Insert Here]

RPE (Figure 3E; time x trial; p = 0.59) and thermal sensation (Figure 3F; time x trial; p = 0.70) increased to a similar extent throughout the EHST between the two trials (Figure 3E; time x trial; p = 0.59). There were no reports (e.g. all 0 scores) of gut discomfort, nausea, total-, upper- or lower- gastrointestinal symptoms for any participant during either the glutamine or placebo trial.

DISCUSSION

The aim of this study was to determine the influence of low-dose (0.3 g·kg·FFM^{-1}) acute oral glutamine supplementation on small intestinal permeability, epithelial injury and bacterial translocation biomarkers in response to exertional-heat stress. The main findings were that low-dose acute oral glutamine supplementation 1-hour before an EHST did not influence small intestinal permeability (serum lactulose/rhamnose) or epithelial injury (plasma I-FABP) in comparison to water-alone. Whilst gastrointestinal bacterial translocation (*Bacteroides*/total 16S DNA) increased following the EHST, this response was also similar between the glutamine and placebo trials. There was no evidence of subjective gastrointestinal symptoms in either trial, whilst whole-body physiological (e.g. T_{core}, heart rate) responses were not influenced by supplementation. Together, these data suggest no benefit of low-dose (0.3 g·kg·FFM^{-1}) acute oral glutamine supplementation to support small intestinal permeability, epithelial injury and bacterial translocation in response to exertional-heat stress.

I-FABP is the prominent biomarker of small intestinal epithelial injury, whereas the DSAT assesses functional gastrointestinal permeability (Bischoff et al., 2014). In the present study, overall mean Δ I-FABP (1.37 ± 1.101 ng·ml^{-1} [53%]) and absolute DSAT (0.023 ± 0.005) responses where comparable in the placebo trial to what has previously been reported by our laboratory applying an identical EHST (Δ I-FABP = 0.20-1.35 ng·ml^{-1} [16-119%]; and DSAT = ~0.020-0.035) (Ogden et al., 2020b, 2020c). This finding was unanticipated given that compared to our previous work, rehydration (12 g·kg·BM^{-1}) and forearm cold water immersion (4 minutes in 15°C) practises were intentionally curtailed in attempt to increase thermoregulatory and cardiovascular strain. However, given that post-EHST hypohydration

(~2% vs ~1% body mass loss), T_{core} (e.g. peak = 38.6 vs. 38.6°C) and heart rate (mean = 145 vs. 150 bpm) were actually similar to our previous studies, the present data are perhaps less surprising (Chantler et al., 2020). For sake of comparison, in previous research where permissive dehydration caused an increased in small intestinal epithelial injury (Costa et al., 2019), these authors were able induce a greater divergence in hydration status between the euhydrated (~0.5% body mass loss) and hypohydrated conditions (~3% body mass loss). Overall, the severity of small intestinal epithelial injury and permeability in the present study is comparable to many previous 60-to-90 minute moderate-intensity (60-70% VO_{2max}) EHST protocols (e.g. Sheahen et al., 2018; Szymanski et al., 2017). It remains to be determined what intensity, duration and mode of exercise causes the greatest disturbance of the gastrointestinal barrier when adopting an exercise protocol that controls for either whole-body physiological strain (e.g. peak T_{core}) or total work performed. Indeed, prolonged duration (≥2 hours), moderate-intensity (~60% VO_{2max}) exertional-heat stress appears to cause the greatest increase in plasma I-FABP concentrations (200 – 500% increase; Costa et al., 2020), however, typically this form of exercise has a lesser influence on GI permeability (e.g. Snipe et al., 2018; Pugh et al., 2019) in comparison to short duration (< 1 hour), higher-intensity (~80% VO_{2max}) exertional-heat stress (Marchbank et al., 2011; Davison et al., 2016). Irrespective of the EHST selected, no laboratory study conducted to date has been able to induce even close-to the severity of small intestinal epithelial injury that was recently reported in marathon runners incapacitated with suspected heat stroke (~1500% increase; Walter et al., 2021), thus questioning the clinical relevance of examining gastrointestinal related risk factors and countermeasures for EHS using sub-clinical laboratory protocols. Furthermore, whilst not the main objective of this study, Gaskell et al. (2021) recently questioned the clinical relevance of gastrointestinal integrity loss (I-FABP, bacterial

translocation, selected cytokines) on exercise-associated gastrointestinal symptoms, concluding in a case-series of symptomatic athletes that gastrointestinal integrity responses are largely normal.

