Title: The effect of cycling in the heat on gastrointestinal-induced damage and

                              neuromuscular fatigue.

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Abstract

Purpose: This study investigated the effect of exercise in the heat on neuromuscular function, gastrointestinal damage, endotoxemia and inflammatory cytokines.

Methods: Eight male cyclists completed two 60 min cycling trials in both hot (HOT: 34.5 ± 0.1 °C and 53 ± 1% relative humidity) and temperate environments (CON: 20.2 ± 0.3 °C and 55 ± 3% relative humidity). The cycling task comprised of alternating 3 min intervals at a moderate-vigorous intensity (50% and 70% of maximum power output; P_{max}) for 30 min, followed by 30 min at moderate intensity (40-50% P_{max}). Neuromuscular function was assessed at pre-, post-exercise and 60 min post-exercise. Circulating levels of endotoxins, inflammatory cytokines and markers of gut permeability and damage were also collected at these time points. Heart rate, core temperature, skin temperature, perceived exertion, thermal sensation and comfort were also measured.

Results: Post-exercise voluntary activation of HOT (87.9% [85.2, 90.8]) was statistically lower (mean difference: -2.5% [-4.5, -0.5], $d = 2.50$) than that of CON (90.5% [87.8, 93.2]). The HOT trial resulted in statistically elevated (+69%) markers of gastrointestinal damage compared to CON (mean difference: 0.424 ng·mL^{-1} [0.163, 0.684, $d = -3.26$]), although this was not observed for endotoxin, other inflammatory markers, or gastrointestinal permeability.

Conclusions: This research provides evidence that short-duration cycling in the heat results in sub-optimal neuromuscular activation and increased expression of gastrointestinal damage markers, without a simultaneous elevation in circulating endotoxins or pro-inflammatory cytokines.

Keywords: thermoregulation, endotoxemia, cycling, central fatigue, hyperthermia.

Abbreviations

½ RT	half relaxation time
CD	contraction duration
CI	credible interval
CLDN-3	claudin 3
CNS	central nervous system
CV	coefficient of variation
ELISA	enzyme-linked immunosorbent assay
EMG	electromyography
HR	heart rate
ICC	intraclass correlation
I-FABP	intestinal fatty acid binding protein
IL-1β	interleukin 1 beta
MCMC	Markov chain Monte Carlo
MD	mean difference
MVC	maximum voluntary contraction
P_{max}	maximal aerobic power output
Pt	peak torque
RPE	Rating of Perceived Exertion
RR	rate of relaxation
RTD	rate of torque development
T_c	core temperature
TNF-α	tumour necrosis factor alpha
TPt	time to peak torque
T_{sk}	mean skin temperature
VA	voluntary activation
VL	vastus lateralis
VM	vastus medialis
VO_{2max}	maximal aerobic capacity

Introduction

Endurance exercise is markedly impaired by heat stress, with a reduction in athletic performance observed in both laboratory and real-world settings (Racinais et al. 2015; Tatterson et al. 2000). The aetiology of heat-related fatigue appears to be multi-factorial and dependent on a complex interplay of different factors including exercise intensity, hydration, health and fitness (Nybo et al. 2014). Exacerbated cardiovascular strain during exercise has traditionally been used to explain declines in exercise performance under heat stress (Nybo et al. 2014). However, this suggestion contrasts the reduced physical capacity observed with a high core temperature (T_c), irrespective of cardiac supply to exercising muscle (Cheung and Sleivert 2004; Nielsen et al. 1993). While there is an apparent inverse relationship between a rising T_c and attenuated CNS drive to the motor neurone pool (Cheung 2007), the proposed critically limiting T_c appears overly simplistic given the evidenced tolerance of >40.0 °C T_c without ill consequence (Byrne et al. 2006; Ely et al. 2009; Racinais et al. 2019). Accordingly, alternative mechanistic processes explaining evoked central fatigue experienced with exercise- and environment-induced hyperthermia remain to be identified (Nybo and González-Alonso 2015; Nybo et al. 2014).

Exercise in the heat results in a redistribution of blood flow from splanchnic regions, leading to death of mucosal epithelial cells that line the intestinal tract (Lambert 2008). Heat also directly effects the integrity of the gastrointestinal barrier and results in the opening of tight junctions (Lambert 2004; Marshall 1998; Moseley et al. 1994). As a consequence of this increased permeability, endotoxins translocate through the weakened intestinal barrier, causing the clinical condition, endotoxemia (Lambert 2008). Endotoxemia elicits a strong immune response and the resultant release of inflammatory signalling cytokines, including tumour necrosis factor alpha (TNF-α) (Lambert 2009). Increased circulating levels of these cytokines have been implicated in signalling the brain to produce symptoms of sickness, such as nausea or malaise (Dantzer 2004). Thus, Vargas and Marino (2014) argue that the production of similar cytokines during exercise may act as a neuro-modulator and result in a manifestation of transient perceptual fatigue, similar to the response that occurs during a disease state.

The exact relationship between this string of factors (i.e., exercise and heat stress, intestinal damage, endotoxin translocation, cytokine release, and the development of fatigue) is currently unknown. However, the neuroinflammatory model of fatigue may explain the decreased exercise performance when competing in a hot environment (Lambert 2008; Nybo et al. 2014; Vargas and Marino 2014). While elevated concentrations of endotoxins and inflammatory cytokines have been evidenced after prolonged exercise in hot conditions

(Bosenberg et al. 1988; Camus et al. 1997; Gill et al. 2015b), the main interest in exertional-endotoxemia and the resultant inflammation has concerned the aetiology of heat illness and injury (Lim and Mackinnon 2006). Instead, we propose that transient endotoxemia and the release of inflammatory cytokines during prolonged exercise in the heat could downregulate neural drive by directly, or indirectly, altering the electrophysiology of central neurons as has been previously demonstrated (Vezzani and Viviani 2015; Vitkovic et al. 2000). Vargas and Marino (2017) recently showed neuroinflammation to alter cortical activity and contribute to fatigue during a 60 min cycle protocol in 35 °C conditions. Unfortunately, the proposed preceding changes in gut permeability and endotoxin concentrations were not reported.

