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Title: Short-term reliability of inflammatory mediators and response to exercise in the heat.

Running Title: Reliability of inflammatory mediators and response to exercise.

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Abstract

Prospective application of serum cytokines, lipopolysaccharide, and heat shock proteins requires reliable measurement of these biomarkers that can signify exercise-induced heat stress in hot conditions. To accomplish this, both short-term (seven day) reliability (at rest, n=12) and the acute responsiveness of each biomarker to exercise in the heat (pre and post 60 min cycling, 34.5^{o}C and 70% RH, n=20) were evaluated. Serum was analysed for the concentration of C-reactive protein (CRP), interleukin (IL-6), heat shock protein 72 (eHSP72), immunoglobulin M (IgM) and lipopolysaccharide (LPS). Test-retest reliability was determined as the coefficient of variation (CV). Biomarkers with the least short-term within-subject variation were IL-6 (19%, ± 20%; CV, ± 95% confidence limits) and LPS (23%, ± 13%). Greater variability was observed for IgM, eHSP72 and CRP (CV range 28-38%). IL-6 exhibited the largest increase in response to acute exercise (95%, ± 11%, p = <0.001) and although CRP had a modest CV (12%, ± 7%) it increased substantially post-exercise (p = 0.02, ES; 0.78). In contrast, eHSP72 and LPS exhibited trivial changes post-exercise. It appears variation of common inflammatory markers after exercise in the heat is not always discernible from short-term (weekly) variation.

Keywords Lipopolysaccharide, heat shock proteins, inflammatory cytokines, heat tolerance.

Introduction

Uncompensable heat stress experienced either passively or in response to exercise in the heat influences a complex network of thermoregulatory, immune, inflammatory and neuromuscular factors (Pyne, Guy, and Edwards, 2014). In extreme cases this inflammation can culminate in multi-organ failure and even death (Singh, Kapoor, and Singh, 2013). In the context of exercise and physical activity, induction of an inflammatory response plays an important role in this process after transient heat can damage the gastrointestinal tract, causing it to become permeable, leading to leakage of harmful bacterial endotoxins from the gut into the circulation (Pyne et al, 2014)..

Exercise-induced endotoxemia has been attributed primarily to lipopolysaccharide (LPS) translocation from the gut into the circulation (Lim, et al., 2009). An abundance of circulating LPS can evoke an inflammatory response, leading to heat shock and overwhelming of anti-LPS mechanisms including the antibody, immunoglobulin M (IgM), (Cohen, Block, Green, Lowell, and Ofek, 1989), and cytokines such as interleukin-6 (IL-6) operating in an anti-inflammatory role (Abbasi et al., 2013). Therefore, when the anti-LPS mechanisms and rate of LPS clearance are inadequate to counter the heat-induced increase of LPS, endotoxemia may ensue.

A rise in extracellular heat shock protein (eHSP) concentration is a consequence of an innate immune response to whole body hyperthermia (Ahlers et al., 2005). In this scenario an acute phase immune response is evoked to counteract heat-induced oxidative stress leading to an increase in leukocyte and eHSP concentrations (Mestre-Alfaro et al., 2012). Numerous studies have demonstrated that non-critical exposure to heat may increase both tolerance to

69 oxidative stress and effectiveness of anti-LPS mechanisms (Pilch et al., 2014; Pyne et al.
70 2014; Yeh, Law, and Lim, 2013).
71
72 Several studies have used blood biomarkers to quantify the magnitude of adaptation to hot
73 environmental conditions, although a comparison of short-term variability in exercise-
74 induced biomarkers has not yet been conducted. This is surprising as there is considerable
75 variation in the magnitude of exercise-induced change to markers such as interleukin (IL)-6,
76 C-reactive protein, LPS and eHSP72 following a bout of exercise in hot conditions (Hailes,
77 Slivka, Cuddy and Ruby, 2011; Lim et al., 2009; Marshal, Campbell, Roberts and Nimmo,
78 2007; Rhind et al., 2004; Wright et al., 2013). As a common length for a short-term heat
79 acclimation protocol for athletes is seven days (Garrett, Rehrer and Patterson, 2011) further
80 investigation into the variation of these biomarkers is warranted. The utility of individual
81 biomarkers may depend on typical variation (noise) under normal conditions, and the
82 magnitude of the response to exercise in the heat (signal). The issue is whether the noise is
83 sufficiently small so as to not mask biologically and/or clinically important changes or
84 differences. While some biomarkers may exhibit substantial short-term variability, they could
85 still be useful if the exercise stimulus produces a sufficiently large signal (response). This is a
86 point often overlooked in the study of reliability of biomarkers.
87 Therefore, it is important to quantify reliable, relevant, and objective outcome measures of
88 the immune and inflammatory responses.
89
90 The aim of this study was to quantify the reliability (short term test re-test reliability) in the
91 concentration of common inflammatory (blood) biomarkers at rest (twice over seven days,
92 Part A). A second aim was to examine the acute response of those biomarkers to an exercise
93 challenge performed in hot and humid conditions (Part B).