Acute high-dose (0.9 g·kg·FFM^{-1}) oral glutamine supplementation has previously been recommended to protect small intestinal permeability and epithelial injury in response to exertional-heat stress (Zuhl et al., 2015; Pugh et al., 2017). However, the practicality of this supplementation regime is questionable, given reports of poor gastrointestinal tolerance (e.g. nausea, bloating) with this dosage in some individuals in the initial hours following ingestion (Ward et al., 2003; Ogden et al., 2020d). To overcome this issue, the present study supplemented participants with a reduced dose of glutamine (0.3 g·kg·FFM^{-1}) 1-hour before exertional-heat stress, which was found to be universally well tolerated in accordance with previous studies that supplemented with this dosage (Ziegler et al., 1990; Ogden et al., 2020d). However, in contrast to the *a priori* hypothesis, low-dose acute oral glutamine supplementation did not improve either small intestinal epithelial injury or permeability in response to exertional-heat stress. In fact, small intestinal permeability and epithelial injury appeared worsened following glutamine supplementation trial with medium and small effect size, respectively. Specifically, serum lactulose/rhamnose was 30% greater with glutamine supplementation and peak plasma I-FABP 18% greater with glutamine supplementation. These outcomes were surprising given that previous literature has consistently demonstrated acute high-dose (0.9 g·kg·FFM^{-1}) glutamine supplementation blunts Δ I-FABP responses by ~15-25%, and small intestinal permeability responses by 40-50%, when ingested either 1 or 2 hours before exertional-heat stress (Zuhl et al., 2015; Pugh et al., 2017; Osborne et al., 2019). Whilst Pugh et al. (2017) previously suggested that the benefits of acute oral glutamine

supplementation on small intestinal permeability and epithelial injury might be dose-dependent in response to exertional-heat stress, the present finding is the first to report a potential adverse effect of acute glutamine supplementation regime on the gastrointestinal barrier in response to exertional-heat stress. Indeed, the effect sizes reported by Pugh et al. (2017) that favour acute low-dose glutamine supplementation on small intestinal permeability (0.25 g·kg·FFM^{-1} dose, *d* = 0.60; 0.5 g·kg·FFM^{-1} dose *d* = 0.50) and epithelial injury (0.25 g·kg·FFM^{-1} dose, *d* = 0.02; 0.5 g·kg·FFM^{-1} dose, *d* = 0.46) in response to exertional-heat stress, are smaller in magnitude than those reported in the present study for small intestinal permeability (*d* = 1.16) and epithelial injury (*d* = 0.42) that conversely favour placebo supplementation. Overall, it is suggested that acute glutamine supplementation in doses *circa* 0.25 – 0.5 g·kg·FFM^{-1} has little influence on small intestinal permeability and epithelial injury in response to exertional-heat stress. Where glutamine has been supplemented in larger doses *circa* 0.9 g·kg·FFM^{-1}, both Pugh et al. (2017; *d* = 0.9) and Zuhl et al. (2015; *d* = 2.0) report strong positive effects.

