

Circulatory endotoxin concentration and cytokine profile in response to exertional-heat stress during a multi-stage ultra-marathon competition

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ABSTRACT

Exertional-heat stress has the potential to disturb intestinal integrity, leading to enhanced permeability of enteric pathogenic micro-organisms and associated clinical manifestations. The study aimed to determine the circulatory endotoxin concentration and cytokine profile of ultra-endurance runners (UER, $n=19$) and a control group (CON, $n=12$) during a five stage 230km ultra-marathon (mean \pm SD: 27h38min \pm 3h55min) conducted in hot and dry environmental conditions (30°C to 40°C and 31% to 40% relative humidity). Body mass and tympanic temperature were measured, and venous blood samples were taken before (pre-stage) and immediately after (post-stage) each stage of the ultra-marathon for the analysis of gram-negative bacterial endotoxin, C-reactive protein, cytokine profile (IL-6, IL-1 β , TNF- α , IFN- γ , IL-10, and IL-1ra), and plasma osmolality. Gastrointestinal symptoms and perceptive thermal tolerance rating were also monitored throughout competition. Mean exercise-induced body mass loss over the five stages ranged 1.0% to 2.5%. Pre- and post-stage plasma osmolality in UER ranged 277 to 282mOsmol/kg and 286 to 297 mOsmol/kg, respectively. Pre-stage concentrations of endotoxin (peak: 21% at Stage 5), C-reactive protein (889% at Stage 3), IL-6 (152% at Stage 2), IL-1 β (95% at Stage 5), TNF- α (168% at Stage 5), IFN- γ (102% at Stage 5), IL-10 (1271% at Stage 3), and IL-1ra (106% at Stage 5) increased as the ultra-marathon progressed in UER; while no changes in CON were observed (except for IL-1 β , 71% at Stage 5). Pre- to post-stage increases were observed for endotoxin (peak: 22% at Stage 3), C-reactive protein (25% at Stage 1), IL-6 (238% at Stage 1), IL-1 β (64% at Stage 1), TNF- α (101% at Stage 1), IFN- γ (39% at Stage 1), IL-10

(1100% at Stage 1), and IL-1ra (207% at Stage 1) concentrations in UER. Multi-stage ultra-marathon competition in the heat resulted in a modest circulatory endotoxaemia accompanied by a pronounced pro-inflammatory cytokinaemia by post-Stage 1, both of which were sustained throughout competition at rest (pre-stage) and after stage completion. Compensatory anti-inflammatory responses and other external factors (i.e., training status, cooling strategies, heat acclimatization, nutrition and hydration) may have contributed towards limiting the extent of pro-inflammatory responses in the current scenario.

Keywords: endurance, running, heat, inflammation, gastrointestinal.

INTRODUCTION

The epithelial lining along the gastrointestinal tract acts as a protective barrier between the internal and external environment, playing a significant role in preventing the penetration of enteric pathogenic microorganisms into portal and systemic circulation (23). Prolonged physical exertion, particularly running exercise, appears to impact upon intestinal epithelial integrity through redistributing blood flow to the working muscles and peripheral circulation (i.e., aiding thermoregulation), inevitably leading to splanchnic hypoperfusion and hypoxia (53, 58). Additionally, alterations to intestinal motility and mechanical trauma (i.e., repetitive jarring associated with running) can further promote intestinal mucosa and epithelial damage and (or) dysfunction (41).

When acute bouts of prolonged strenuous exercise are performed in hot (>30°C) environmental conditions, enhanced thermoregulatory strain, increased body water losses and accompanying hypovolaemia are commonly observed (62), and have the potential to further promote splanchnic hypoperfusion and disruption to intestinal epithelial integrity (24, 57). Such perturbations have been linked to increased intestinal permeability of localised gram-negative bacteria (e.g., terminal ileum colonized lipopolysaccharide), primarily due to deterioration of the protective mucosal lining and widening of

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epithelial tight junction spaces. Subsequently, this leads to endotoxaemia and a responsive cytokinaemia (5, 6). Endotoxin induced cytokinaemia has previously been implicated in the aetiology of heat stroke and septic shock (26, 34); whereas its potential link to autoimmune disease, gastrointestinal disease, and chronic fatigue in high risk individuals (i.e., genotype predisposition) is of current research interest (7, 29, 43). In extreme cases, where by systemic endotoxin proliferation is evident, over-exaggerated immune activation (i.e., innate immune cell proliferation and function, and cytokine responses) and increased pro-coagulant factors results in tissue hypoperfusion, intravascular coagulation, endothelial injury, and the end-point being refractory shock (19).

It is well established that moderate levels of exercise elicit favourable changes in cytokine profile; such as suppression of low-grade inflammation and enhanced anti-inflammatory cytokine responses (37, 59). Indeed, the classical cytokine response to moderate exercise in thermoneutral conditions (body temperature increase $<1^{\circ}\text{C}$) results in raised circulatory interleukin (IL)-1 receptor antagonist (ra), IL-10, and muscle derived IL-6 concentrations; while pro-inflammatory cytokine IL-1 β and tumour necrosis factor (TNF)- α responses are generally minimal (59). For example, 2 hours of running at 75% of maximum oxygen uptake ($\text{VO}_{2\text{max}}$) in 20°C ambient temperature resulted in an approximate 150%, 570%, and 1490% increase in IL-1ra, IL-10, and IL-6, respectively; while no changes in IL-1 β and TNF- α were observed (11).

On the contrary, excessive strenuous exercise (e.g., long distance running), especially in hot ambient conditions (body temperature increase $>1^{\circ}\text{C}$), results in enhanced enteric endotoxin translocation and a cytokine-mediated systemic inflammatory response similar to the cytokine profile of an acute infectious episode (e.g., sepsis, trauma, fever) (3, 15, 22, 47). Previous laboratory controlled studies have observed elevated pro- (e.g., TNF- α) and anti-inflammatory (e.g., IL-10) cytokine production after prolonged exercise in hot (32°C to 40°C) compared with thermoneutral (15°C to 22°C) environmental conditions (9, 42, 47, 49) indicating ambient temperature plays a crucial role in the degree of cytokinaemia observed after physical exertion. Such systemic endotoxin and cytokine responses have also been associated with symptomatic manifestations of gastrointestinal symptoms; a commonly observed feature in individuals exposed to prolonged periods of exertional-heat stress (22, 23, 33, 36, 56). Taking into account that previous research has predominantly focused on single bouts of exertional-heat stress, to date, it is still unclear the extent to which consecutive days of exertional-heat stress may impact on circulatory endotoxin and cytokine responses along the duration of exposure. Moreover, despite differences in sex and training status influencing such responses to acute exertional-heat stress through hormonal and adaptive factors (3, 47, 59, 61), it is unknown whether these sub-groups respond differently to consecutive repetitive exposure.

During exertional-heat stress, endotoxin induced systemic cytokinaemia appears to be a key feature in the aetiology of exertional-heat illnesses (i.e., exertional-heat stroke) (26) with fatalities being acknowledged as resulting from systemic inflammatory response syndrome (SIRS), a condition known as a whole body inflammatory state (39). For example, fatal incidence of heat stroke in military personnel during infantry training in hot ambient conditions were reported to be due to

septic shock, in which SIRS was a key feature (32, 39). From a practical perspective, given the substantial growth of ultra-endurance sports worldwide over the past decade and the environmental extremes of these events, competing in multi-stage ultra-endurance competition exposes ultra-endurance athletes to consecutive days of exertional-heat stress. This population may thus be predisposed to sub-clinical (e.g., gastrointestinal symptoms) and clinical (e.g., exertional-heat illnesses, sepsis, autoimmune diseases, gastrointestinal diseases, chronic fatigue) manifestations potentially originating from intestinal mucosa and epithelial damage and dysfunction. Indeed, mild circulatory endotoxaemia, cytokinaemia, and gastrointestinal symptoms have been reported after marathon (6) and Ironman triathlon (22) events, which were also associated with decrements in overall performance (38).

To date, exercise immunology research in ultra-endurance sports is limited (30), with no research exploring and tracking intestinal permeability of endotoxins and cytokine profile during multi-stage ultra-marathon. Besides the consecutive days of exertional-heat stress, such events are also accompanied by additional stressors that have previously been acknowledged as predisposing factors in the aetiology of fatal incidence of heat stroke and SIRS (2, 32, 39, 60). These include inadequate recovery opportunities, sleep deprivation, and acute periods of compromised hydration and (or) nutritional status (12, 13). Moreover, the predominant characteristics of ultra-endurance runners generally observed (e.g., recreationally active population, not acclimatised to environmental conditions, training status suboptimal for degree of physical exertion required, high body fat, high motivation, and situation of compromised immune status) are also reported to be aetiological predisposing factors (60).

The aims of the current study were to: 1) determine circulatory endotoxin concentration and cytokine profile of ultra-endurance runners throughout a five days (five stages) multi-stage ultra-marathon competition conducted in hot and dry environmental conditions; 2) determine the relationship between these responses with gastrointestinal symptoms and perceptive thermal tolerance rating; and 3) determine if sex and training status influence responses. Taking into account the consecutive days of exertional-heat stress, limited recovery time in-between stages, and acute periods of compromised hydration and (or) nutritional status throughout a multi-stage ultra-marathon competition, it was hypothesised that endotoxaemia would be seen by post-Stage 1 and progressively increase (both pre- and post-stage) along the ultra-marathon, which would be mirrored by a cytokinaemic response. It was also hypothesised that a correlation between circulatory responses with severe gastrointestinal symptoms (positive) and perceptive thermal tolerance rating (negative) would be seen. Additionally, it was hypothesised that no difference in responses would be seen between the sexes, and that faster runners with higher training status would show lower responses.

METHODS

Setting

The study was conducted during the 2011 Al Andalus Ultimate Trail (www.alandalus-ut.com), held during the 11th to

15th of July, in the region of Loja, Spain (Table 1). The multi-stage ultra-marathon was conducted over five stages (five consecutive days) totalling a distance of 230 km over a variety of terrains; predominantly off-road trails and paths, but also included steep and narrow mountain passes, and occasional road. Running intensity averaged 8.0, 8.1, 7.1, 7.0, and 7.5 metabolic equivalents (METs) (SenseWear 7.0, BodyMedia Inc., Pittsburgh, PA, USA) from Stages 1 to 5, respectively. Sleeping arrangements along the course included a combination of outdoor tent and village sports hall accommodation [sleep duration (mean \pm SD) 8h10min \pm 0h43min, 7h50min \pm 0h36min, 8h32min \pm 0h51min, 8h18min \pm 1h05min; and sleep quality (rating scale, 1= very poor to 10= very good) 6 \pm 2, 3 \pm 2, 5 \pm 2, 3 \pm 1, from Stages 1 to 4, respectively].

resided in countries with cold or thermo-neutral environmental conditions ($\leq 20^{\circ}\text{C}$). No participant reported any incidence of illness and/or infection in the 12 weeks leading up to the ultra-marathon.