The present study aimed to examine the effect of exercise in the heat on neuromuscular function and exercise-induced endotoxemia. It was hypothesised that cycling in a hot environment would induce central fatigue, observable through reduced post-exercise voluntary activation. Further, exercise in the heat would increase the level of circulating endotoxins, inflammatory cytokines and markers of gastrointestinal permeability and damage, when compared to the control condition.

Methods

Participants

Eight trained male cyclists (age: 28 ± 5 years; height 182 ± 7 cm; body mass: 76 ± 10 kg; relative VO_{2max}: 58 ± 7 mL·kg^{-1}·min^{-1}; P_{max}: 394 ± 35 W), volunteered to participate in this study. All participants cycled at least twice per week and were classified as trained or well-trained athletes (performance level 3 or 4) (De Pauw et al. 2013). Participants were non-smokers, free of any injury or illnesses and reported no history of gastrointestinal issues or diseases. All participants were informed of all study requirements before they provided verbal and written consent. University Human Research Ethics Committee approval of this project was attained before the commencement of any testing.

Participants completed an initial familiarisation trial followed by two experimental trials. During the initial familiarisation trial, participants were extensively familiarised with all neuromuscular assessments and procedures that would be undertaken during subsequent experimental trials. Participants also completed a maximal aerobic capacity (VO_{2max}) test via expired gas analysis (TrueOne 2400; ParvoMedics, Salt Lake City, USA) on a cycle

ergometer (Excalibur Sport; Lode, Groningen, Netherlands) using a 25 $W \cdot min^{-1}$ step protocol. The power of the highest, completed stage was taken as the participant's maximal power output (P_{max}).

Exercise Protocol

Experimental trials consisted of a 60 min cycling task on a cycle ergometer (Excalibur Sport, Lode, Netherlands), comprised of 10 x 3 min intervals, alternating between 50% and 70% P_{max}, followed by 30 min at a self-selected power between 40-50% P_{max}. This exercise protocol was utilised as it rapidly elevated T_c (Δ 0.039 $°C \cdot min^{-1}$) when compared to a traditionally fixed load at 50% for 60 min (Δ 0.020 $°C \cdot min^{-1}$; unpublished observations). Similar duration cycling protocols have previously reported elevated markers of gut damage and permeability (van Wijck et al. 2011) and increased levels of inflammatory cytokines (Gray et al. 2009; Vargas and Marino 2017). While the exercise prtocol was identical, environmental conditions differed between the two trials, with one trial undertaken in the heat (HOT: 34.5 $\pm$ 0.1 °C and 53 $\pm$ 1% relative humidity) and the other in a temperate environment (CON: 20.2 $\pm$ 0.3 °C and 55 $\pm$ 3% relative humidity). The trials were completed in a counter-balanced and randomised order, matched for time of day ($\pm$ 2 h), and separated by $\geq$ 7 days. Participants abstained from caffeine and alcohol for 12 h, and strenuous exercise for 48 h, before each experimental trial. Physical activity, food and fluid intake were diarised for the 24 h before the first experimental trial and replicated for the subsequent trial.

Upon arrival for each experimental trial, participants provided urine and blood samples before undertaking a pre-exercise neuromuscular assessment. Participants were asked to nude weigh with a voided bladder, before being instrumented (i.e., rectal and skin thermistors, heart rate monitor) and dressing in cycling apparel (i.e., bib, socks and cycling shoes). Participants were seated for 5 min in a climate-controlled laboratory to provide baseline data before entering the climate chamber and undertaking the cycling task. Upon completion of the cycling task, participants immediately completed a neuromuscular assessment and provided a blood sample. A towel-dried post-exercise nude weigh was completed to allow for calculation of fluid loss. Participants then consumed 250 mL of water and rested for 60 min in a temperature laboratory environment before a final neuromuscular assessment and blood sample collection.

Physiological Measures

Heart rate (HR) values were recorded using a chest strap (Polar Electro Oy, Kempele Finland) and software (Polar Team², Kempele Finland). Core temperature was measured via a flexible rectal thermistor (449H; Henleys Medical, Hertfordshire, UK) inserted ~12 cm past the anal sphincter. Skin temperature was measured from conductive skin thermistors (EU-UU-VL5–0; Grant Instruments, Cambridge, UK) affixed to the skin (Leuko Sportstape Premium; Beiersdorf, Hamburg, Germany) at four separate sites: right shin, right scapula, posterior neck, and posterior left hand. Mean skin temperature (T_{sk}) was calculated according to the four-site formula published by the International Organization for Standardization (ISO 9886 2004). T_c and T_{sk} thermistors were connected to a data logger (Squirrel SQ2020; Grant Instruments, Cambridge, UK) and computer, which recorded every 5 min while cycling. Hydration status was assessed upon arrival using a mid-stream urine sample to measure urine specific gravity (PAL-10S; Atagi Ci. Ltd, Tokyo, Japan) and fluid loss was calculated via pre- to post-exercise nude body mass changes using a set of calibrated scales (WB-110AZ; Tanita Corp., Tokyo, Japan).

Perceptual measures

Borg's Rating of Perceived Exertion (RPE) scale (Borg 1970) was used to measure perceived exercise intensity. Thermal sensation was recorded using a 16 point scale (0 = 'unbearably cold' to 8 = 'unbearably hot') (Young et al. 1987), and thermal comfort was measured using a 4 point scale (1 = 'comfortable' to 4 = 'very uncomfortable') (Gagge et al. 1967). These perceptual measures were recorded at baseline and then every 5 min during the cycling task.

Neuromuscular function and voluntary activation

The neuromuscular function of the right knee extensors were assessed pre-, post- and 1 h post-exercise using a Biodex Systems 3 Dynamometer (Biodex Medical Systems, Shirley, New York, USA). Participants were secured in an upright position via straps across the chest, waist and right thigh. The lever fulcrum was aligned with the right lateral epicondyle and the right lower leg attached to the lever arm via a strap positioned 10 mm proximal to the lateral malleolus.