In comparison to previous research reporting positive results with high-dose oral glutamine supplementation on small intestinal permeability and epithelial injury , there are several potential mechanisms that may explain why low-dose glutamine supplementation offered no protection of the gastrointestinal barrier in the present study. First, the dose and/or timing of glutamine ingestion might have been insufficient for meaningful induction of key protective mechanisms. These include: intracellular heat stroke protein (I-HSP) expression (Singleton and Wischmeyer, 2006); epithelial cell proliferation (Rhoads et al., 1997); glutathione biosynthesis (Harward et al., 1994); and gastrointestinal TJ protein expression (Li et al., 2004). Whilst these mechanisms could not be examined in the present

474 study due to the infeasibility of collecting intestinal tissue, previous *in vitro* studies using
475 intestinal epithelial cells have shown dose-dependent benefits of glutamine supplementation
476 on these mechanisms inside 1-hour (Wischmeyer et al., 1997). Second, implementation of the
477 serum lactulose/rhamnose dual-sugar abruption test at a single timepoint following probe
478 ingestion is potentially confounded by the influence of prior glutamine ingestion on gastric
479 emptying rate (Du et al., 2018). Whilst this effect is theoretically controlled through
480 paracellular/transcellular molecular probe ratio correction in 5-hour urine samples
481 (Bjarnason et al., 1995), the time-courses of absorption of low/high molecular weight probes
482 in the blood is not necessarily identical (Sequeira et al., 2014). Third, previous studies
483 conducted trials following an overnight fast, where the placebo supplement was non-calorific
484 (Zuhl et al., 2015, Pugh et al., 2017). Given regular macronutrient ingestion improves
485 intestinal vascular perfusion during exertional-heat stress (Snipe et al., 2017), previous
486 favourable responses with glutamine supplementation might simply be attributable to the
487 dose-dependent effects with higher energy provision *per se*. Finally, acute glutamine (1 ml of
488 2% solution) increased mortality when injected into the duodenum of mice at the onset of
489 75-minutes controlled ischemia-reperfusion injury (Omata et al., 2007). In this study, the
490 adverse effects reported with glutamine supplementation were hypothesised to be attributed
491 to an enhanced priming of circulating myeloid cells (CD11b expression) and spontaneous
492 production of reactive oxygen species production. These responses could provide a potential
493 mechanism for glutamine worsening small intestinal permeability and epithelial injury.

494 Bacterial DNA is an emerging biomarker to assess gastrointestinal bacterial
495 translocation through high-sensitivity conserved 16S gene sequencing (Paisse et al., 2016).
496 Compared with traditional bacterial translocation biomarkers (e.g. endotoxin), bacterial DNA

assessment is less susceptible to analytical issues surrounding both: (1) exogenous contamination, given the ability to target phyla/species with high gastrointestinal specificity (e.g. *Bacteroides*); and (2) liability to hepatic clearance, given that concentrations are largely similar between portal and systemic blood (Mortensen et al., 2013). Furthermore, to reduce the influence of potential covariates on Bacteroides DNA concentrations, such as the efficacy of DNA extraction, DNase concentrations, immune function and sample contamination, correction for total 16S DNA has previously been recommended (March et al., 2019). In the present study, mean *Bacteroides*/total 16S DNA concentrations increased by 89 ± 217% following the EHST in the placebo trial. Previous evidence has reported comparable basal *Bacteroides*/total 16S DNA ratios (~0 – 1.0), though large inter-individual variability in responses were evident (March et al., 2019; Ogden et al., 2020b, 2020c). For example, in a previous report from our laboratory, *Bacteroides*/total 16s DNA ratio increased by 183 ± 336% in participants with low aerobic fitness (VO_{2max} < 50 ml·kg·min^{-1}), but was unchanged (-6 ± 45%) in participants with high aerobic fitness (VO_{2max} > 60 ml·kg·min^{-1}) following a similar EHST to that applied in the present study (Ogden et al., 2020c). Thus, gastrointestinal bacterial translocation assessed using the *Bacteroides*/total 16s DNA ratio, is elevated following brief subclinical exertional-heat stress in most individuals. The standalone measurement of plasma total 16S DNA was unchanged in response to exertional-heat stress in the current study, which is concordant with previous research from our laboratory using a similar EHST (Ogden et al., 2020b, 2020c).