Oral anti-inflammatory agents

The use of non-steroidal anti-inflammatory drugs (NSAIDs) and other anti-inflammatory agents amongst UER included: paracetamol (500-1000mg), ibuprofen (400mg), cocodamol (500-1000mg), Celebrex (200mg), and fish oils (1-2g omega 3 fatty acids). Anti-inflammatory agent usage by UER was n= 2, n= 5, n= 3, n= 4, and n= 6 from Stage 1 to 5, respectively. No form of oral anti-inflammatory agents were used by n= 13 UER and CON (n= 12) throughout the ultra-marathon.

Table 1: Multi-stage ultra-marathon characteristics, including stage times and average speed of ultra-endurance runners.

	Distance (km)	Altitude (m)	Ambient Temperature ($^{\circ}\text{C}$)	Ambient Relative Humidity (%)	Running Time (hours:minutes) and Speed (km/h)	Predominant Course Terrain
Stage 1	37	503 to 1443	30 to 32	31 to 32	4:41 \pm 0:37 7.9 \pm 1.1	Off-road trails and paths, steep and narrow mountain passes.
Stage 2	48	830 to 1338	30 to 34	32 to 33	6:56 \pm 1:03 6.9 \pm 1.1	Off-road trails and paths, steep and narrow mountain passes, and occasional road.
Stage 3	38	689 to 1302	32 to 38	35 to 37	4:51 \pm 0:42 7.8 \pm 1.2	Off-road trails and occasional road.
Stage 4	69	671 to 1152	32 to 40	31 to 33	7:11 \pm 1:12 9.6 \pm 1.7	Off-road trails and road.
Stage 5	38	473 to 1065	36 to 40	37 to 40	4:42 \pm 0:43 8.1 \pm 1.2	Off-road trails and road.

Mean \pm SD or range (n= 19).

Participants

After ethical approval from Coventry University Ethics Committee that conforms with the 2008 Helsinki declaration for human research ethics, 19 out of the total 69 ultra-endurance runners (UER) who entered this ultra-marathon competition volunteered to participate in the study [males n= 13: age 41 \pm 8 y, height 1.77 \pm 0.05 m, body mass 76 \pm 7 kg, body fat mass 14 \pm 5%; females n= 6: age 49 \pm 4 y, height 1.65 \pm 0.05 m, body mass 62 \pm 6 kg, body fat mass 21 \pm 3%]. For comparative purposes, 12 individuals who accompanied the ultra-runners (on-location for pre- and post-stage measurements and sampling, slept at the ultra-marathon location with the competition participants, but drove around the course spectating the event) along the ultra-marathon course, but did not compete (absence of exertional stress), volunteered to participate in the study as part of the control (CON) group [males n= 5: age 41 \pm 10 y, height 1.76 \pm 0.10 m, body mass 76 \pm 14 kg, body fat mass 18 \pm 5%; females n= 7: age 31 \pm 13 y, height 1.60 \pm 0.04 m, body mass 62 \pm 13 kg, body fat mass 25 \pm 7%]. All participants arrived at location ≤ 48 hours prior to the start of Stage 1. Only n= 2 participants resided in countries with hot ambient conditions similar to those of the race location ($\geq 30^{\circ}\text{C}$ T_{amb}) at the time of competition; the remaining participants

Study design and data collection

Following participant recruitment and informed consent, a preliminary session was completed to determine baseline body mass, height, and body fat mass. Height was measured by a wall-mounted stadiometer. Baseline body mass was determined using calibrated electronic scales (BF510, Omron Healthcare, Ukyo-ku, Kyoto, Japan) placed on a hard levelled surface. Waist and hip circumferences were measured using a standard clinical tape measure by trained researchers, in accordance with ISAK international standards for anthropometric assessment. Body mass and circumference measures were used when conducting multifrequency bioelectrical impedance analysis (MBIA; Quadscan 4000, Bodystat, Douglas, Isle of Man, UK) to estimate body composition. The current ultra-marathon was semi self-sufficient, whereby participants (including CON) planned and provided their own foods and fluids (except plain water) along the five days of competition. Participants' equipment and sustenance was transported to each stage section by the race organisation. *Ad libitum* water was provided by the race organisers during the rest phase throughout competition. Additionally, aid stations along the running route were situated approximately 10 km apart, and only provided plain water, fruit (oranges and watermel-

on), and electrolyte supplementation that was used by $n=9$ UER as per manufacturer's instruction (2.46 ml/896 ml fluid; Elete electrolyte add-in, Mineral Resources International, South Ogden, Utah, US). Participants were advised to adhere to their programmed habitual dietary practices throughout the entire competition.

Each day, for five consecutive days, running stages commenced at either 08:00 or 09:00. Within the hour prior to the start of each running stage, body mass measurement was determined using calibrated electronic scales placed on a hard levelled surface. Participants were then required to sit in an upright position for 10 minutes before tympanic temperature (T_{tym} ; Braun Thermoscan, Kronberg, Germany) was determined and whole blood collected. To determine T_{tym} , participants were asked to position their head in the Frankfort plane and avoid head movement until T_{tym} measurement was completed. A disposable thermometer tip cover was placed on the sensor; the right auricle was then gently pulled up and back before the sensor was insertion into the right external auditory canal for five seconds, without touching the tympanic membrane. All measurement techniques and samples were consistently conducted and collected in a large partitioned research field tent (four sections, 3 m x 3 m) or sports hall facility. Body mass was re-measured in those participants who needed to urinate prior to the stage start.

Immediately post-stage and before any foods or fluids could be consumed, body mass and T_{tym} were measured, followed by whole blood collection. For consistency, the order, positioning and technique of measurements and sampling were similar pre- and post-stage for all stages, and were taken by the same trained researcher throughout. At the end of each competition day (20:00 to 22:00) on Stages 1 to 4, researchers explored severe gastrointestinal symptoms (38) and perceptive thermal tolerance rating (20) through a rating scale (gastrointestinal symptoms: "no symptoms" to "extremely bad symptoms" and thermal tolerance rating: "cool" to "unbearable hot"). Exertional-heat illness symptoms were verified by a qualified Sports Physician.

Dietary analysis and hydration status

At the end of each competition day on Stages 1 to 4, trained dietetic researchers conducted a standardised structured interview (dietary recall interview technique) on participants to ascertain total daily food and fluid ingestion. Energy and water intake through foods and fluids were analysed on Dietplan 6 dietary analysis software (v6.60, Forestfield Software, Horsham, West Sussex, UK). A comprehensive description of the dietary assessment and analysis technique used can be viewed in Costa et al. (12, 13). Pre- and post-stage body mass values were used to determine exercise-induced body mass change. Pre- and post-stage plasma osmolality (P_{Osmol}) was determined from 50 μl lithium heparin plasma in duplicate by freeze-point osmometry (Osmomat 030, Gonotec, Berlin, Germany). The coefficient of variation for P_{Osmol} was 3.5%.

Blood sample collection and analysis

Whole blood samples were collected by venepuncture without venostasis from an antecubital vein using a 21G butterfly syringe into one lithium heparin (6 ml, 1.5 IU/ml heparin; Becton Dickinson, Oxford, UK) and one K_3EDTA (6 ml, 1.6 mg/ml of K_3EDTA ; Becton Dickinson, Oxford, UK) vacutain-

er tube. Blood samples were immediately centrifuged and plasma aliquoted into Eppendorf tubes and stored frozen initially at -20°C during the ultra-marathon competition, prior to transferring to -80°C storage after completion of the experimental procedure. Whole blood haemoglobin concentration and haematocrit were used to estimate changes in plasma volume (P_V) relative to pre-Stage 1. Haemoglobin concentration and hematocrit content of K_3EDTA blood samples (100 μl) were determined using an automated cell counter (Coulter ACT Diff, Beckman Coulter, USA) immediately after sample collection. All blood parameters were corrected for changes in P_V (14).

Circulatory concentrations of C-reactive protein (CRP) (eBioscience, Hatfield, UK), IL-6, TNF- α , IL-1 β , IFN- γ , IL-10, and IL-1ra (Invitrogen, Carlsbad, US) were determined by ELISA using K_3EDTA plasma as per manufacturer's instructions. Gram-negative bacterial endotoxin concentration was determined by limulus amoebocyte lysate (LAL) chromogenic endpoint assay using K_3EDTA plasma (HIT302, Hycult Biotech, Uden, Netherlands) as per manufacturer's instructions. In short, 20 μl of sample was diluted in 380 μl of endotoxin-free water, and then incubated at 75°C for 10 minutes. Once at room temperature, 50 μl of standards, blank, positive control, and samples were added to plate wells in duplicate. To enhance assay validity, background plate reading without LAL reagent was performed at OD 405nm. 50 μl LAL reagent was then added. Plate was covered and incubated at 22°C for 30 minutes, followed by reading at OD 405nm. Concentration was calculated by plotting the absorbance against standards in a linear regression curve and eliminating background error. The assay was performed using endotoxin-free and depyrogenated consumables in a sterile laboratory. Each plasma variable was analysed on the same day, with standards and controls on each plate, and each participant assayed on the same plate. The intra-assay coefficient of variation for plasma variables analysed was $\leq 5.5\%$. In CON, blood-borne indices were determined on pre-Stages 1, 3 and 5 only.

Data analysis

Data in text and tables are presented as mean \pm standard deviation (SD). Due to commonly large individual variation in immunological responses to exercise (59), data in figures (% change) are presented as individual participant responses. Prior to data analysis, outlying values for all variables were detected through box-plot analysis (SPSS v.20, Illinois, US). Participants that presented consistent outlying values throughout the ultra-marathon were removed. The data were examined using a two-way (stage x time) repeated-measures ANOVA (Friedman for gastrointestinal symptoms and perceptive thermal tolerance rating) (SPSS v.20, Illinois, US); except for energy, macronutrient, and water intake that was examined using a one-way ANOVA. Assumptions of homogeneity and sphericity were checked, and then appropriate adjustments to the degrees of freedom were made using the Greenhouse-Geisser correction method. Significant main effects were analysed using a post hoc Tukey's HSD test. For comparative purposes, a two-way ANOVA was also applied to sub-group analysis [UER vs CON, sexes (total and body mass corrected values), oral anti-inflammatory agent administration, and running speed (slow runners (SR, $n=11$), who completed the entire distance of the ultra-marathon using a mixture of walk-

ing and running (overall mean speed <8 km/h) and fast runners (FR, n= 8), who completed the majority of the ultra-marathon distance running (overall mean speed ≥8 km/h)]. Pearson’s coefficient correlation analysis was used to assess the associations between endotoxin with C-reactive protein and cytokine profile. Spearman’s rank correlation analysis was used to assess the associations between blood-borne variables with self-reported gastrointestinal symptoms and perceptible thermal tolerance rating. The pro- to anti-inflammatory balance was determined by calculating the IL-1β:IL-10 and TNF-α:IL-10 ratios. The acceptance level of significance was set at P < 0.05.