Assessment of voluntary activation (VA) was achieved via stimulation of the right femoral nerve using reusable self-adhesive gel electrodes (Pals; Axelgaard Manufacturing Co. Ltd., Fallbrook, CA). An anode electrode (3.2 cm diameter) was placed 3 cm distal to the inguinal ligament bordering the femoral triangle, and a cathode electrode (5 x 9 cm) was positioned on the medio-posterior aspect of the right upper thigh, on the border of the

gluteal fold. The current applied to the nerve was driven by a Digitimer DS7AH stimulator (Digitimer Ltd., Welwyn Garden City, Hertfordshire, England) using a single square-wave pulse with a 500 µs width. A resting twitch ramp was undertaken at the start of each experimental trial to determine the required current for maximal stimulation. The final current was then increased by an additional 20% to ensure supramaximal stimulation of the femoral nerve (Saboisky et al. 2003).

Participants performed a standardised warm-up and rested for two minutes before completing a set of 5 x 5 s maximum voluntary isometric knee extensor contractions (MVC) at 90° knee flexion, with a 30 s rest between each repetition. During each MVC, participants received strong verbal encouragement and exhortation as well as visual feedback of torque production. Upon observing a plateau in voluntary torque during each MVC, the primary investigator manually triggered a single femoral nerve stimulation. A further stimulation was also triggered following the completion of each MVC, providing evoked twitch torque properties (Shield and Zhou 2004). This neuromuscular assessment was repeated immediately post- and 1 h post-exercise.

VA was calculated using the twitch interpolation technique as previously described by Allen et al. (1995). Before calculating VA, each MVC torque trace was visually assessed to ensure it demonstrated a clear plateau in voluntary contraction torque. MVCs that failed this criterion were rejected and removed from the mean VA calculations. VA was assessed using the formula: VA (%) = (1 − interpolated twitch torque/resting control twitch torque) * 100. Peak voluntary isometric torque was taken as the mean torque value of the 25 ms preceding delivery of the electric stimulus. The peak torque value recorded in the 100 ms period following the stimulus was considered the superimposed torque value. These neuromuscular assessments of MVC torque and VA are reliable, with calculated ICCs of 0.94 and 0.94, respectively, in our laboratory.

Evoked twitch contractile properties

Evoked twitch properties were assessed from the resting twitch delivered following the completion of each MVC, as described previously (Cannon et al. 2008; Shield and Zhou 2004). Twitch data were averaged across the five repetitions for each time point and analysed for peak twitch torque (Pt; defined as the peak torque during the evoked twitch); time to peak torque (TPt; time from the first rise in torque above baseline to peak torque); half relaxation time (½ RT; time taken for torque to reduce by half of the peak torque value); contraction duration (CD; time to peak torque plus half relaxation time); rate of torque development (RTD; slope of twitch-torque

curve from onset to peak torque); rate of relaxation (RR; slope of twitch-torque curve peak torque to half relaxation time).

Surface electromyography (EMG)

Surface knee extensor EMG data were recorded from the vastus medialis (VM) and vastus lateralis (VL) of the right leg during MVC assessments. The 30 x 22 mm surface electrodes (Ambu Blue Sensor N-00-S; Ambu A/S, Ballerup, Denmark) were positioned over the visual mid-point of the respective muscle bellies, and an additional earth electrode was attached to the lateral femoral epicondyle. The electrodes were positioned with an inter-electrode distance of 20 mm and orientated parallel with the muscle fibres. All sites were shaved, abraded and cleaned before electrode placement. Electrodes remained attached to the participant during the length of the testing trial to ensure consistency in placement. Raw EMG data was sampled with dynamometer data at 1 kHz through a 16-bit PowerLab 26T AD unit (AD Instruments, Sydney, Australia) (amplification=1000; common mode rejection ratio=110 dB), bandpass filtered between 20-500 Hz and stored for later analysis. EMG data were quantified via the root-mean-square method with a triangular Bartlett sliding window of 100 ms (100 data points) using LabChart 8.0 software (AD Instruments, New South Wales, Australia). The mean EMG signal amplitude over the 500 ms period before stimulation was calculated and averaged across all repetitions for each time point (Cannon et al. 2007). Mean post- and 1 h post-exercise EMG amplitudes were then normalised to mean values obtained during pre-exercise MVC (Burden and Bartlett 1999). The 60 ms before each peak root-mean-square EMG value was excluded to remove the confounding effect of the stimulation artefact.

Biochemical markers and analysis

Pre-, post- and 1 h post-exercise samples were drawn from an antecubital venipuncture using a butterfly needle (21G, BD, North Ryde, Australia) and serum Vacutainer tubes (BD, North Ryde, Australia). After clotting at room temperature, samples were centrifuged at 3500 revolutions·min^{-1} for 10 min at 4 ˚C, pipetted into pyrogen-free microtubes, and frozen at -20 ˚C for a maximum of 8 months. Participants were seated upright in a phlebotomy chair for all venepunctures. Serum concentrations of circulating cytokines (i.e., TNF-α and interleukin 1 beta; IL-1β) were determined using quantitative sandwich enzyme-linked immunosorbent assays (ELISA) as per the manufacturer instructions (elisakit.com, Melbourne, Australia). Intra-assay precision was calculated for IL-1β (CV = 13.2%) and TNF-α (CV= 5.6%). Absorbance was read at 450 nm with a 570 nm wavelength subtraction and corrected for blank control wells (SpectroStar Nano; BMG Labtech, Germany).

Intestinal fatty acid binding protein (I-FABP) serum levels were quantified using a commercially available ELISA kit (CV= 7.0%; Human FABP2, RayBiotech, Norcross, GA, USA) and read at 450 nm with correction for blank control wells. Serum claudin 3 (CLDN-3) concentration was assessed with a commercially available ELISA kit (CV= 17.1%; Human Claudin-3, Cusabio, Wuhan, China). Absorbance was read at 450 nm with a 570 nm wavelength subtraction.