Whilst several studies have previously demonstrated acute oral glutamine supplementation can support small intestinal permeability and epithelial injury in response to exertional-heat stress, few studies have assessed whether these benefits translate into

reduced downstream gastrointestinal bacterial translocation. In addressing this gap in the literature, the present study reported no difference in gastrointestinal bacterial translocation measured using both total 16S DNA and *Bacteroides*/total 16S DNA between the glutamine and placebo trials following exertional-heat stress. This null effect of glutamine supplementation on gastrointestinal bacterial translocation was perhaps not surprising given that glutamine did not simultaneously protect either small intestinal epithelial injury or permeability. Alternatively, there is some rationale that glutamine supplementation might have improved microbial neutralisation capacity independent of translocation, which might explain the observed large effect size for glutamine in blunting total 16S DNA concentrations following exertional-heat stress. For example, in experimental rodent sepsis models, glutamine supplementation enhanced: lymphocyte function in gut-associate lymphoid tissue (Manhart et al., 2001); luminal immunoglobulin concentrations (Fan et al., 2018); and hepatic integrity upon pathological insult (Cruzat et al., 2014). Of the previous studies assessing the influence of glutamine supplementation on small intestinal permeability and epithelial injury around exertional-heat stress, only Zuhl et al. (2015) attempted to monitor gastrointestinal bacterial translocation through assessment of plasma endotoxin concentration. Unfortunately, endotoxin concentrations were unchanged following exertional-heat stress in this study, thus making it unfeasible to assess the efficacy of glutamine supplementation on gastrointestinal bacterial translocation (Zuhl et al., 2015). Therefore, the present study provides the first direct evidence that acute-oral glutamine supplementation does not reduce gastrointestinal bacterial translocation in response to exertional-heat stress.

Despite administering a tightly controlled methodological design, the present results were not without limitations. First, the EHST only evoked moderate disturbance of small

intestinal permeability, epithelial injury and bacterial translocation. A previous systematic review outlined an exercise induced T_{core} threshold of 38.6°C for gastrointestinal barrier integrity loss (lactulose/rhamnose, I-FABP and endotoxin) to be commonplace (>50% incidence) and of 39.0°C for gastrointestinal barrier integrity loss to be universal (100% incidence; Pires et al., 2017). In this study, participants only exceeded the 38.6°C threshold in 11/20 trials, whilst only 3/20 exceeded the 39.0°C threshold. Notwithstanding this limitation, the present EHST was chosen as it has robust ecological validity in representing group-based military work-rest guidance (Spitz et al., 2012) and previously was severe enough to induce gastrointestinal bacterial translocation (Ogden et al., 2020c). It is recommended that future research should examine the efficacy of nutritional countermeasures on gastrointestinal barrier biomarkers during physically arduous field activities (e.g. military selection tests, wildland firefighting) where participants are exposed to a greater severity of heat strain than is achievable in the laboratory. Second, *Bacteroides* DNA had poor analytical reliability (mean duplicate CV = 24.5%), which resulted from a large proportion of samples being close to the assays minimum level of detection. Future analysis should consider assessment of whole-blood samples, given that bacterial 16S DNA concentrations in the buffy coat far exceed that of plasma (Paisse et al., 2016). Third, a standardised diet was not prescribed in the days prior to experimental trials to minimise participant burden. The full impact of this lack of dietary standardisation is unclear, though previous studies report little effect of manipulating either short-term dietary gluten (Lis et al., 2015), carbohydrate (Moncada-Jimenez et al., 2009) or fermentable oligo- di- mono- saccharide and polyol (FODMAP; Gaskell et al., 2021a) availability on small intestinal permeability, epithelial injury or bacterial translocation biomarkers in response to fasted exercise. Finally, implementation of an isocaloric placebo or standardised pre-trial breakfast would have reduced concerns regarding the extraneous

influence of macronutrient provision on examined biomarkers. The decision to utilise a non-calorific placebo was selected to ensure consistency with previous research (Zuhl et al., 2014, 2015; Pugh et al., 2017; Osborne et al., 2019). It is recommended that future studies look to examine the efficacy of high-dose glutamine supplementation alongside standardised dietary guidance for athletic populations.