Materials and methods

Experimental Design

Part A: Short-term reliability of serum biomarkers.

This phase of the study was designed to examine the weekly variation in venous blood of selected biomarkers in a non-exercise context and was conducted over 14 days (Figure 1). The seven days preceding the first test day were used as a "lead-in" period and participants were instructed to abstain from partaking in moderate -high intensity physical activity for the duration of the study period. Participants then had venous blood drawn on two occasions seven days apart. Venous blood was drawn in a seated position prior to and immediately following the heat stress test. Blood was sampled approximately 2 h post-prandial at a similar time of day (morning) to limit diurnal variation. At the beginning of the lead-in period all participants undertook a baseline evaluation of maximum oxygen uptake ($\dot{V}O_{2max}$) using an incremental treadmill running test to exhaustion. A seven day controlled lead-in or baseline period was used to ensure that the participants were not suffering from any residual inflammatory effects of exercise or illness prior to taking part in this study. Participants were instructed to maintain a similar dietary intake and (light) activity levels for 24 h preceding each venous blood sample.

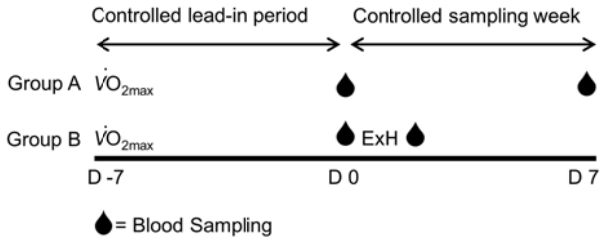


Figure 1. Schematic illustration of the experimental procedures showing that blood was sampled at D 1 (day one, Part A and Part B) and D 7 (Day seven, Part A). ExH; Exercise in the heat intervention (Part B).

Part B: Acute response of serum biomarkers to exercise in the heat.

This phase of the study examined the acute response of biomarkers to exercise performed in the heat. To aid robust evaluation of biomarkers free from influence of prior exercise, this part of the study also contained a seven day lead-in period prior to assessment. At baseline, all participants performed an incremental test to exhaustion for the assessment of $\dot{V}O_{2max}$ on a cycle ergometer - the same modality as the subsequent heat stress test protocol. As before, all participants were required to abstain from moderate-high intensity exercise for the remainder of the seven day lead-in period prior to further assessment of pre- to post-exercise evaluation of biomarker activity. The exercise in the heat test occurred seven days after baseline evaluation of $\dot{V}O_{2max}$. Venous blood was drawn in a seated position prior to and immediately following the heat stress test. Blood was sampled approximately 2 h post-prandial at a similar time of day for all participants (morning) to limit diurnal variation.

Participants

Participants in Part A of this study (short-term variation) comprised twelve healthy moderately-trained males (age 24.3±4.1 years, $\dot{V}O_{2max}$ 52.0±2.7 ml.kg.min^{-1}, height 1.78±0.09 m, mass 73.9±8.5 kg, mean ±SD). Part B participants (acute response to exercise in the heat intervention) comprised twenty males (age 24.6±3.7 years, $\dot{V}O_{2max}$ 43.2±5.4 ml.kg.min^{-1}, height 1.78±0.07 m, mass 83.5±11.0 kg). All participants completed a pre-screening medical questionnaire the screened for the use of immunomodulating medications (none were present). After explanation of the study procedures, benefits and risks, participants provided written informed consent before inclusion in the project. This study was approved by the James Cook University Human Research Ethics Committee and conformed to the guidelines set forth by the Helsinki Declaration. Participants in Part A were also required to complete a daily physical activity diary for the duration of the study so that any

exercise undertaken could be quantified for intensity and duration. All participants were also required to self-report any symptoms of illness, inflammation, or soreness.