RESULTS

Energy, macronutrient, and water ingestion

A difference in total daily energy intake was seen between stages in UER and CON (P < 0.001; Table 2). Total daily protein and carbohydrate intakes were higher (P < 0.001) in UER compared with CON at various stages of the ultra-marathon. Rate of carbohydrate intake during running did not differ between stages in UER. No difference in total daily water intake through foods and fluids was seen between stages in UER and CON (Table 2). Total daily water intake through foods and fluids was higher in UER on all stages compared with CON (P < 0.001). Rate of water intake through foods and fluids during running did not differ between stages in UER.

Body mass, plasma osmolality and volume change

Pre- and post-stage body mass did not significantly alter throughout competition in UER (pre-Stage 1: 71.7 ± 9.5 kg to pre-Stage 5: 71.2 ± 9.2 kg; and post-Stage 1: 69.8 ± 8.9 kg to post-Stage 5: 69.6 ± 9.5 kg) and CON (pre-Stage 1: 67.4 ± 15.0 kg to pre-Stage 5: 67.0 ± 14.8 kg. Stage 1 (2.5%) resulted in a greater exercise-induced body mass loss compared

with Stages 2 to 5 in UER (2.0%, 1.0%, 2.2%, and 2.2%, respectively; P < 0.001). Pre-stage (range: 277 to 282 mOsmol/kg) and post-stage (range: 286 to 297 mOsmol/kg) P_{Osmol} did not differ between stages in UER. Pre-stage P_{Osmol} did not differ from CON throughout the ultra-marathon. Pre- to post-stage increases in P_{Osmol} (P < 0.001) were observed on all stages in UER. Relative to pre-Stage 1, resting pre-stage P_V increased significantly (P < 0.001) by Stage 2 (7.0 ± 1.4%) and peaked at Stage 5 (22.7 ± 2.0%) in UER; while no significant change in P_V was observed in CON. UER presented greater P_V change at pre-Stages 3 and 5 compared with CON (P < 0.001).

Tympanic temperature

Tympanic temperature (T_{tymp}) was within normal range pre- (overall mean: 36.3 ± 0.4°C) and post-stage (overall mean 37.0 ± 0.3°C) in UER. Pre-stage T_{tymp} gradually decreased (P = 0.003) in UER as the ultra-marathon progressed (pre-Stage 1: 36.5°C and pre-Stage 5: 36.0°C). No change in pre-stage T_{tymp} (36.7 ± 0.5°C) was observed for CON throughout the ultra-marathon. Pre- to post-stage increase (0.7°C; P < 0.001) in T_{tymp} was also observed in UER throughout the ultra-marathon. No difference in T_{tymp} was observed for sub-group comparisons.

Circulatory gram-negative bacterial endotoxin concentration

Pre-stage circulatory endotoxin concentration gradually increased (P < 0.001) in UER as the ultra-marathon progressed (Table 3, Figure 1A), peaking at Stage 5 (21%). No change in pre-stage circulatory endotoxin concentration was observed between Stages 1, 3, and 5 for CON. Pre- to post-stage increase (P = 0.001) in circulatory endotoxin concentration was also observed in UER throughout the ultra-marathon (Table 3, Figure 1B). No difference in circulatory endotoxin concentration was observed for sub-group comparisons.

Table 2: Energy, macronutrient, and water intake (through foods and fluids) of a control group and ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment.

	Stage 1		Stage 2		Stage 3		Stage 4	
	UER	CON	UER	CON	UER	CON	UER	CON
Total daily intake								
Energy (MJ/day)	16.3 ± 4.4 ^{aa}	10.4 ± 1.4	14.6 ± 4.2 [†]	12.6 ± 1.1 [†]	13.7 ± 4.0 [†]	12.1 ± 1.7	15.2 ± 5.4 ^{aa}	12.6 ± 1.9 [†]
Protein (g/day)	123 ± 42 ^{aa}	54 ± 8	109 ± 33 ^{aa}	77 ± 28	92 ± 31 ^{aa}	68 ± 6	106 ± 40 ^{aa}	81 ± 27
Carbohydrate (g/day)	590 ± 189 ^{aa}	400 ± 58	527 ± 190	487 ± 37	516 ± 145	469 ± 47	534 ± 184	484 ± 45
Fat (g/day)	116 ± 51	75 ± 12	106 ± 39	85 ± 10	93 ± 45	82 ± 27	120 ± 47	84 ± 23
Water (L/day)	7.5 ± 1.5 ^{aa}	2.8 ± 0.3	6.8 ± 2.9 ^{aa}	3.4 ± 0.2	6.6 ± 1.6 ^{aa}	3.3 ± 0.6	6.5 ± 2.8 ^{aa}	3.7 ± 0.7
During running								
Total carbohydrate (g)	121 ± 72 [‡]		146 ± 60 [‡]		137 ± 61 [‡]		195 ± 91	
Carbohydrate intake rate (g/h)	27 ± 16		23 ± 10		29 ± 13		28 ± 14	
Total water (L)	3.7 ± 1.0 [‡]		4.3 ± 1.9		3.6 ± 1.5 [‡]		4.4 ± 1.7	
Water intake rate (ml/h)	819 ± 277		693 ± 269		797 ± 331		721 ± 256	

Mean ± SD: ultra-endurance runners (UER, n= 19) and control group (CON, n= 12). [†] P < 0.05 vs Stage 1, [‡] P < 0.05 vs Stage 4, ^{aa} P < 0.01 vs CON.

Table 3: Circulatory endotoxin, C-reactive protein concentrations, and plasma cytokine profile of a control group and ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment.

	Stage 1		Stage 2		Stage 3		Stage 4		Stage 5	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Gram-negative endotoxin (EU/ml)										
UER	2.8 ± 0.3	3.2 ± 0.8 [§]	3.0 ± 0.4	3.1 ± 0.5	2.9 ± 0.4	3.5 ± 0.8**	3.3 ± 0.5 ^{††}	3.6 ± 0.6*	3.4 ± 0.5 ^{††}	3.6 ± 0.9
CON	3.0 ± 0.2				3.0 ± 0.4				3.1 ± 0.5	
C-reactive protein (µg/ml)										
UER	1.1 ± 1.7	1.6 ± 2.4	7.4 ± 5.3 ^{††}	8.8 ± 5.4	10.0 ± 5.7 ^{††aa}	9.6 ± 5.9	9.2 ± 5.9 ^{††}	10.0 ± 6.7*	8.8 ± 5.6 ^{††aa}	11.0 ± 6.4*
CON	1.4 ± 0.7				1.3 ± 0.8				1.3 ± 0.8	
IL-6 (pg/ml)										
UER	8.2 ± 4.5	27.9 ± 23.4**	20.8 ± 18.5 ^{††}	20.7 ± 14.8	20.7 ± 16.8 ^{††aa}	25.3 ± 24.3**	19.2 ± 14.1 ^{††}	21.7 ± 12.6**	18.2 ± 11.6 ^{††aa}	23.4 ± 13.1**
CON	7.5 ± 2.5				5.5 ± 7.1				6.5 ± 5.7	
IL-1β (pg/ml)										
UER	0.6 ± 0.3	1.0 ± 0.3**	1.1 ± 0.4 ^{††}	1.1 ± 0.4	1.2 ± 0.4 ^{††}	1.2 ± 0.4	1.1 ± 0.3 ^{††}	1.4 ± 0.4**	1.2 ± 0.4 ^{††}	1.4 ± 0.4*
CON	0.7 ± 0.2				1.2 ± 0.2 [†]				1.3 ± 0.5 ^{††}	
TNF-α (pg/ml)										
UER	3.1 ± 2.9	6.3 ± 5.0**	6.1 ± 4.5 ^{††}	6.6 ± 3.7	6.9 ± 4.4 ^{††aa}	6.1 ± 3.8*	6.5 ± 4.2 ^{††}	8.1 ± 4.3**	7.1 ± 3.8 ^{††aa}	8.3 ± 5.0
CON	1.3 ± 0.4				1.8 ± 0.7				2.3 ± 0.7	
IFN-γ (IU/ml)										
UER	9.3 ± 5.5	12.9 ± 6.0**	15.2 ± 6.8	16.9 ± 5.7	16.7 ± 6.7 [†]	15.2 ± 5.2	16.2 ± 7.2 [†]	19.9 ± 8.3**	18.8 ± 10.0 ^{††}	22.7 ± 9.9**
CON	16.8 ± 5.5				14.3 ± 2.0				16.8 ± 5.1	
IL-10 (pg/ml)										
UER	0.7 ± 0.6	7.9 ± 10.1**	7.0 ± 10.8 ^{††}	7.9 ± 9.1	9.0 ± 10.2 ^{††aa}	8.0 ± 9.4	9.0 ± 12.2 ^{††}	9.3 ± 10.1	8.2 ± 11.2 ^{††aa}	10.9 ± 15.0*
CON	0.6 ± 0.1				1.4 ± 0.3				1.4 ± 0.7	
IL-1ra (pg/ml)										
UER	22.9 ± 8.0	70.3 ± 28.1**	39.8 ± 12.4 ^{††}	61.0 ± 39.8**	45.5 ± 20.6 ^{††aa}	53.9 ± 19.0*	37.9 ± 14.7 ^{††}	56.3 ± 30.4**	47.1 ± 22.4 ^{††aa}	63.2 ± 28.1**
CON	23.4 ± 7.3				36.4 ± 9.2				33.1 ± 9.3	

Mean ± SD: ultra-endurance runners (UER, n= 19) and control group (CON, n= 12). ^{††} P < 0.01 and [†] P < 0.05 vs pre-Stage 1, ^{**} P < 0.01 and ^{*} P < 0.05 vs respective pre-stage, [§] P = 0.058 vs respective pre-stage, ^{aa} P < 0.01 vs CON.

Plasma C-reactive protein concentration

Pre-stage plasma CRP concentration increased (P < 0.001) by Stage 2 in UER, and remained elevated thereafter (Table 3, Figure 2A), peaking at Stage 3 (889%). No change in pre-stage plasma CRP concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 (P < 0.001). Pre- to post-stage increase (P = 0.014) in plasma CRP concentration was also observed in UER throughout the ultra-marathon (Table 3, Figure 2B). Plasma CRP concentration was observed to be higher (P < 0.001) in males (pre-stage: 8.9 ± 3.6 µg/ml, post-stage: 10.1 ± 4.0 µg/ml) compared with females (pre-stage: 4.2 ± 2.6 µg/ml, post-stage: 4.3 ± 2.5 µg/ml) throughout the ultra-marathon. This difference was also observed when corrected for body mass (P < 0.001). No differences in other sub-group comparisons were observed.