This study assessed the influence of low-dose (0.3 g·kg·FFM^{-1}) acute oral glutamine supplementation on small intestinal permeability, epithelial injury and bacterial translocation biomarkers in response to subclinical exertional-heat stress. The glutamine bolus was well-tolerated, with no adverse gastrointestinal symptoms responses reported for any participant. Neither small intestinal permeability (serum lactulose/rhamnose) or epithelial injury (plasma I-FABP) were improved with glutamine supplementation in response to subclinical exertional-heat stress. In fact, large- and small- effects were observed that showed glutamine worsened small intestinal permeability and epithelial injury in comparison to placebo supplementation. Therefore, unlike previous research on this topic supplementing with high-dose (0.9 g·kg·FFM^{-1}) acute oral glutamine, these findings show that when supplemented in lower doses, glutamine has no protective benefit on small intestinal permeability and epithelial injury during subclinical exertional-heat stress. Downstream gastrointestinal bacterial translocation assessed via *Bacteroides*/total 16S DNA responses increased following the EHST, however, there was no clear impact of glutamine supplementation. Given this response, it is speculatively recommended that the meaningfulness of heightened gastrointestinal barrier disturbance following glutamine supplementation would not translate into an increased risk of clinical complications during more severe exertional-heat stress (e.g. exertional-heat stroke). The peak thermoregulatory strain induced in the present study was sub-clinical (e.g.

peak T_{core} = 38-39.5°C), whereby it is recommended that future research should examine the efficacy of nutritional countermeasures in more physically demanding field-based circumstances. These findings do not support the use of acute low-dose oral glutamine supplementation to protect small intestinal permeability, epithelial injuryand prevent bacterial translocation in response to subclinical exertional-heat stress.

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Table 1. Participant demographic characteristics