Serum endotoxin levels were detected using a commercially-available, quantitative kinetic chromogenic *Limulus* amebocyte lysate (LAL) assay kit (CV= 12.7%; Lonza, Walkersville, MD, USA). Samples were analysed according to the manufacturer's instructions. Briefly, the *E.* coli 055:B5 endotoxin standard (Lot Number: 0000547137) was reconstituted with reagent water to the volume stated on the supplied Certificate of Analysis and vigorously vortexed for 15 minutes to yield a 50 EU·mL^{-1} stock solution. Tenfold serial dilutions of this standard (5, 0.5, 0.05, 0.005 EU·mL^{-1}) were prepared in duplicate using the provided sterile water (Lonza, Walkersville, MD, USA). Each solution was vortexed for at least 1 min between dilutions and sterile tips (Biopur epTIPS; Eppendorf AG, Germany) were used for all pipetting to reduce carry-through contamination of the standards. Samples were thawed, diluted at 1:5 with sterile water (Lonza, Walkersville, MD, USA) in endotoxin-free microtubes (GoldenGate Bioscience, Claremont, CA, USA) and heated in a heat block for 15 min at 75 °C to inactivate endotoxin-neutralising agents. 100 μL of each sample, standard and negative controls were then pipetted into 96-well plate and incubated in a microplate reader (SpectroStar Nano; BMG Labtech, Germany) for 10 min at 37 °C before the 100 μL of LAL reagent was added to each well. The spectrophotometer immediately read the optical density of the plate at 405 nm and then every 61 s for 118 cycles. The reaction time in seconds for each well to increase absorbance 0.2 units above baseline was determined. A log/log (reaction time/concentration) linear correlation of each standard was calculated (r = -0.991). The slope and y-intercept from this formula were then used to calculate the unknown endotoxin concentration of the samples, corrected for the dilution factor.

Statistical analysis

Data were analysed using linear mixed modelling in a Bayesian framework (Mengersen et al. 2016). Exploratory data plots were inspected for normality before Markov chain Monte Carlo (MCMC) procedures were used to generate posterior predicted values, using the 'rjags' and 'R2jags' packages in the statistical software, R. Specifically, 50k iterations with an initial 1k burn-in were thinned by a factor of 10 to generate 5k posterior values.

Models utilised vague prior distributions (mean 0, precision 0.001) with a random intercept for each participant. Fixed model parameters included *time*, *condition*, and *time x condition* and the final model for each analysis was chosen following comparison of extracted deviance information criterion values.

Mean and 95% credible interval [CI] values were calculated for each posterior parameter, with a comparison between conditions or time points calculated as the mean difference (MD) and associated 95% CI. Evidence of statistical effect or difference was accepted when a 95% CI did not include 0. Cohen's *d* effect sizes were used to evaluate the magnitude of all statistically different comparisons and were categorised as small (0.2), moderate (0.5) and large (0.8) (Cohen 1988).

Results

Exercise protocol

Workload changes (i.e., a reduction from 50% P_{max} constant exercise load to 40% P_{max} between the 30-45 min) were replicated each participant during the subsequent experimental trial. No difference in the completed workload was observed between conditions.

Neuromuscular function

No statistical difference in pre-exercise MVC torque (MD: 3 N·m [-11, 18]) or VA (MD: 0.3% [-1.3, 1.9]) was observed between conditions (Table 1). Both conditions reported statistically lower post-exercise MVC torque than respective pre-exercise values (CON MD: -24 N·m [-38, -8], d = 3.09; HOT MD: -27 N·m [-43, -11], d = 1.44; Figure 1), although no difference in MVC torque was observed between conditions at this time point (MD: 7 N·m [-9, 23]). Post-exercise VA followed a similar pattern, with reductions compared to pre-exercise values (CON MD: -1.9% [-3.6, -0.1], d = 2.08; HOT MD: -4.8% [-6.7, -2.8], d = 4.82) observed for both conditions. Further, post-exercise HOT VA values were found to be statistically lower than CON (MD: -2.5% [-4.5, -0.5]; d = 2.50). There was some evidence of a statistical condition difference in MVC torque 1 h post-exercise, with HOT impairing MVC torque values compared to CON (MD: -19 N·m [-35, -3]; d = 2.42), and both conditions were decreased compared to pre-exercise values (MD: -35 to -19 N·m [-51, -4], d = 2.46 – 4.47). There was some evidence that VA remained impaired in HOT compared to CON 1 h post-exercise (MD: -1.7% [-3.5, 0.1], d = 1.92), and was also lower than within-condition pre-exercise values (MD: -3.5% [-5.3, -1.6], d = 3.75).

Evoked twitch contractile properties and EMG

Cycling for 60 min, regardless of environmental condition, resulted in a statistical reduction in Pt torque compared to pre-exercise values (MD: -12 to -12 N·m [-19, -6], d = 3.42 – 3.80; Table 1), although this difference disappeared 1 h post-exercise for both conditions (MD: -5 to -2 N·m [-11, 4]). There was no evidence of a statistical condition difference at any time point for Pt. Cycling in HOT resulted in statistically lower post-exercise ½ RT and CD values compared to baseline (d = 2.01 – 2.75), although no other time, condition or interaction effects were observed (Table 1). There was no evidence of any time, condition or time x condition interaction factor effects for TPt, RTD or RR (Table 1).