Plasma interleukin-6 concentration

Pre-stage plasma IL-6 concentration increased (P = 0.006) by Stage 2 (152%) in UER and remained elevated thereafter (Table 3, Figure 3A). No change in pre-stage plasma IL-6 concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 (P < 0.001). Pre- to post-stage increase (P < 0.001) in plasma IL-6 concentration was also observed in UER (Table 3, Figure 3B). Post-stage plasma IL-6 concentration was observed to be higher (P = 0.054) in males (26.7 ± 20.5 pg/ml) compared with females (17.6 ± 5.7 pg/ml) throughout the ultra-marathon. However, when corrected for body mass no substantial difference was observed. There was also a tendency for higher (P = 0.094) pre-stage plasma IL-6 concentration in SR (20.4 ± 15.2 pg/ml) compared with FR (13.3 ± 5.4 pg/ml) throughout the ultra-marathon. No difference in plasma IL-6 concentration was observed for oral anti-inflammatory administration.

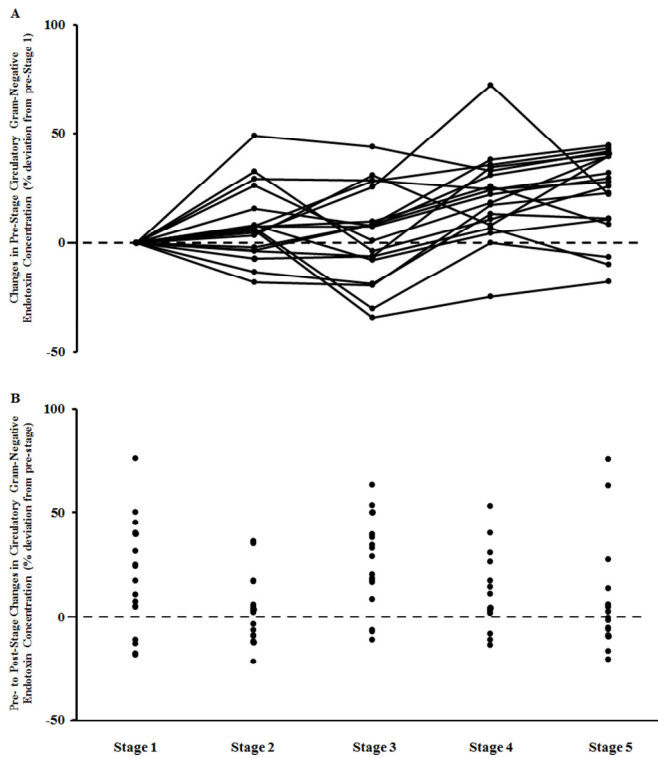


Figure 1. Individual changes in pre-stage resting (A) and pre- to post-stage (B) circulatory gram-negative endotoxin concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (•; n=19).

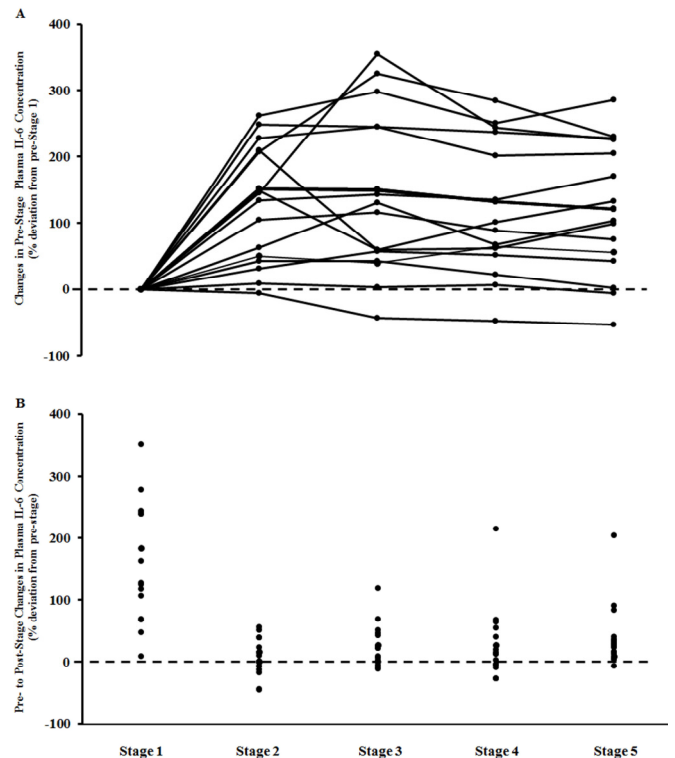


Figure 3. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma IL-6 concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (•; n=19). Additional individual responses outside the figure range for pre- to post-stage changes; Stage 1, n=1 at 605%, n=1 at 704%, and n=1 at 1205%

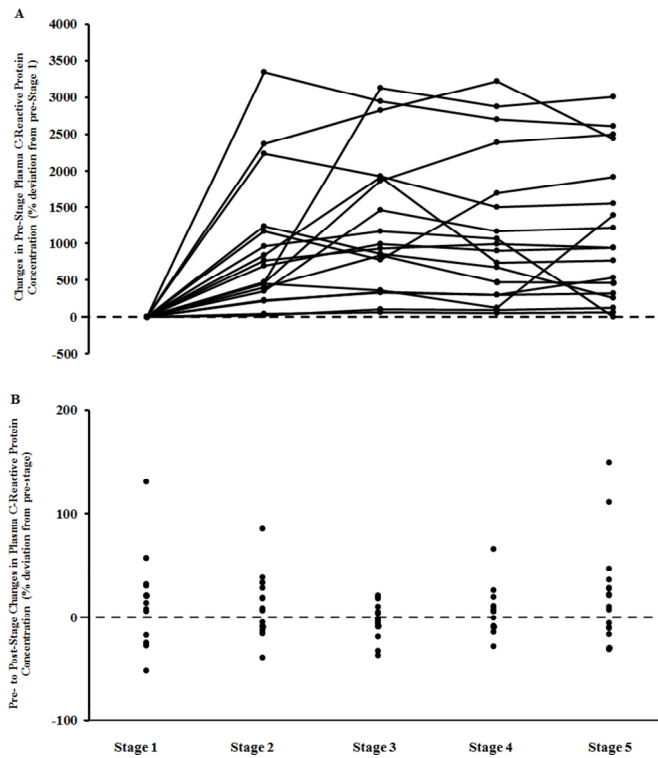


Figure 2. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma C-reactive protein concentration of ultra-endurance runner participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (•; n=19). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 1, n=1 at 1827%; Stage 2, n=1 at 429% and n=1 at 620%; and Stage 4, n=1 at 1192%

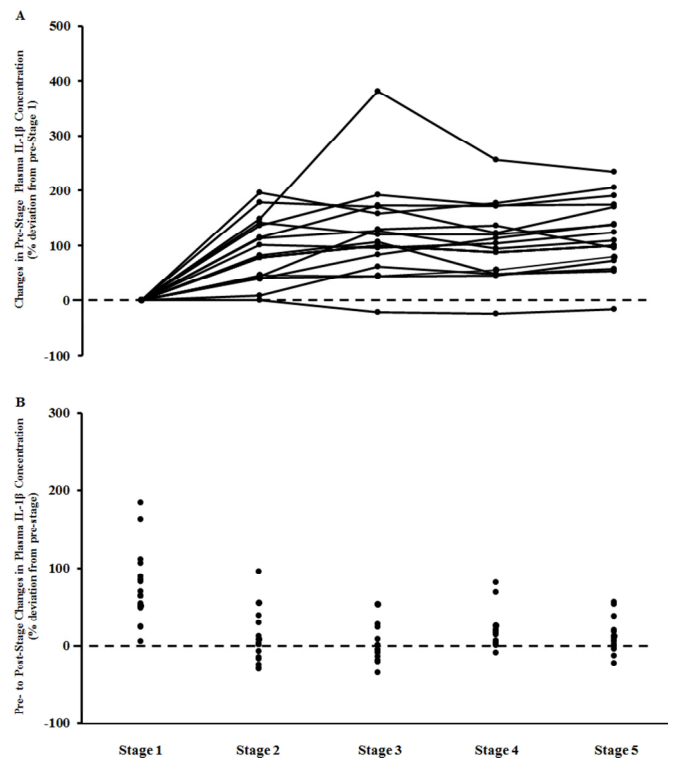


Figure 4. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma IL-1β concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (•; n=19).

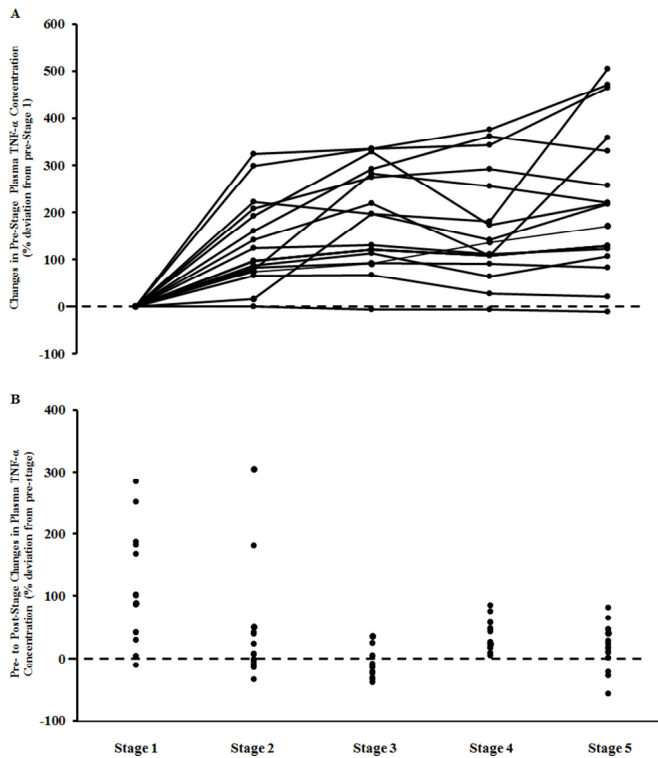


Figure 5. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma TNF- α concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (\bullet ; n=19).