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

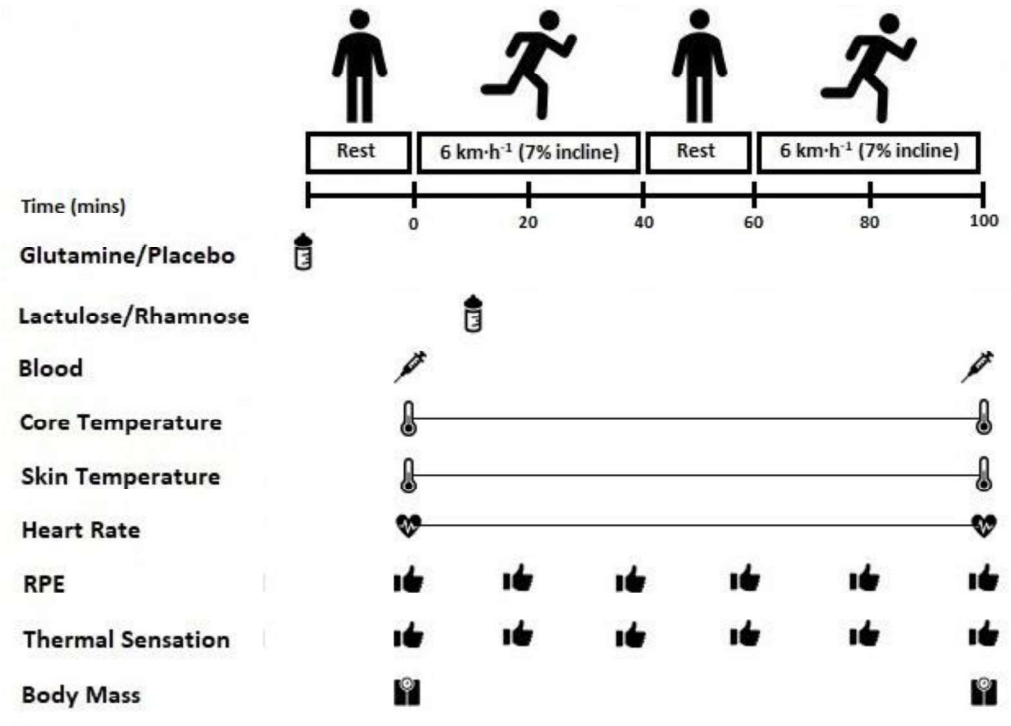
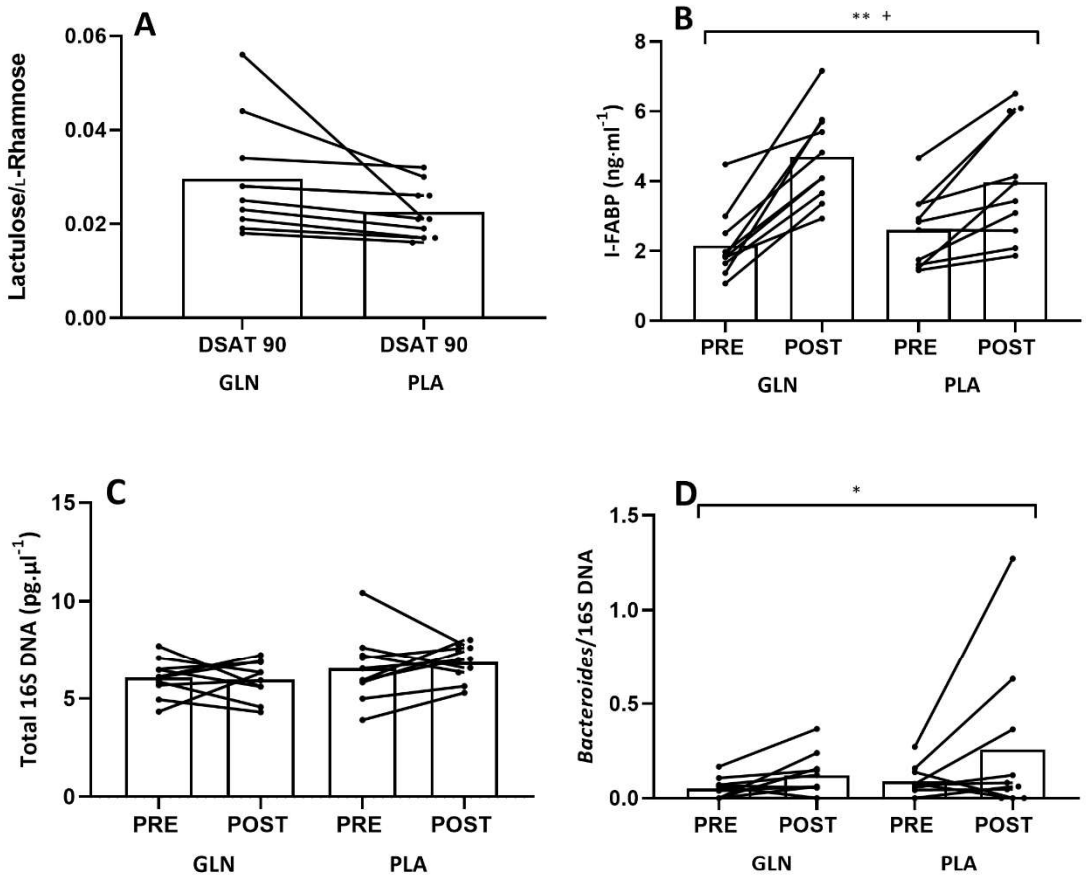


Figure 1. Schematic illustration of the experimental measurement timings



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828 **Figure 2.** Gastrointestinal biomarker responses to exertional-heat stress: (A) = L/R ratio at 90
829 minutes; (B) I-FABP; (C) = total 16S DNA; and (D) *Bacteroides*/total 16S DNA. Overall effect
830 of time (*$p \leq 0.05$; ** $p \leq 0.01$).