Blood collection

For both Parts A and B, blood was drawn via a 22g needle from a prominent superficial forearm vein located at the antecubital fossa, and drained directly into an 8.5 ml sterile serum separator Vacutainer tube containing a clot activator and gel for serum separation (Beckton and Dickson, USA). Samples were refrigerated at 4^{o}C for 30 min to allow clotting and then centrifuged at 1000 x *g* at 6^{o}C for 10 min (Rotina 420R, Hettich, Germany). Serum was removed and stored in 400 µl aliquots frozen immediately for a maximum of three months at -80^{o}C for later analysis. Levels of IL-6 (Quantikine HS600B, R&D Systems, United States), inducible eHSP72 (HSP72;ADI-EKS-715, Enzo Life Sciences, United States), IgM (AB137982, Abcam PLC, United Kingdom), CRP (hsCRP Immunoassay kit 11190, Oxis International, United States), and LPS (HIT302, Hycult, Biotechnology, Netherlands) were analysed in duplicate by ELISA according to the manufacturer's instructions. The manufacturer stated intra-assay precision was <10% for all assays. Additionally, the in-house intra- and inter-assay coefficient of variations were calculated and are provided in Table 1.

Table 1. Intra- and inter-assay variability

Biomarker	Intra-assay CV	Inter-assay CV
eHSP70	2.2, ± 2.7 %	11.9, ± 7.1 %
LPS	4.2, ± 2.9%	17.3, ± 20.2 %
IL-6	4.7, ± 3.6 %	15.4, ± 15.6 %
IgM	3.1, ± 1.9 %	8.2, ± 5.5 %
CRP	4.1, ± 4.6 %	22.4, ± 11.6

Biomarkers presented as intra- and inter-assay mean coefficient of variation (CV), ± 95% CI. eHSP72; extracellular heat shock protein. LPS; lipopolysaccharide. IL-6; interleukin-6. IgM; immunoglobulin M. CRP; C-reactive protein.

160 *Exercise in the heat protocol (Part B)*

161 Participants in Part B undertook an exercise test involving three submaximal workloads of 10
162 min duration (50%, 60% and 70% $\dot{V}O_{2max}$) on a cycle ergometer followed by a 5 km time trial
163 (TT) at 35^{o}C and 70% relative humidity (RH) (VeloTron Dynafit Pro and Velotron Coaching
164 Software, Racermate, United States). Three min rest was given between submaximal
165 workloads and five min rest was given prior to the start of the TT. Participants undertook
166 approximately 40 min of exercise and were exposed to the hot humid environment for 60-65
167 min. Briefly, the submaximal workloads required the participants to cycle at a fixed wattage
168 between 85-95 rpm. During the TT the participants were able to self-select their gearing and
169 informed of their rpm and distance every 500m. Participants were not aware of their gear,
170 speed, or time elapsed during the TT. A standardised warm-up of 5 min cycling at 40% of
171 $\dot{V}O_{2max}$ followed by dynamic stretching was undertaken prior to the 50% workload. Heart rate
172 (RS400, Polar Elektro, Finland), and core temperature (T_c) (ttec 501-3, software version 10.1,
173 Nordex Pty Ltd, Australia; MEAS 449 1RJ rectal temperature thermistor, measurement
174 specialities, United States) were sampled at 5s intervals. Fluid intake (water, ad libitum) and
175 rating of perceived exertion (Borg RPE 6 – 20) were recorded throughout the test (Borg,
176 1970). Nude dry body mass was recorded pre and post exercise and body mass was
177 normalised for fluid loss and expressed as a percentage change.
178
179 *Statistical Analysis*

180 The concentration of each biomarker is presented as mean ± SD. Biomarker reliability was
181 calculated as a coefficient of variation (CV) both within- and -between subjects at day o and
182 day 7 and presented as mean %CV ± 95% confidence limits (CL). Day 0 to day seven and
183 pre- to post-exercise changes in biomarker concentrations were analysed with paired t-tests
184 and significance was accepted if p was <0.05. Effect sizes for changes in biomarker

concentrations were also calculated. The expected reference change, or signal, was estimated for each biomarker as 0.2 x between-subject standard deviation.