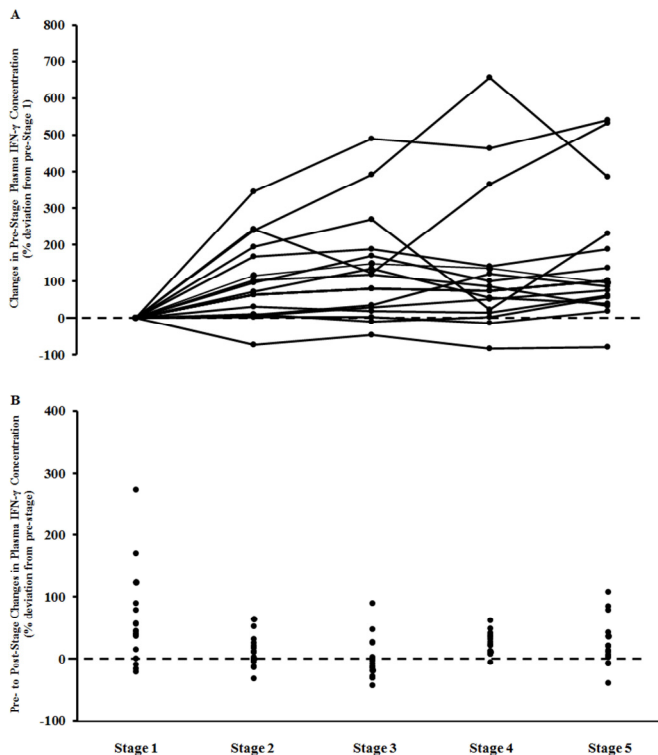


Figure 6. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma IFN- γ concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual-endurance runner responses (\bullet ; n=19). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 2, n=1 at 533%; and Stage 5, n=1 at 511%

Plasma interleukin-1 β concentration

Pre-stage plasma IL-1 β concentration increased ($P < 0.001$) by Stage 2 in UER (Table 3, Figure 4A) and remained elevated thereafter, peaking at Stage 5 (95%). While an unexpected increase was also observed for CON ($P < 0.001$), whereby plasma IL-1 β concentration increased by Stage 3 and remained elevated there after. Pre- to post-stage increase ($P < 0.001$) in plasma IL-1 β concentration was also observed in UER (Table 3, Figure 4B). Pre- and post-stage plasma IL-1 β concentration was observed to be lower ($P = 0.014$) in males (pre-stage: 1.0 ± 0.2 pg/ml, post-stage: 1.1 ± 0.2 pg/ml) compared with females (pre-stage: 1.2 ± 0.4 pg/ml, post-stage: 1.3 ± 0.4 pg/ml) throughout the ultra-marathon. However, when corrected for body mass no substantial difference was observed. There was a tendency for higher ($P = 0.054$) pre-stage plasma IL-1 β concentration in SR (1.1 ± 0.2 pg/ml) compared with FR (0.9 ± 0.5 pg/ml) throughout the ultra-marathon. No difference in plasma IL-1 β concentration was observed for oral anti-inflammatory administration.

Plasma tumour necrosis factor- α concentration

Pre-stage plasma TNF- α concentration increased ($P < 0.001$) at Stage 2 in UER (Table 3, Figure 5A) and remained elevated thereafter, peaking at Stage 5 (168%). No change in pre-stage plasma TNF- α concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($P < 0.001$). Pre- to post-stage increase ($P < 0.001$) in plasma TNF- α concentration was also observed in UER (Table 3, Figure 5B). Pre- and post-stage plasma TNF- α concentration was observed to be lower ($P < 0.001$) in males (pre-stage: 4.8 ± 1.8 pg/ml, post-stage: 6.1 ± 2.8 pg/ml) compared with females (pre-stage: 8.4 ± 6.1 pg/ml, post-stage: 9.3 ± 6.1 pg/ml) throughout the ultra-marathon. This difference was also observed when corrected for body mass (pre-stage: $P < 0.001$, post-stage: $P = 0.001$). Pre-stage plasma TNF- α concentration was observed to be higher ($P = 0.016$) in SR (6.9 ± 1.8 pg/ml) compared with FR (4.7 ± 6.1 pg/ml) throughout the ultra-marathon. No difference in plasma TNF- α concentration was observed for oral anti-inflammatory administration.

Plasma interferon- γ concentration

Pre-stage plasma IFN- γ concentration increased ($P < 0.001$) at Stage 3 in UER (Table 3, Figure 6A), peaking at Stage 5 (102%). No change in pre-stage plasma IFN- γ concentration was observed for CON. Pre- to post-stage increase ($P < 0.001$) in plasma IFN- γ concentration was also observed in UER (Table 3, Figure 6B). Pre-stage plasma IFN- γ concentration was observed to be higher ($P = 0.016$) in SR (16.7 ± 6.4 IU/ml) compared with FR (13.2 ± 9.2 IU/ml) throughout the ultra-marathon. No differences in other sub-group comparisons were observed.

Plasma interleukin-10 concentration

Pre-stage plasma IL-10 concentration increased ($P = 0.011$) by Stage 2 in UER, and remained elevated thereafter (Table 3, Figure 7A), peaking at Stage 3 (1271%). No change in pre-stage plasma IL-10 concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($P < 0.001$). Pre- to post-stage increase ($P = 0.020$) in plasma IL-10 concentration was also observed in UER (Table 3, Figure 7B). Pre- and post-stage plasma IL-10

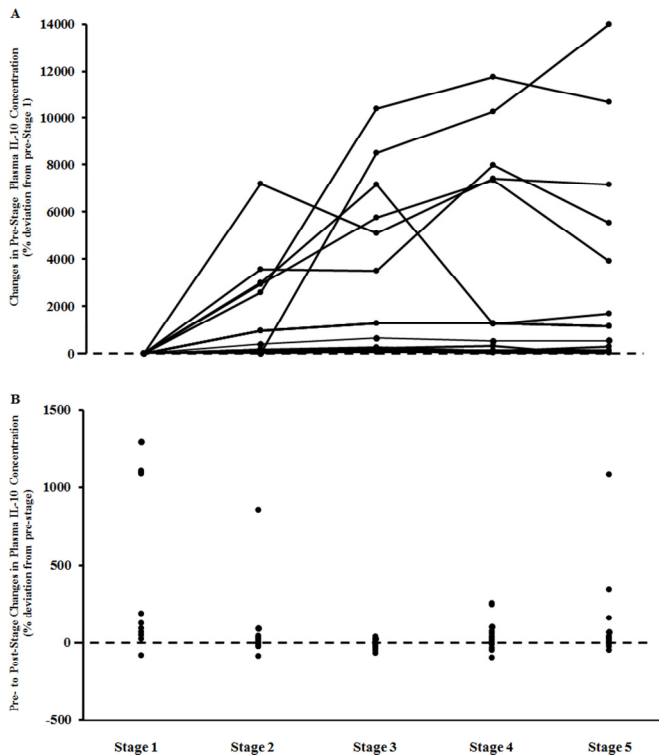


Figure 7. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma IL-10 concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (*; n=19). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 1, n=1 at 2305%, n=1 at 3781%, n=1 at 4761%, and n=1 at 6226%; and Stage 3, n=1 at 1810%.

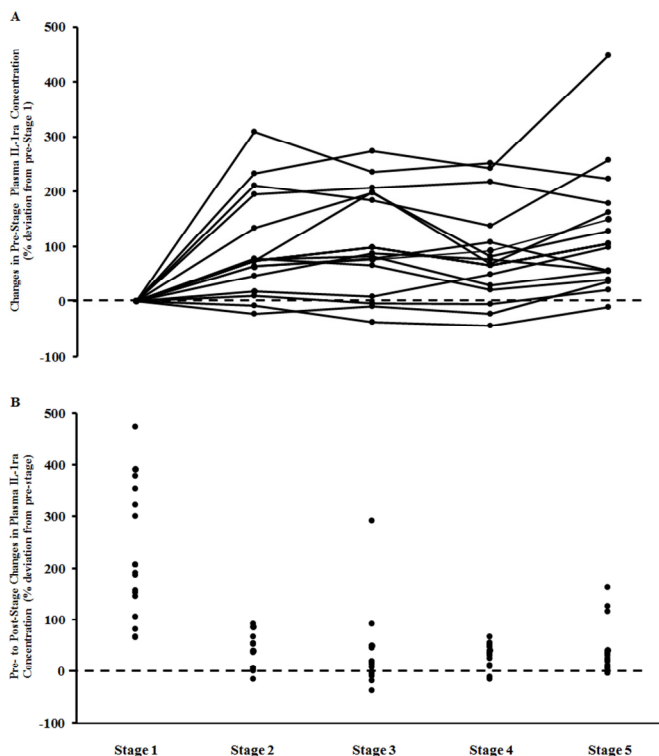


Figure 8. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma IL-1ra concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (*; n=19). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 2, n=1 at 509%; and Stage 4, n=1 at 696%

concentration was observed to be lower ($P < 0.001$) in male ultra-runners (pre-stage: 4.1 ± 4.1 pg/ml, post-stage: 6.3 ± 7.6 pg/ml) compared with female ultra-runners (pre-stage: 12.6 ± 13.6 pg/ml, post-stage: 14.2 ± 14.3 pg/ml) throughout the ultra-marathon. This difference was also observed when corrected for body mass (pre-stage: $P < 0.001$, post-stage: $P = 0.001$). No differences in other sub-group comparisons were observed.

Plasma interleukin-1 receptor antagonist concentration

Pre-stage plasma IL-1ra concentration gradually increased ($P < 0.001$) in UER as the ultra-marathon progressed (Table 3, Figure 8A), peaking at Stage 5 (106%). No change in pre-stage plasma IL-1ra concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($P < 0.001$). Pre- to post-stage increase ($P < 0.001$) in plasma IL-1ra concentration was also observed in UER throughout the ultra-marathon (Table 3, Figure 8B). No difference in plasma IL-1ra concentration was observed for sub-group comparisons.

Pro-inflammatory to anti-inflammatory cytokine ratio

Pre-stage IL-1 β :IL-10 and TNF- α :IL-10 ratios decreased in UER as the ultra-marathon progressed, but failed to reach significance ($P > 0.05$). Ratios in UER did not differ from CON pre-Stages 3 and 5. No change in pre- to post-stage IL-1 β :IL-10 and TNF- α :IL-10 ratios were observed in UER. No differences in sub-group comparisons were observed for ratios.

Gastrointestinal symptoms and thermal tolerance rating

Gastrointestinal symptoms were a common feature amongst UER sampled for endotoxin and cytokine responses; with 58% reporting at least one severe gastrointestinal symptom (including 33% of sampled UER reporting nausea) during competition, while no gastrointestinal symptoms were reported by CON. No differences in the reported rates of severe gastrointestinal symptoms were observed between stages in UER. Perceptive thermal tolerance rating in UER improved as the ultra-marathon progressed ($P = 0.005$), with no change in CON. Additionally, no heat related illnesses were observed in UER and CON throughout the ultra-marathon.