Relative %EMG output for post-exercise VL and VM was statistically reduced compared to baseline values, regardless of condition ($d = 1.92 - 4.55$, Table 1). Further, VM EMG remained impaired 1 h post-exercise for both conditions (MD: -13 to -12 [-24, 0], $d = 2.06 - 2.31$). While no evidence of a condition difference was observed for VL at any time point, post-exercise VM EMG values for HOT were found to be statistically lower than CON (MD: -11% [-23, 0]; $d = 1.95$).
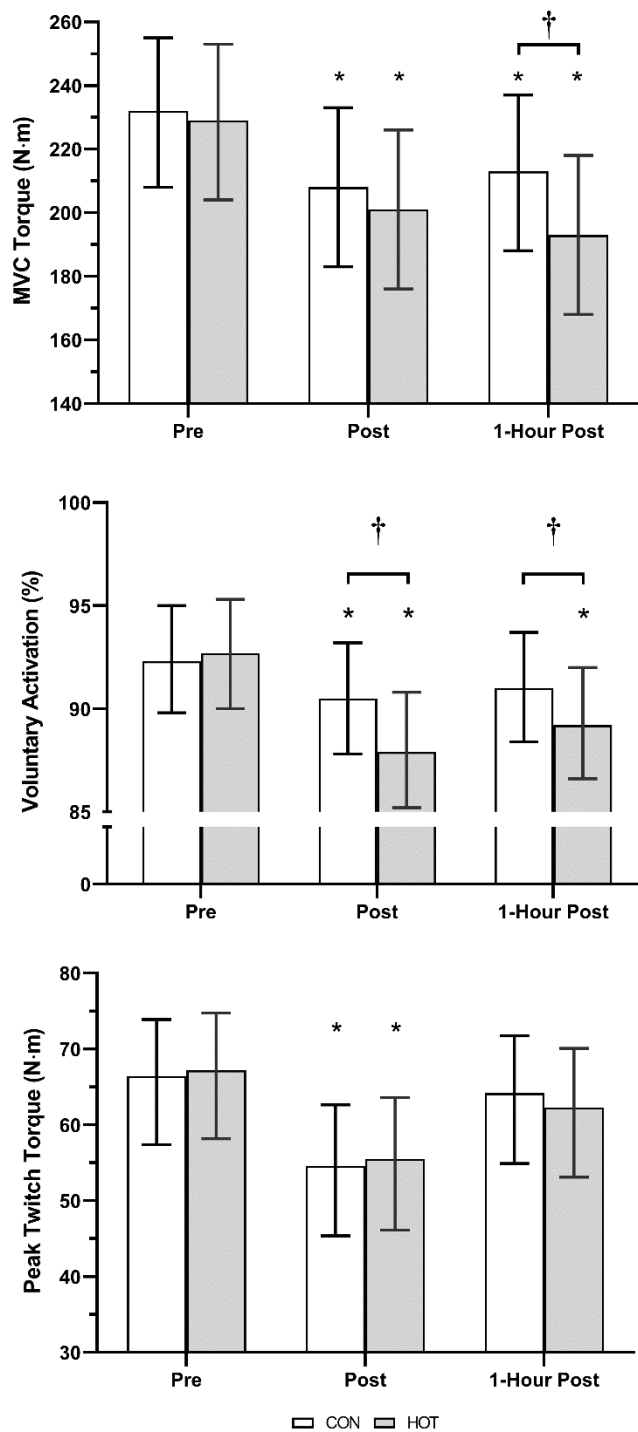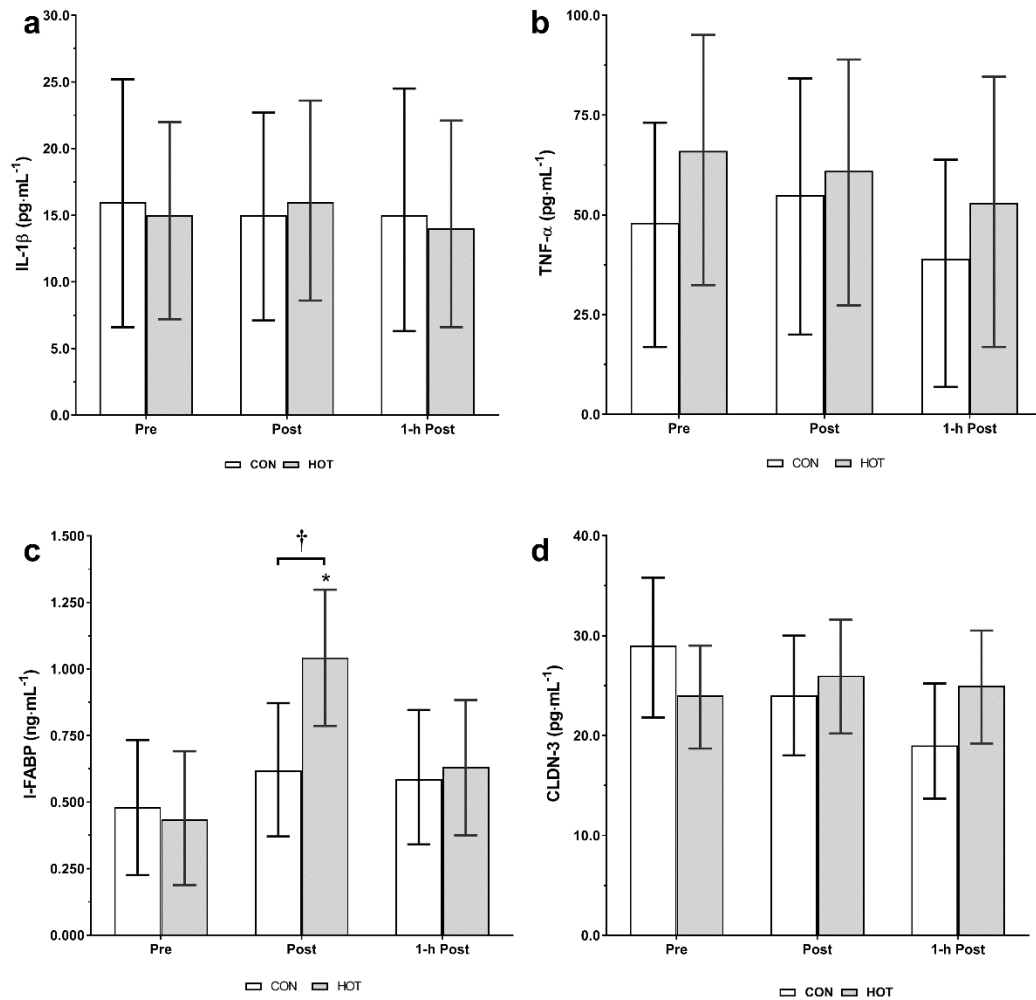
Figure 1. Posterior predicted mean [95% CI] for MVC torque (N·m), VA (%) and Pt torque (N·m) at pre, post and 1 h post-exercise in HOT and CON. * indicates a statistical time difference compared to pre-exercise values within a condition; † indicates a statistical condition difference at a respective time point.