The criteria to interpret the magnitude of ES were: trivial (0–0.19), small (0.20–0.49), medium (0.50–0.79) and large (0.80 and greater) (Cohen, 1992).The signal to noise ratio score was determined by dividing the reference effect size (signal) by the within-subject test-retest reliability (noise). The utility of a biomarker was considered 'good' if the expected signal was greater than the noise, or 'unclear' where the signal was less than the noise. A minimum of eight participants was deemed sufficient to detect the smallest worthwhile change between means assuming the reference change was approximately twice the magnitude of the typical error of measurement, with a Type I error of 5% and Type II error of 20%. Biomarker concentrations and curve fit was performed using GraphPad Prism Version 6.03 (GraphPad Software Inc, United States) according to the manufacturer instructions. Statistical analyses were performed in IBM SPSS Statistics Version 20 (IBM, United States).

Results

Part A: Short-term biomarker reliability

The biomarker with the lowest within-subject coefficient of variation over the 7 day assessment period (day 0 to day 7) was IL-6 (CV; 19% ± 20%, mean ± 95% CI, ES; 0.16,). CRP had the highest CV (38% ± 21%) with a substantially lower level of serum concentration (ES; -0.28) after seven days (Table 2), although none of the biomarkers changed significantly over this period ($p>0.05$). A comparison of the within-subject variability for each biomarker with an expected reference change is detailed in Table 2. Biomarkers that displayed a good signal to noise ratio were IL-6 and CRP. The expected signal for LPS, IgM and eHSP72 was less than that of the typical noise estimated in this analysis. In-house quality control procedures indicated that this variation was not due to a

problems with the assay itself, and therefore the biomarkers were categorised as having unclear or poor reliability (Table 2).

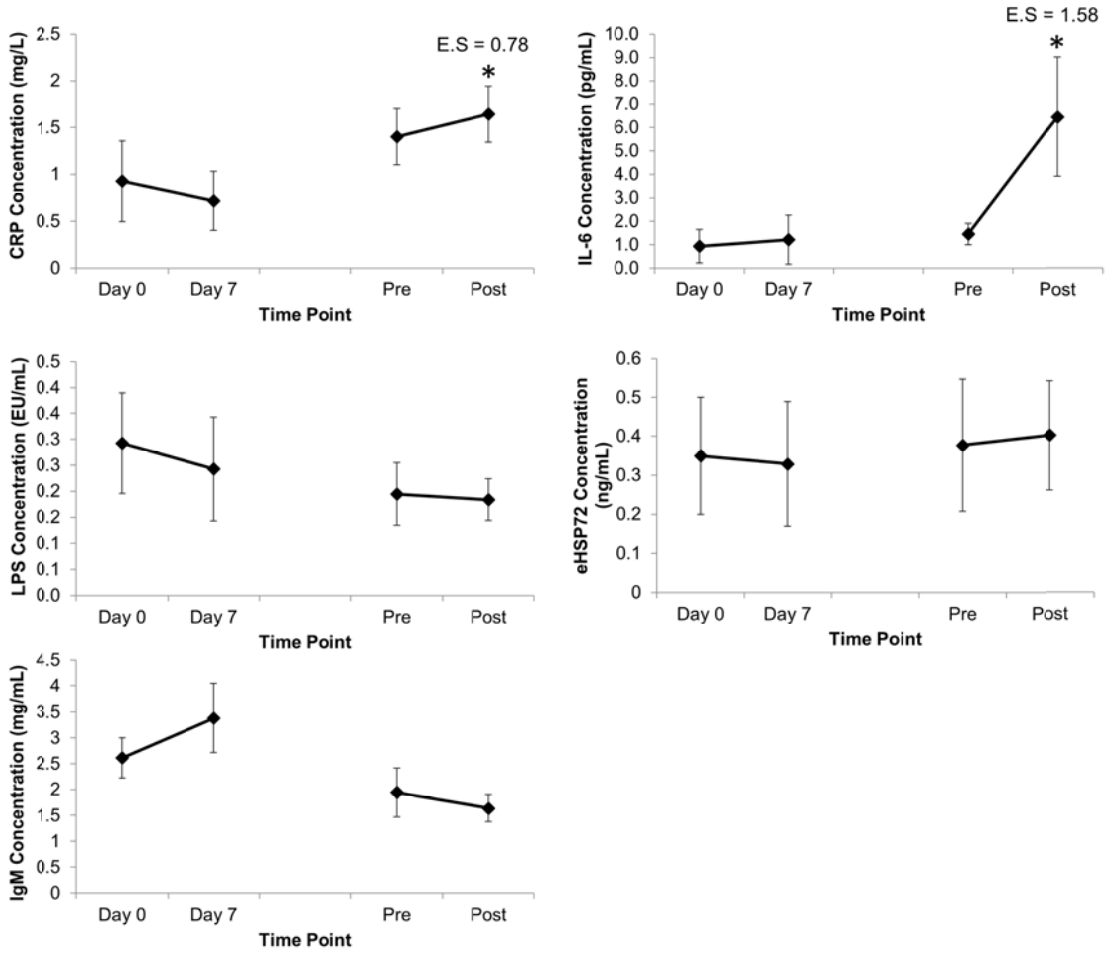
Table 2. Coefficient of variation both within (day zero to day seven) and between subjects with inferences to the reliability and usefulness (signal to noise) of selected biomarkers