Correlation analysis

Small but significant positive correlations were observed between pre-stage circulatory gram-negative bacterial endotoxin concentration with pre-stage plasma CRP ($r = 0.343$, $P = 0.001$), IL-6 ($r = 0.246$, $P = 0.019$), IL-1 β ($r = 0.305$, $P = 0.003$), TNF- α ($r = 0.370$, $P < 0.001$), IFN- γ ($r = 0.282$, $P = 0.007$), IL-10 ($r = 0.309$, $P = 0.003$), and IL-1ra ($r = 0.268$, $P = 0.011$) concentrations; and between post-stage circulatory endotoxin concentration with post-stage plasma CRP concentration ($r = 0.213$, $P = 0.043$). No correlations were observed between circulatory gram-negative bacterial endotoxin and plasma cytokine concentrations with severe gastrointestinal symptoms (including nausea). A strong relationship between perceptive thermal tolerance rating and severe gastrointestinal symptoms was observed ($r = -0.665$, $P < 0.001$), whereby lower perceptive tolerance rating to heat was associated with greater reports of severe gastrointestinal symptoms in UER. However, no correlations were observed between circulatory gram-negative bacterial endotoxin and

plasma cytokine concentrations with perceptive thermal tolerance rating.

DISCUSSION

The current study aimed to determine circulatory endotoxin concentration and cytokine profile of ultra-endurance runners throughout a multi-stage ultra-marathon competition conducted in hot and dry environmental conditions; and determine the relationship between these responses with severe gastrointestinal symptoms and perceptive thermal tolerance rating. Findings confirm that consecutive days of exertional-heat stress resulted in a modest and sustained rise in both resting and post-stage circulatory gram-negative bacterial endotoxin concentration. Despite overnight recovery between stages, results show that pyrogenic pro-inflammatory cytokines (i.e., IL-6, IL-1 β , TNF- α , and IFN- γ) increased in response to exertional-heat stress and remained elevated at rest throughout competition. These responses however were counteracted by compensatory anti-inflammatory cytokine responses that predominated throughout the ultra-marathon (1, 48). Strength of the findings is supported by the control group showing no change in circulatory endotoxin concentration and cytokine profile (except IL-1 β) throughout the ultra-marathon period.

Although the characteristics of the cytokine responses are similar to that observed during an acute infectious episode and in accordance with the aetiology of exertional-heat illnesses (i.e., exertional-heat stroke and SIRS), no diagnosis of heat related illnesses by a qualified Sports Physician were established in UER along the ultra-marathon. Severe gastrointestinal symptoms reported by UER were generally high; but in contrast to our hypothesis, no relationship between severe gastrointestinal symptoms with circulatory gram-negative endotoxin and plasma cytokine concentrations were observed. Thermal strain (T_{lymp} and perceptive thermal tolerance rating) appeared to improve as the ultra-marathon progressed, and is in accordance with heat acclimatization (10). A strong relationship between severe gastrointestinal symptoms and perceptive thermal tolerance rating was evident, whereas no relationship between perceptive thermal tolerance rating with circulatory gram-negative endotoxin and plasma cytokine concentrations were observed. Even though ultra-endurance runners presented no heat related medical issues during the current study, the endotoxin and cytokine responses observed provide a novel and valuable insight into a potentially high risk situation, whereby individuals with predisposition and multiple risk factors for deranged pro-inflammatory and compensatory anti-inflammatory responses are likely to develop consequential clinically significant issues. For example, the degree of cytokinaemia observed has been linked to the aetiology of heat stroke, septic shock, autoimmune disease, gastrointestinal disease, and chronic fatigue (7, 34, 26, 29, 43).

Plasma CRP concentration, which is normally low and undetectable in the circulation of healthy populations, is an acute phase reactant that dramatically rises in the presence of inflammation (e.g., induced by trauma, bacterial infection, and (or) inflammatory responses). In the current study, plasma CRP concentrations of UER peaked by Stage 2 and remained elevated thereafter; while no change in CON was observed.

These responses are similar to that observed during a six-days (total distance: 468 km) endurance mountain bike event (44), whereby plasma concentrations were significantly elevated at rest for the duration of the event. However, the level of rise in plasma CRP concentration was less pronounced in comparison to levels (1.9 to 18.4 mg/L) reported in ultra-endurance athletes after six-days of track based running race totalling 622 km (16), and after the 246 km Spartathlon ultra-marathon race (0.65 to 97.3 mg/L) (27). Taking into account the responsive nature of CRP to general inflammation, the wide variations in plasma concentrations observed in the current and previous ultra-endurance studies likely reflect multi-influen-tial stimulating factors, such as intestinal originated bacterial endotoxin leakage into circulation ($r = 0.343$, $P = 0.001$) and soft tissue damage (e.g., exertional rhabdomyolysis) (8). It is possible that persistent elevations in CRP at rest in UER may contribute to progressive perceptions of fatigue and subsequent impaired performance over the given time course (31, 43). Interestingly, on this occasion, male ultra-runners showed high plasma CRP concentration throughout competition compared with female ultra-runners, suggesting greater general inflammatory presence in males. The reason for this observation is unclear; it is however likely to be attributed to muscle originated responses (50), since greater plasma IL-6 concentrations concomitant with lower IL-1 β and TNF- α responses were observed in male ultra-runners compared with female ultra-runners. Due to practical limitations in monitoring parameters after competition (i.e., ultra-endurance athletes returning to country of origin after cessation of the ultra-marathon), the current study was not able to determine the recovery time course of CRP. However, such responses have been shown to remain elevated above pre-exercise values for a considerable period of time (i.e., up to nineteen-days after an Ironman triathlon event)(31), suggesting time course for full recovery of altered inflammatory status is considerably delayed.

In comparison with previous endurance and ultra-endurance studies observing mild (e.g., marathon, 160 km ultra-marathon, and Ironman distance triathlon: 5 to 15 pg/ml) (6, 22) and substantial (e.g., 89.4 km ultra-marathon whereby 81% of runners had concentrations >100 pg/ml and an ultra-distance triathlon reporting 81 to 294 pg/ml) (3, 4) increases in circulatory endotoxin concentrations, the current ultra-marathon resulted in modest increases in post-stage circulatory endotoxin concentrations throughout competition (i.e., 30 pg/ml average increase from pre- to post-stage, with the highest individual increase observed at 92pg/ml). A novel finding was the gradual increases in resting levels as the ultra-marathon progressed (i.e., 60pg/ml average increase from Stage 1 to 5, with 32% of UER showing concentrations >100 pg/ml and the highest individual increase observed at 130 pg/ml), possibly attributed to a delayed and sustained intestinal leakage upon exercise cessations, which is accompanied by splanchnic reperfusion (57). The cumulative affect observed as the ultra-marathon progressed suggests a reduced tolerance for exertional-heat stress induced endotoxin leakage, subsequent to anti-endotoxin antibodies not restoring to their optimal level on consecutive occasions (25). For example, depressed anti-endotoxin antibodies have been reported after a marathon race, which remained below pre-exercise values for 24 hours (6). More over, a100-fold range difference in

endotoxin neutralizing capacity in plasma has been observed between individuals (61), likely associated with training adaptations (3). Indeed, higher circulating concentrations of endotoxin and anti-endotoxin antibodies have been observed in untrained compared with trained individuals (22, 47).

The proposed gained adaptation to endotoxin tolerance in trained individuals is likely attributed to repetitive endotoxin challenge resulting from exercise-stress inducing endotoxin intestinal leakage and subsequent “self-immunisation” (3, 4). Therefore during the current ultra-marathon, it is possible that the experience level of ultra-runners and frequent endotoxin exposure induced as part of their competition preparation may have resulted in training adaptations favouring an attenuated circulatory endotoxin peak along competition (i.e., ultra-runners developing adaptations that enhance resistance and resilience to enteric pathogenic endotoxin exposure); such plausibility, however, warrants investigation. Favourable adaptations would reduce the risk of developing clinically significant issues associated with endotoxaemia and subsequent cytokinaemia during consecutive days of exertional-heat stress with or without additional stressors. Conversely, inadequate training and not being physically prepared for such an extreme event would potentially increase the risk. Even though no differences in endotoxin was seen between running speeds, pre-stage plasma IL-6, IL-1 β , TNF- α , and IFN- γ concentrations were higher in SR throughout the ultra-marathon compared with FR; potentially suggesting greater intestinally originated endotoxin exposure above clearance capacity in less trained ultra-runners. This explanation however also warrants further investigation (e.g., role of intestinally originated endotoxin in training adaptations- immune competence), and may provide valuable findings into the role of endotoxin leakage in physiological adaptations to exercise stress, especially in environmental extremes. Moreover, it has also been suggested that plasma endotoxin concentrations may reach equilibrium during endurance exercise, whereby endotoxin influx from the gastrointestinal tract into circulation matches endotoxin clearance by anti-endotoxin antibodies (5, 47); which may in part explain why only modest fluctuations in circulatory endotoxin concentrations were observed.

The current study observed increases in resting pre-stage and pre- to post-stage plasma IL-6, IL-1 β , TNF- α , and IFN- γ concentrations that remained elevated throughout competition; while no change in CON was observed (except for IL-1 β). The cytokine profile of the current study mirrors that of an acute infectious episode, and is similar to pro-inflammatory cytokine responses seen after endotoxin (e.g., lipopolysaccharide) infusion in both animal (17) and human (55) models. These results are in accordance with previous endurance based (e.g., marathon) experimental designs observing modest increases in plasma IL-6, IL-1 β , and TNF α concentrations (35, 51); which were also accompanied by compensatory anti-inflammatory responses (i.e., increase in plasma IL-10 and IL-1ra concentrations). The current ultra-marathon also resulted in substantial increases in resting pre-stage and pre- to post-stage anti-inflammatory cytokines that remained elevated throughout competition to a similar degree as compensatory anti-inflammatory syndrome (1, 48). It is possible the anti-inflammatory properties of IL-10, with adjunct IL-1ra, may have restricted the magnitude of pro-inflammatory cytokine production along competition. Interestingly, no dif-

ferences in pro- and anti-inflammatory cytokine responses were observed between UER that ingested and did not ingest oral anti-inflammatory agents. This observation may suggest that exposure to exertional-heat stress induced by the event far outweighs any impact of inconsistent use of low dose anti-inflammatory medication on cytokine responses, and questions the efficacy of such inconsistent administration of anti-inflammatory pharmaceutical agents within medical management of ultra-runners during extreme events.

In well trained individuals, where exertional-heat stress is better tolerated (10), anti-inflammatory responses predominated, offsetting potential clinically significant episodes associated with cytokinaemia. It is however concerning that inadequately trained individuals may not present such competent anti-inflammatory responses, and may be a prime risk population for developing heat illness from immune aetiology (i.e., exertional-heat stroke, SIRS) (25, 32, 39). Indeed, SR presented a higher resting pro-inflammatory cytokine profiles compared with FR. It also needs to be taken into consideration that SR were on the course routes for a greater amount of time than FR; and thus SR may have been exposure to greater volumes of exertional-heat stress and a time-dependant effect on cytokine production during recovery may produce delayed anti-inflammatory responses in SR. Moreover, an age difference existed between UER and CON, but only in the female participants. It is well established that immune responses decline with age. Depressed responses are commonly observed in the elderly population, with and without medical issues, compared with the healthy adult population (59). It is, however, unlikely that the healthy recreational middle-aged ultra-endurance female population of the current study would present altered immune responses due to their age.