Biochemical markers and analysis

A rise in markers of gastrointestinal damage markers (I-FABP) was observed in HOT, with statistically higher (+140% mean) post-exercise values compared to baseline (MD: 0.608 ng·mL^{-1} [0.345, 0.861], d = -4.67; Table 2). Evidence of a condition difference at this time point was also observed, with post-exercise HOT reporting statistically higher (+69% mean) levels of I-FABP than CON (MD: 0.424 ng·mL^{-1} [0.163, 0.684]; d = 3.26), although this difference disappeared by 1 h post-exercise (Figure 2). In contrast, the exercise in CON was not found to alter circulating I-FABP levels above pre-exercise levels (Table 2). No parameter (i.e., *time*, *condition or interactions*) was statistically different for either inflammatory cytokine (IL-1β and TNF-α), intestinal permeability (CLDN-3) or endotoxin.

Figure 2. Posterior predicted mean [95% CI] serum concentrations of inflammatory cytokines (IL-1β and TNF-α), markers of intestinal damage (I-FABP) and permeability (CLDN-3) at pre, post and 1 h post-exercise in HOT and CON. * indicates a statistical time difference compared to pre-exercise values within a condition; † indicates a statistical condition difference at a respective time point

Physiological and perceptual measures

Evidence of *time*, *condition* and *time x condition* interaction effects were observed for all physiological (HR, T_c and T_{sk}) and perceptual (RPE, thermal sensation and thermal comfort) measures (Table 3). While both HR and T_c were similar at baseline, a statistical difference between conditions was observed from 5 min and 10 min, respectively, and this condition difference persisted for the remainder of the exercise task. Statistically higher T_{sk}, RPE, thermal sensation and thermal comfort values were observed for every time point in HOT, when compared to CON. Analysis of pre-exercise USG ($\beta_{CONDITION}$: 0.005 [-0.032, 0.040]) and body mass ($\beta_{CONDITION}$: 0.30 [-0.27,

0.88]) revealed no statistical differences between conditions at baseline. Body mass loss was statistically greater following HOT, compared to CON (MD: -0.66 kg [-0.86, 0.44], d = -6.28).

Discussion

This study investigated the effect of exercise in the heat on neuromuscular function, exertional-endotoxemia and inflammation. It was hypothesised that the development of mild endotoxemia and the subsequent transient release of inflammatory cytokines could potentially downregulate neural drive, and therefore voluntary torque output, during exercise in the heat (Gill et al. 2015a; Lambert 2008; Vargas and Marino 2014). However, 60 min of cycling was found to attenuate MVC torque, regardless of the environmental condition, equally. While central fatigue was observed following exercise in both conditions, HOT resulted in a greater impairment in post-exercise VA, compared to CON (d = 2.50; Figure 1). Peripheral fatigue was similar between conditions, despite a faster half-relaxation time and shorter contraction duration in the heated muscles compared to baseline. Intestinal damage was observed to be statistically higher following exercise in HOT than CON (d = -3.26). In contrast to the study hypothesis and the reported intestinal damage, there was no evidence of a statistical difference for either inflammatory cytokine (Table 2). Nevertheless, these findings align with the observed absence of endotoxin translocation, which was postulated as the initial driving mediator of the pro-inflammatory cytokine response. In summary, the findings from this study suggest that reductions in MVC torque following cycling in the heat appear to principally stem from diminished CNS drive to the musculature with partial contribution from peripheral fatigue; i.e., distal to the neuromuscular junction.

Périard and colleagues (2011) reported similar reductions in voluntary force production to that seen here (Figure 1) following a 40 km cycling time trial in hot and cool temperatures. The authors noted that exercise in the heat resulted in only a small decrease in VA, but considerable peripheral fatigue, highlighting the failure of the muscular contractile properties, in particular, a shorter half-relaxation time. The present study observed a similar finding for peripheral fatigue, with evidence of a statistical reduction in post-exercise peak twitch torque for both conditions, despite alterations in HOT muscle properties (i.e., faster ½RT and CD; Table 1). One possible explanation may be that temperature-induced changes in muscle contractile properties could be briefly surmounted by a transient increase in motoneurone discharge rates to preserve fusion (Todd et al. 2005). We speculate that a brief upregulation in motor unit firing during the short-duration of an MVC (5 s) in the current study could potentially explain the similar level of voluntary torque production between conditions.

The termination of fixed-pace exercise at a specific critical T_c or the anticipatory reduction in power output to avoid reaching this critical threshold has been suggested in multiple studies (Nielsen et al. 1993; Tucker et al. 2004). However, Nybo and González-Alonso (2015) recently argued that a causative association between elevated T_c and fatigue may be overly simplistic, as studies have reported some athletes continuing to exercise without adverse performance or health complications, despite a T_c that exceeds the 'cut-off' of 40°C (Byrne et al. 2006; Ely et al. 2009). While it should be recognised that elevated T_c may be a driving factor behind exercise cessation and the development of fatigue in certain situations, alternate mechanisms and process that may induce fatigue during exercise in the heat should also be considered. Therefore, by building upon work by Lambert (2008) and Marshall (1998), we hypothesised that the development of exertional endotoxemia and subsequent release of pro-inflammatory cytokines could potentially down-regulate neural drive to the skeletal muscle.

Prolonged exercise in the heat has been shown to place considerable strain on the circulatory system and sufficiently impair blood flow to the gut to cause hypoxia of the epithelial tissue (Lambert 2009). The subsequent cell death compromises the integrity of the intestinal wall and permits translocation of endotoxins into the circulation, mediating an immune response and the production of inflammatory cytokines (Jeukendrup et al. 2000; Pals et al. 1997). Inflammatory cytokines such as IL-1β and TNF- α, have been linked with the manifestation of transient fatigue and effort-related motivation (Vargas and Marino 2014). The exact mechanism(s) by which inflammatory cytokines may produce these symptoms is currently equivocal. However, it has been theorised that peripherally-produced cytokines could influence afferent feedback via neuronal modulation of the vagus nerve or by signalling communication cells located in the circumventricular organs of the brain (Dantzer 2004; Vezzani and Viviani 2015). The blood-brain barrier normally blocks the translocation of peripherally-produced inflammatory cytokines. However, saturable transport systems (Dantzer 2004) or a rise in hyperthermia-induced barrier permeability (Sharma and Hoopes 2003) may permit cytokines to cross into the brain and directly regulate neuronal output (Vezzani and Viviani 2015; Vitkovic et al. 2000). While the role of pro-inflammatory cytokines on brain neural output is complex and multifaceted, elevated levels of these molecules following exercise, or in patients with neuro-immune diseases (Morris et al. 2016), have been evidenced to underpin mental fatigue and the sensations of illness (Ament and Verkerke 2009; Robson-Ansley et al. 2004).