		Noise		Signal		Signal to Noise	
Biomarker	Concentration Day 0	Within-subject CV Day 0 to Day 7	Between-subject CV Day 0	Within-subject E.S	Pre to Post E.S	Ratio Score	Inference
eHSP72	0.35 ± 0.07 ng/mL	37%, ± 23%	62%	-0.67	0.08	0.12	Unclear
LPS	0.29 ± 0.04 EU/mL	23%, ± 13%	41%	-0.55	-0.06	0.11	Unclear
IL-6	0.94 ± 0.45 pg/mL	19%, ± 20%	153%	0.16	1.58	9.88	Good
IgM	2.56 ± 0.29 mg/mL	28%, ± 17%	261%	0.73	-0.42	0.57	Unclear
CRP	0.93 ± 0.72 mg/L	38%, ± 21%	93%	-0.28	0.78	2.78	Good

Biomarker concentrations are presented as mean ± SD, within-subject coefficient of variation (CV) is presented as mean, ± 95% CI. E.S; Effect size (Cohen's d), trivial (0–0.19), small (0.20–0.49), medium (0.50–0.79) and large (0.80 and greater). Within-subject effect size was calculated from the typical change in the mean (raw units) of the measured parameter from day 0 to day 7. Ratio score was calculated by dividing the pre to post effect size by the within-subject effect size and was considered 'good' if the expected signal was greater than the noise, or 'unclear' where the signal was less than the noise. CRP; C-reactive protein. eHSP72; extracellular heat shock protein. IL-6; interleukin-6. LPS; lipopolysaccharide. IgM; immunoglobulin M.

214 *Part B: Acute responses of blood biomarkers to exercise in the heat*

215 Blood biomarkers with the largest pre- to post-exercise change were IL-6 ($p < 0.001$) and
216 CRP ($p = 0.02$). The blood biomarkers least sensitive to change following the exercise in the
217 heat exposure were IgM, LPS and eHSP72 ($p > 0.05$). The exhaustive nature of the exercise
218 task was confirmed with high levels of physiological and perceptual stress (Table 3). Changes
219 in mean blood biomarker concentration in addition to effect sizes pre-to-post exercise in the
220 heat are presented in Figure 2.