The recovery time course of cytokine responses after the ultra-marathon was not determined on this occasion due to practical limitations; however previous ultra-endurance studies (e.g., long-distance triathlon and ultra-marathon running) have observed variations in cytokine responses during the recovery period. For example, IL-6 and TNF- α returned to baseline by 24 hours after a 50 km ultra-marathon (28); whilst IL-6 returned to baseline values 16 hours, with no significant changes observed in TNF- α , after a long-distance triathlon (22). Furthermore, on cessation of two endurance events of similar duration (long-distance triathlon and 100 km run), IL-6, IL-10, and IL-1ra peaked after competition, returning to baseline values seven days after the events (18). Whereas after a long distance triathlon, IL-6 remained elevated on day one (345%) and day five (79%); while IL-10 was elevated on day one (37%), declining by 4% below pre-competition concentrations on day five (31). These observations suggest the time course for full recovery of altered cytokine profile in response to extreme events are considerably delayed, and may play a role in the aetiology of undefined underperformance and chronic fatigue syndromes (29, 43). The potential role of extreme event induced immune perturbations initiating autoimmune disease in individual with predisposition warrants attention, since chronic elevations in cytokine responses are reported in many autoimmune condition (e.g., systemic lupus erythematosus, fibromyalgia, myalgic encephalomyelitis, idiopathic inflammatory myopathies, arthritic conditions, and inflammatory bowel diseases) (7, 29, 52, 63).

Despite amplified cytokine responses similar to that of an acute infectious episode and in accordance with the aetiology of heat-related illnesses, none of the current $n=19$ UER were diagnosed with heat related illnesses. Previously, only $n=1$ ultra-endurance runner competing in the five-days 2010 Al Andalus Ultra-Trail race suffered heat-related problems (46), reported to be due to ultra-runners experience (e.g., training status), the hot environmental conditions, and the nature of the race course (e.g., limited shade availability). Perceptive thermal comfort rating improved as the competition progressed in the sampled population, and likely reflected heat acclimatization as evidenced by P_V increases and reductions in T_{lymp} as the ultra-marathon progresses, with no changes in CON being observed (10, 13). Interestingly, the two ultra-runners that originating from countries with hot ambient conditions at the time of competition showed similar circulatory endotoxin and cytokine responses to the main cohort, with substantial increases in P_V indicative of heat acclimatization still being observed in these ultra-runners (pre-stage 1 to pre-stage 5: ultra-runner 1 = 30.4% and ultra-runner 2 = 24.9%); suggesting exertional stress is an essential key feature of heat adaptations (10). In view of the unique and challenging characteristics of ultra-marathon competitions (i.e., prolonged physical exertion, sleep deprivation, environmental extremes, acute periods of under-nutrition and hypohydration) and associated factors (i.e., training status, inadequate rest, tolerated injury and trauma) having the potential to disturb intestinal integrity and promote cytokine-mediated inflammatory responses, the maintenance of hydration status in the majority of runners, thermoregulatory-induced adaptations, and cooling behaviours throughout competition may have contributed to improved heat tolerance despite prolonged exposure to exertional-heat stress (2, 10, 13, 54).

The systemic endotoxin and cytokine responses seen in the current study have previously been associated with symptomatic manifestations of gastrointestinal symptoms, commonly associated with prolonged exposure to exertional-heat stress (22, 23, 36, 38, 40, 56). For example, gastrointestinal symptoms, such as nausea and vomiting, have been observed in endurance athletes presenting endotoxaemia after an Ironman triathlon event (22). In contrast to previous studies, no associations between gastrointestinal symptoms with circulatory endotoxin and plasma cytokine concentrations were observed on this occasion. However, a strong relationship ($r=-0.665$) between severe gastrointestinal symptoms and perceptive thermal tolerance rating was confirmed ($P < 0.001$). These results suggest that severe gastrointestinal symptoms likely originate from heat stress during exercise, potentially through splanchnic hypoperfusion and hypoxia (i.e., exercising in the heat creating greater redistribution of blood flow away from the splanchnic area) (53, 57, 58). Such physiological changes in splanchnic blood flow, which have symptomatic outcomes, likely lead to disturbances in intestinal mucosal and epithelial integrity that enhances local enteric endotoxin leakage and subsequent cytokinaemia; and not necessarily that endotoxaemia and cytokinaemia induced gastrointestinal symptoms.

To date, it is still unknown how the degree of exertional stress, with or without environmental extremes and between different exercise modes, impacts overall gastrointestinal integrity. Additionally, does the nutritional and hydration status before exertional stress, and the changes that occurs to sta-

tus during physical exertion, influence the degree of gastrointestinal disturbance? Conducting a set of controlled laboratory experiments assessing varying ambient temperatures, exercise intensities, durations and modes whilst assessing gastrointestinal integrity measures (57) would contribute substantially to the current knowledge base and provide a foundation to investigate potential strategies to overcome gastrointestinal complications associated with exertional-heat stress. For example, dietary strategies during physical exertion, development of gut training protocols, functional foods, heat acclimation protocols, external pre-cooling (e.g., cold water bath or cooling vest) and (or) during physical exertion internal cooling (e.g., cold beverages) are proposed strategies that may attenuate exertional-heat stress induced gastrointestinal perturbations. Indeed, due to gut plasticity, there is potential for the gastrointestinal tract to adapt to a challenge load ('training the gut') (21). Whereas, previous investigations have demonstrated favourable effect of prebiotic oligosaccharides (e.g., inulin and oligofructose) and probiotic bacteria (e.g., *Lactobacillus casei* and *Bifidobacterium*) on markers of gastrointestinal integrity; albeit within inflammatory diseases of the gut (45). Knowledge into the impact of such biotics on gut integrity during exertional-heat stress is, however, scarce. Anecdotal evidence during the current study highlighted that ultra-runners who consistently consumed commercial probiotic product in the week leading up to the ultra-marathon presented no incidence of gastrointestinal symptoms; suggesting further controlled investigation is needed to confirm any beneficial effects of biotics on gastrointestinal integrity in response to exertional-heat stress.

CONCLUSION

In conclusion, multi-stage ultra-marathon competition in the heat resulted in a modest circulatory endotoxaemia accompanied by a pronounced pro-inflammatory cytokinaemia and compensatory anti-inflammatory responses. No incidences of exertional-heat illnesses were evident throughout competition. Even though severe gastrointestinal symptoms were reported, no relationships with blood borne indices were identified. The expected exacerbated cytokine responses were possibly attenuated by the maintenance of hydration status in the majority of runners, and as well thermoregulatory-induced adaptations and behaviours adopted by participants. The findings from the current study suggest that appropriate informed training (e.g., physically trained to complete the required distance in environmental extremes) and competition preparation (e.g., effective and evidence-based heat acclimation protocols, hydration maintenance and (or) cooling strategies) may help prevent significant exertional-heat related sub-clinical and clinical manifestations from occurring in high risk ultra-endurance runners competing in extreme events.

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REFERENCES

- Adib-Conquy M, and Cavaillon JM. Compensatory anti-inflammatory response syndrome. *ThrombHaemost* 101: 36-47, 2009.
- American College of Sports Medicine, Armstrong LE, Casa DJ, Millard-Stafford M, Moran DS, and Pyne SW. American College of Sports Medicine position stand exertional heat illness during training and competition. *Med Sci Sports Exerc* 39:556-572, 2007.
- Bosenberg AT, Brock-Utne JG, Gaffin SL, Wells MT, and Blake GT. Strenuous exercise causes systemic endotoxemia. *J ApplPhysiol*65: 106-108, 1988.
- Brock-Utne JG, Gaffin SL, Wells MT, Gathiram P, Sohar E, James MF, Morrell DF, and Norman RJ. Endotoxemia in exhausted runners after a long-distance race. *S Afr Med J* 73: 533-536, 1988.
- Camus G, Nys M, Poortmans JR, Venneman I, Monfils T, Deby-Dupont G, Juchmes-Ferir A, Deby C, Lamy M, and Duchateau J. Endotoxemia production of tumor necrosis factor alpha and polymorphonuclear neutrophil activation following strenuous exercise in humans. *Eur J ApplPhysiol* 79: 62-68, 1988.
- Camus G, Poortmans J, Nys M, Deby-Dupont G, Duchateau J, Deby C, and Lamy M. Mild endotoxaemia and the inflammatory response induced by a marathon race. *ClinSci* 92: 415-422, 1997.
- Caradonna L, Amati L, Magrone T, Pellegrino NM, Jirillo E, and Caccavo D. Enteric bacteria, lipopolysaccharide and related cytokines in irritable bowel disease: Biological and clinical significance. *J Endotoxin Res* 6: 205-214, 2000.
- Clarkson PM. Exertional rhabdomyolysis and acute renal failure in marathon runners. *Sports Med* 37: 361-363, 2007.
- Cosio-Lima LM, Desai BV, Schuler PB, Keck L, and Scheeler L. A comparison of cytokine responses during prolonged cycling in normal and hot environmental conditions. *JSports Med* 2: 7-11, 2011.
- Costa RJS, Crockford MJ, Moore JP, and Walsh NP. Heat acclimation responses of an ultra-endurance running group preparing for hot desert based competition. *EurJSport Sci*14: S131-S141, 2014.
- Costa RJS, Richardson K, Adams F, Birch T, Bilzon JJJ, and Walsh NP. Effects of immediate post-exercise carbohydrate ingestion with and without protein on circulating cytokine balance. *J Sport Sci*29: S99-S100, 2011.
- Costa RJ, Swancott AJM, Gill S, Hankey J, Scheer V, Murray A, and Thake CD. Compromised energy and macronutrient intake of ultra-endurance runners during a multi-stage ultra-marathon conducted in a hot ambient environment. *Int J Sports Sci* 3: 51-61, 2013.
- Costa RJS, Teixeira A, Rama L, Swancott A, Hardy L, Lee B, Camões-Costa V, Gill S, Waterman J, Barrett E, Freeth E, Hankey J, Marczak S, Valero E, Scheer V, Murray A, and Thake D. Water and sodium intake habits and status of ultra-endurance runners during a multi-stage ultra-marathon conducted in a hot ambient environment: An observational study. *Nutr J* 12: 13(1-16), 2013.
- Dill DB, and Costill DL. Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J ApplPhysiol* 37: 247-248, 1974.
- Fehrenbach E, and Schneider ME. Trauma-induced systemic inflammatory response versus exercise-induced immunomodulatory effects. *Sports Med* 36: 373-384, 2006.
- Fallon KE. The acute phase response and exercise. The ultra-marathon as prototype exercise. *Clin J Sport Med* 11: 38-43, 2001.
- Givalois L, Dornand J, Mekaouche M, Solier MD, Bristow AF, Ixart G, Siaud P, Assenmacher I, and Barbanel G. Temporal cascade of plasma levels surges in ACTH, corticosterone, and cytokines in endotoxin-challenged rats. *Am J Physiol* 267:R164-R170, 1994.
- Gomez-Merino D, Drogou C, Guezennec CY, Burnat P, Bourrilhon C, Tomaszewski A, Milhau S, and Chennaoui M. Comparison of systemic cytokine responses after a long distance triathlon and a 100-km run: relationship to metabolic and inflammatory processes. *Eur Cytokine Netw*17: 117-124, 2006.
- Hodgin KE, and Moss M. The epidemiology of sepsis. *Curr Pharm Des* 14: 1833-1839, 2008.
- Hollies NRS, and Goldman RF. Psychological scaling in comfort assessment. In Hollies NRS and Goldman RF (eds.), *Clothing comfort: Interaction of thermal, ventilation, construction, and assessment factors*, pp. 107-120. Ann Arbor Science Publishers Inc, 1977.
- Jeukendrup AE, and McLaughlin J. Carbohydrate ingestion during exercise: effects on performance, training adaptations and trainability of the gut. *Nestle NutrInst Workshop Ser*69:1-17, 2011.
- Jeukendrup AE, Vet-Joop K, Sturk A, Stegen JHJC, Senden J, Saris WHM, and Wagenmakers AJM. Relationship between gastro-intestinal complaints and endotoxaemia, cytokine release and the acute-phase reaction during and after a long-distance triathlon in highly trained men. *ClinSci* 98: 47-55, 2000.
- Lambert GP. Intestinal barrier dysfunction, endotoxemia, and gastrointestinal symptoms: the 'canary in the coal mine' during exercise-heat stress? *Med Sport Sci* 53: 61-73, 2008.
- Lambert GP. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. *J AnimSci* 87:E101-E108, 2009.
- Leon LR, and Helwig BG. Heat stroke: role of the systemic inflammatory response. *J ApplPhysiol* 109: 1980-1988, 2010.