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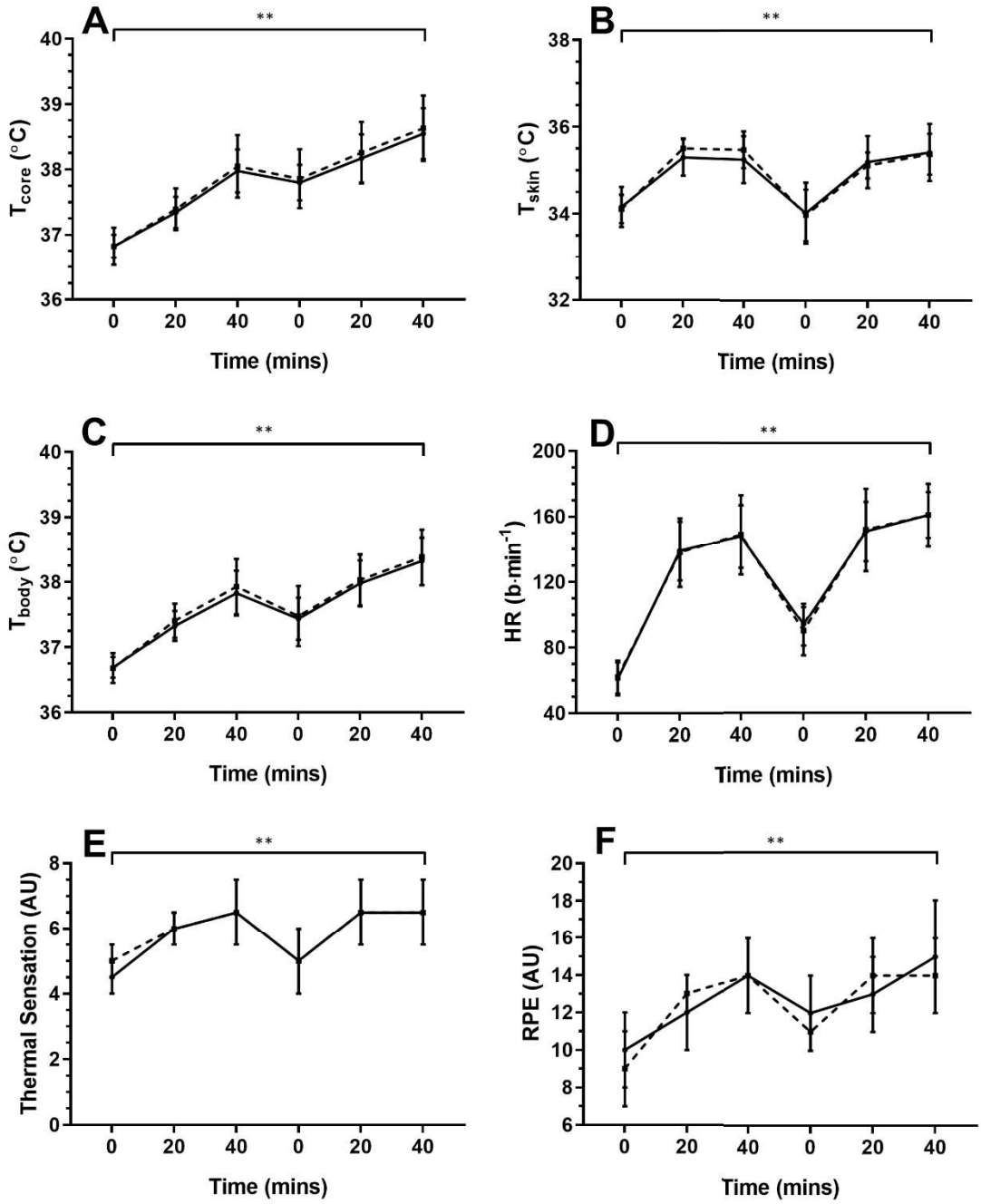

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833 **Figure 3.** Whole-body physiological responses to exertional-heat stress: (A) = core
834 temperature; (B) = mean skin temperature; (C) = mean body temperature; (D) = heart rate;
835 (E) = thermal sensation; and (F) = rate of perceived exertion. Solid line = glutamine, broken
836 line = placebo. Overall effect of time (*$p \leq 0.05$; ** $p \leq 0.01$).

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