Although intestinal blood flow was not directly measured, the cycling protocol resulted in considerable cardiovascular and thermal strain (Table 3). In contrast to previous research, intestinal permeability was unchanged between conditions and time points (van Wijck et al. 2011). One possible explanation for this unexpected result may be the use of serum biomarker CLDN-3 to assess intestinal permeability, as opposed to the more commonly utilised technique of ingestible sugar probes (Marchbank et al. 2011; Pals et al. 1997) that requires prolonged post-trial urine collection (i.e. 4-5 h post-exercise). While claudin-3 expression has been proposed as a non-invasive method of assessing intestinal barrier dysfunction (Grootjans et al. 2010; Thuijls et al. 2010), only one previous exercise science study has utilised this marker (Yeh et al. 2013), with the authors reporting considerably higher expression of CLDN-3 (i.e., 6500-8500 pg·mL^{-1}) than observed in the present study (19-29 pg·mL^{-1}). The exact cause of this difference is difficult to ascertain, particularly as clinical research utilising cardiac surgery patients has previously reported urinary CLDN-3 levels in the range of 20-170 pg·mL^{-1} (Habes et al. 2017).

Exercise in HOT was found to result in increased (+140% mean) serum levels of I-FABP, a marker of gastrointestinal damage, indicating that the intestinal barrier was compromised. This observation was similar to the gastrointestinal damage recently reported (March et al. 2017; Pugh et al. 2017), despite differing environmental conditions and exercise protocols (i.e., running) compared to the present study. While running has been linked with increased mechanical tearing of tight junctions within the intestinal tract (Lim and Mackinnon 2006; van Nieuwenhoven et al. 2004), the observed increase in I-FABP (Figure 2) during the present study suggests that sufficient thermal and physiological strain was experienced to induce intestinal damage in-line with previous literature (March et al. 2017; Pugh et al. 2017), despite the use of cycling.

Despite this rise in intestinal damage following exercise in the heat, endotoxin concentrations were not observed to increase for either condition statistically, or reach a level classified as mild endotoxemia (>5 pg·mL^{-1}, corresponding to approximately 0.05-0.10 EU·mL^{-1}). Similarly, no evidence supported a statistical effect of time, condition or interaction for any of the inflammatory cytokines. This may have been due to the comparatively short exercise duration (60 min) or modality (cycling) which are in contrast to the ultra-endurance events (Gill et al. 2015a; Ng et al. 2008) or treadmill protocols (Shing et al. 2014; Yeh et al. 2013) utilised in previous exertional-endotoxemia research. Alternatively, the variable half-life, detection and expression of endotoxins and cytokines in the blood may not have aligned with the venepuncture collection time points. Further, Gnauck, Lentle and

Kruger (2016) identified multiple confounding issues which can influence the quantification of endotoxin, including the vacutainer material, false positives from β-glucan contamination, and neutralisation by inhibitory components of a blood sample. Dilution (1:5) and heating of samples (75 °C for 15 min) was utilised in the current study to reduce the sequestration of endotoxins by binding proteins in serum. However, a spike recovery of only 23-26% indicated continued inhibition of the samples. Thus, we suggest that the low level of endotoxins found here (undetectable to 0.011 EU·mL^{-1}) may not accurately reflect the true endotoxin response during cycling in the heat.

Conclusion

The present study proposed that exercise in the heat, and the resultant translocation of endotoxins into systemic circulation, could potentially alter motivation and drive the development of central fatigue via the release of pro-inflammatory cytokines. In alignment with the study hypothesis, strenuous exercise in hot environmental conditions resulted in increased intestinal damage. However, the low endotoxin concentration and attenuated inflammatory cytokine response in HOT was an unexpected finding and in contrast to previous research (Jeukendrup et al. 2000). Exercise in the heat was also observed to reduce knee extensor torque and VA, indicating the development of central fatigue, when compared to a temperate condition, which aligns with the findings of Nybo and Nielsen (2001). Interestingly, this attenuation in central drive occurred during brief MVCs, as opposed to the sustained contractions utilised in other studies (Périard et al. 2011; Todd et al. 2005). How impairments in maximal isometric contraction force translate to submaximal, dynamic exercise performance remains to be elucidated. Future investigations should reassess the possibility of an association between transient exertional-endotoxemia and hyperthermia-induced fatigue, with a view to implementing prophylactic strategies that may preserve neural drive and potentially resulted in improved exercise performance.

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Table 1.

Posterior predicted mean [95% credible interval] for pre-, post and 1-h post-exercise for neuromuscular variables in HOT or CON.

Variable	Pre-Exercise		Post-Exercise		1 Hour Post-Exercise	
	CON	HOT	CON	HOT	CON	HOT
MVC Torque (N·m)	232 [208, 255]	229 [204, 253];	208 [183, 233]*	201 [176, 226]*;	213 [188, 237]*	193 [168, 218]*†;
		d: 0.40 [-1.53, 2.39]		d: 0.85 [-1.15, 2.83]		d: 2.42 [0.42, 4.41]
VA (%)	92.3 [89.8, 95.0]	92.7 [90.0, 95.3];	90.5 [87.8, 93.2]*	87.9 [85.2, 90.8]*†;	91.0 [88.4, 93.7]	89.2 [86.6, 92.0]*†;
		d: -0.41 [-2.36, 1.54]		d: 2.50 [0.48, 4.45]		d: 1.92 [-0.09, 3.87]
EMG VL (%)	-	-	81 [71, 92]*	86 [75, 97]*;	94 [83, 100]	84 [73, 95];
				d: -0.64 [-2.58, 1.31]		d: 1.41 [-0.56, 3.36]
EMG VM (%)	-	-	86 [78, 95]*	75 [66, 83]*†;	88 [79, 96]*	87 [78, 96]*;
				d: 1.95 [0.00, 3.94]		d: 0.03 [-1.97, 1.97]
Pt (N·m)	66 [57, 74]	67 [58, 75];	55 [45, 63]*	55 [46, 64]*;	64 [55, 72]	62 [53, 70];
		d:-0.26 [-2.24, 1.72]		d: -0.26 [-2.22, -1.74]		d: -0.65 [-1.35, 2.59]
TPT (ms)	65 [59, 72]	62 [55, 69];	65 [58, 71]	57 [50, 64];	64 [57, 71]	57 [51, 64];
		d: 1.14 [-0.85, 3.31]		d: 2.53 [0.55, 4.51]		d: 2.18 [0.25, 4.15]
1/2 RT (ms)	52 [41, 63]	50 [39, 61];	42 [31, 52]	36 [25, 47]*;	61 [50, 72]	55 [44, 66];
		d: 0.32 [-1.67, 2.28]		d: 0.83 [-1.17, 2.77]		d: 0.83 [-1.19, 2.75]