26. Lim CL, and Mackinnon LT. The roles of exercise-induced immune system disturbances in the pathology of heat stroke: the dual pathway model of heat stroke. *Sports Med* 36: 39-64, 2006.
27. Margeli A, Skenderi K, Tsironi M, Hantzi E, Matalas AL, Vretou C, Kanavakis E, Chrousos G, and Papassotiropoulos I. Dramatic elevations of interleukin-6 and acute-phase reactants in athletes participating in the ultra-distance foot race spartathlon: severe systemic inflammation and lipid and lipoprotein changes in protracted exercise. *J Clin Endocrinol Metab* 90: 3914-3918, 2005.
28. Mastaloudis A, Morrow JD, Hopkins DW, Devaraj S, and Traber MG. Antioxidant supplementation prevents exercise-induced lipid peroxidation, but not inflammation in ultramarathon runners. *Free Radical Biol Med* 36: 1329-1341, 2004.
29. Morris G, Berk M, Galecki P, and Maes M. The Emerging Role of Autoimmunity in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/cfs). *Mol Neurobiol* 49: 741-756, 2014.
30. Murray A, and Costa RJS. Born to run. Studying the limits of human performance. *BMC Medicine* 10:(76) 1-3, 2012.
31. Neubauer O, Konig D, and Wagner K. Recovery after an Ironman triathlon: sustained inflammatory responses and muscular stress. *Eur J Appl Physiol* 104: 417-426, 2008.
32. O'Connor FG, Casa DJ, Bergeron MF, Carter R, Deuster P, Heled Y, Kark J, Leon L, McDermott B, O'Brien K, Roberts WO, and Sawka M. American College of Sports Medicine round table on exertional heat stroke. Return to duty/return to play: Conference Proceedings. *Curr Sports Med Rep* 9: 314-321, 2010.
33. Øktedalen O, Lunde OC, Opstad PK, Aabakken L, and Kvernebo K. Changes in the gastrointestinal mucosa after long-distance running. *Scand J Gastroenterol* 27: 270-274, 1992.
34. Opal SM. Endotoxins and other sepsis triggers. *Contrib Nephrol* 167: 14-24, 2010.
35. Ostrowski K, Rohde T, Asp S, Schjerling P, and Pedersen BK. Pro and anti-inflammatory cytokine balance in strenuous exercise in humans. *J Physiol* 515: 287-291, 1999.
36. Peters HP, Bos M, Seebregts L, Akkermans LM, van Berge Henegouwen GP, Bol E, Mosterd WL, and de Vries WR. Gastrointestinal symptoms in long-distance runners, cyclists, and triathletes: prevalence, medication, and etiology. *Am J Gastroenterol* 94: 1570-1581, 1999.
37. Peterson AMW, and Pedersen BK. The anti-inflammatory effect of exercise. *J Appl Physiol* 98: 1154-1162, 2005.
38. Pfeiffer B, Stellingwerff T, Hodgson AB, Randell R, Pöttgen K, Res P, and Jeukendrup AE. Nutritional intake and gastrointestinal problems during competitive endurance events. *Med Sci Sports Exerc* 44: 344-351, 2012.
39. Rav-Acha M, Hadad E, Epstein Y, Heled Y, and Moran DS. Fatal exertional heat stroke: a case series. *Am J Med Sci* 328: 84-87, 2004.
40. Rehrer NJ, Brouns F, Beckers EJ, Frey WO, Villiger B, Riddoch CJ, Menheere PP, and Saris WH. Physiological changes and gastro-intestinal symptoms as a result of ultraendurance running. *Eur J Appl Physiol Occup Physiol* 64: 1-8, 1992.
41. Rehrer NJ, and Meijer GA. Biomechanical vibration of the abdominal region during running and bicycling. *J Sports Med Phys Fitness* 31: 231-234, 1991.
42. Rhind SG, Gannon GA, Shepard RJ, Buguet A, Shek PN, and Radomski MW. Cytokine induction during exertional hyperthermia is abolished by core temperature clamping: neuroendocrine regulatory mechanisms. *Int J Hyperthermia* 20: 503-516, 2004.
43. Robson P. Elucidating the unexplained underperformance syndrome in endurance athletes: the interleukin-6 hypothesis. *Sports Med* 33, 10: 771-781, 2003.
44. Robson-Ansley P, Barwood M, Canavan J, Hack S, Eglin C, Davey S, Hewitt J, Hull J, and Ansley L. The effect of repeated endurance exercise on IL-6 and sIL-6R and their relationship with sensations of fatigue at rest. *Cytokine* 45: 111-116, 2009.
45. Sator BR. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterol* 126: 1620-1633, 2004.
46. Scheer BV, and Murray A. Al Andalus Ultra Trail: An observation of medical interventions during a 219-km, 5-Day ultramarathon Stage Race. *Clin J Sport Med* 21: 444-446, 2011.
47. Selkirk GA, McLellan TM, Wright HE, and Rhind SG. Mild endotoxemia, NF- κ B translocation, and cytokine increase during exertional heat stress in trained and untrained individuals. *Am J Physiol Regul Integr Comp Physiol* 295: 611-623, 2008.
48. Shubin NJ, Monaghan SF, and Ayala A. Anti-inflammatory mechanisms of sepsis. *Contrib Microbiol* 17: 108-124, 2011.
49. Starkie RL, Hargreaves M, Rolland J, and Febbraio MA. Heat stress, cytokines, and the immune response to exercise. *Brain Behav Immun* 19: 404-412, 2005.
50. Steensberg A, van Hall G, Osada T, Sacchetti M, Saltin B, and Pedersen BK. Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *J Physiol* 529: 237-242, 2000.
51. Suzuki K, Nakaji S, Yamada M, Liu Q, Kurakake S, Okamura N, Kumae T, Umeda T, and Sugawara K. Impact of a competitive marathon race on systemic cytokine and neutrophil responses. *Med Sci Sports Exerc* 35: 348-355, 2003.
52. Thomas JL. Helpful or harmful? Potential effects of exercise on select inflammatory conditions. *Phys Sportsmed* 41: 93-100, 2013.
53. ter Steege RW, and Kolkman JJ. Review article: the pathophysiology and management of gastrointestinal symptoms during physical exercise, and the role of splanchnic blood flow. *Aliment Pharmacol Ther* 35: 516-28, 2012.
54. Thomas DR, Cote TR, Lawhorne L, Levenson SA, Rubenstein LZ, Smith DA, Stefanacci RG, Tangalos EG, Morley JE, and the Dehydration Council. Understanding clinical dehydration and its treatment. *J Am Med Dir Assoc* 9: 292-301, 2008.
55. van Deventer SJ, Buller HR, ten Cate JW, Aarden LA, Hack CE, and Sturk A. Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. *Blood* 76: 2520-2526, 1990.
56. van Leeuwen PAM, Boermeester MA, Meyer APS, Westorp RIC, Houdijk APJ, Ferwerda CC, and Cuesta MA. Clinical significance of translocation. *Gut* 35: S28-S34, 1994.
57. van Wijck K, Lenaerts K, Grootjans J, Wijnands KP, Poeze M, van Loon LJ, Dejong CHC, and Buurman WA. Physiology and pathophysiology of splanchnic hypoperfusion and intestinal injury during exercise: strategies for evaluation and prevention. *Am J Physiol* 303: G155-G168, 2012.

58. vanWijck K, Lenaerts K, van Loon LJ, Peters WH, Buurman WA, andDejong CH. Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. *Plos One* 6(E22366): 1-9, 2012.
59. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, Fleshner M, Green C, Pederson BK, Hoffman-Goetz L, Rogers CJ, Northoff H, Abbasi A, and Simon P. Position statement. Part one: immune function and exercise. *ExercImmunol Rev* 17: 6-63, 2011.
60. Walsh NP, Gleeson M, Pyne DB, Nieman DC, Dhabhar FS, Shephard RJ, Oliver SJ, Bermon S, andKajeniene A. Position statement. Part two: maintaining immune health. *ExercImmunolRev* 17: 64-103, 2011.
61. Warren HS, Novitsky TJ, Ketchum PA, Roslansky PF, Kania S, and Siber GR. Neutralization of bacterial lipopolysaccharides by human plasma. *J ClinMicrobiol* 22: 590-595, 1985.
62. Wendt D, van Loon LJ, andLichtenbelt WD. Thermoregulation during exercise in the heat: strategies for maintaining health and performance. *Sports Med* 37: 669-682, 2007.
63. Woolley N, Mustalahti K, Mäki M, and Partanen J. Cytokine gene polymorphisms and genetic association with coeliac disease in the Finnish population. *Scand J Immunol* 61: 51-56, 2005.