CD (ms)	113 [96, 128]	109 [92, 124]; d: 0.67 [-1.28, 2.58]	103 [86, 119]	90 [72, 105]*; d: 1.95 [-0.01, 3.93]	122 [105, 137]	108 [90, 123]; d: 2.03 [0.01, 3.96]
RTD (N·m·s^{-1})	961 [914, 1010]	979 [920, 1041]; d: -0.72 [-2.64, 1.26]	939 [846, 1004]	967 [887, 1047]; d: -0.79 [-2.75, 1.24]	974 [910, 1038]	991 [916, 1069]; d: -0.52 [-2.44, 1.44]
RR (N·m·s^{-1})	704 [654, 756]	712 [644, 782]; d: -0.28 [-2.25, 1.74]	705 [632, 778]	719 [622, 819]; d: -0.36 [-2.31, 1.58]	688 [616, 759]	690 [592, 790]; d: -0.06 [-2.01, 1.93]

* indicates a statistical time difference compared to pre-exercise values in the same condition; † indicates a statistical condition difference at the same time point. Cohen's d effect size [95% credible interval] is presented for condition parameter comparisons.

MVC: maximum voluntary contraction; VA: voluntary activation; EMG: electromyography; VL: vastus lateralis; VM: vastus medialis; Pt; peak twitch torque; TPt; time to peak torque; ½ RT: half relaxation time; CD: contraction duration; RTD: rate of torque development; RR: rate of relaxation.

Table 2.

Posterior predicted mean [95% credible interval] for pre-, post and 1-h post-exercise for blood markers in HOT or CON.

Variable	Pre-Exercise		Post-Exercise		1 Hour Post-Exercise	
	CON	HOT	CON	HOT	CON	CON
Endotoxin (EU·mL^{-1})	0.009 [n.d., 0.042]	0.009 [n.d., 0.043]; d: -0.07 [-1.99, 1.91]	0.010 [n.d., 0.045]	0.011 [n.d., 0.046]; d: -0.12 [-2.07, 1.90]	0.011 [n.d., 0.045]	0.010 [n.d., 0.044]; d: 0.08 [-1.86, 2.04]
IL-1β (pg·mL^{-1})	14.6 [7.2, 22]	15.9 [6.6, 25.2]; d: -0.25 [-2.23, 1.65]	16.2 [8.6, 23.6]	14.8 [7.1, 22.7]; d: 0.26 [-1.71, 2.27]	14.3 [6.6, 22.1]	15.5 [6.3, 24.5]; d: -0.20 [-2.09, 1.82]
TNF-α (pg·mL^{-1})	48.2 [16.9, 73.1]	66.2 [32.4, 95.1]; d: -1.61 [-3.56, 0.32]	55.4 [20.0, 84.2]	61.4 [27.3, 88.9]; d: -0.50 [-2.53, 1.47]	38.9 [6.9, 63.8]	53.4 [16.9, 84.6]; d: -1.05 [-3.03, 0.92]
I-FABP (ng·mL^{-1})	0.481 [0.226, 0.733]	0.435 [0.188, 0.691]; d: 0.35 [-1.60, 2.35]	0.619 [0.371, 0.872]	1.043 [0.786, 1.298]*†; d: -3.26 [-5.26, -1.26]	0.587 [0.341, 0.846]	0.633 [0.375, 0.883]; d: -0.34 [-2.31, 1.58]
CLDN-3 (pg·mL^{-1})	24 [19, 29]	29 [22, 36]; d: -1.26 [-3.21, 0.74]	26 [20, 32]	24 [18, 30]; d: 0.57 [-1.38, 2.53]	25[19, 30]	19 [14, 25]; d: 1.52 [-0.40, 3.50]

* indicates a statistical time difference compared to pre-exercise values in the same condition; † indicates a statistical condition difference at the same time point.
Cohen's d effect size [95% credible interval] is presented for condition parameter comparisons.

EU: endotoxin units; n.d.: not detected; IL-1β: interleukin 1 beta; TNF-α: tumour necrosis factor alpha; I-FABP: intestinal fatty acid binding protein; CLDN-3: claudin 3.

Table 3.

Posterior predicted values [95% credible interval] for physiological and perceptual measures during exercise.

Variable	CON	HOT
Mean HR (beats·min^{-1})	139 [132, 145]	159 [152, 165]
Peak HR (beats·min^{-1})	165 [157, 172]	182 [174, 188]
Mean T_c (°C)	38.2 [38.0, 38.3]	38.6 [38.5, 38.8]
Peak T_c (°C)	38.5 [38.4, 38.7]	39.5 [39.3, 39.7]
Mean T_{sk} (°C)	31.1 [30.9, 31.4]	35.5 [35.3, 35.7]
Peak T_{sk} (°C)	32.0 [31.7, 32.3]	35.9 [35.7, 35.2]
Mean RPE (AU)	12 [12, 13]	16 [16, 17]
Mean Thermal sensation (AU)	4 [4, 5]	7 [6, 7]
Mean Thermal comfort (AU)	2 [1, 2]	3 [3, 3]

HR: heart rate; T_c : core temperature; T_{sk}: skin temperature; RPE: rating of perceived exertion; AU = arbitrary units.