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222 **Figure 2.** Serum biomarker concentrations presented as mean ± SD from Part A (Short-term;
223 Day 1 and Day 7) and Part B (Exercise in the heat; Pre and Post). * = significantly different
224 from pre concentration. CRP; C-reactive protein. eHSP72; extracellular heat shock protein.
225 IL-6; interleukin-6. LPS; lipopolysaccharide. IgM; immunoglobulin M. E.S = Effect size
226 (Cohen's d), trivial (0-0.19), medium (0.20-0.49), and large (0.80 and greater).

Table 3. Physiological and perceptual responses to the exercise task in the heat

Measure	Group B
5km TT time (s)	626 ± 100
Peak HR (bpm)	187 ± 5
Mean HR (bpm)	160 ± 19
Peak core temperature (^{o}C)	38.9 ± 0.2
Reduction in body mass (%)	1.7 ± 0.3
End point RPE (units)	17 ± 1

Data is presented as mean ± SD. TT; time trial. HR; heart rate. RPE; rating of perceived exertion

227

228 **Discussion**

229 The biomarker IL-6 exhibited the smallest within-subject short-term variation (19%) and the
230 greatest acute pre- to post-exercise change in the heat (4.5 fold change). For the other
231 biomarkers, the short-term resting variation was similar to that of pre- to post-exercise
232 evaluations in the heat, indicating minimal alteration to an acute bout of exercise. It appears
233 only some biomarkers are potentially useful for the purpose of reliably quantifying acute
234 physiological responses in healthy active individuals to hot environmental conditions that
235 elicit modest rises in T_c.

236 Even in a resting state, considerable weekly variation was evident for each variable. The
237 cytokine IL-6 exhibited the least within-subject variability of 19% whereas other biomarkers
238 such as CRP varied by 38%. The magnitude of this variation is considered concurrently with
239 the expected change in response to an exercise challenge or a period of training, and can be
240 used to inform the decision making process on effects of heat stress (Table 2). Quantifying
241 variation is an inherent part of studying biological systems and can yield important
242 information when seeking to determine whether or not intervention-induced change in a
243 measured parameter is meaningful.

The exercise presented to the participants resulted in a mean core temperature rise of 1.5^{o}C above baseline levels and the duration of heat exposure was 65 mins, of which 40 mins was dedicated exercise. Although concentrations of IL-6 and the acute phase protein CRP were elevated following exercise, other biomarkers indicative of heat stress such as LPS and eHSP72 did not rise significantly from pre-exposure levels. Serum concentration of IgM also did not rise but instead there was a small 15% reduction in circulation following the exercise bout. It seems plausible that a modest reduction in IgM concentration post exercise reflects the anti-LPS properties of this antibody in response to mild heat stress. This observation is consistent with the findings of Camus et al. (1998), but not of Hailes et al. (2011) and Lim et al. (2009). The exercise stimulus elicited a response from non-specific pro- and anti-inflammatory blood biomarkers, however it was not sufficient to cause further inflammatory processes associated with heat stress in healthy, moderately trained males.

The significant increase of IL-6 concentration post-exercise may not signify heat stress per se, but rather the stress invoked by the exercise demand itself. IL-6 can be released into the circulation following various pathological events such as physical exercise, trauma, sepsis, and thermal injury (Moldoveanu , Shephard, and Shek, 2000). There are few studies that have investigated IL-6 as a blood biomarker during exhaustive exercise in the heat, although Selkirk and colleagues (2008) observed a large increase following 2h of exhaustive walking in protective clothing in very hot and humid conditions. However, similar effects have been detected following exercise in the absence of a significant heat load. Moldoveanu and colleagues (2000) reported a twenty-fold increase in plasma IL-6 concentrations following 3h of exercise at 60-65% of peak oxygen uptake in a thermo-neutral environment - this change is similar in magnitude to that reported by Selkirk et al. (2008).

The large within-subject variation observed for CRP (38%) raises the question of its suitability as a meaningful biomarker. However, in this study, the biomarker noise (short-

term, within-subject variability) was less than that of the signal (response to the exercise task) and there was a medium increase in CRP concentration pre- to post-exercise ($p = 0.02$, ES; 0.78). Serum levels of CRP can increase rapidly during the acute phase of an inflammatory process (Pepys and Hirschfield, 2003), but this is a non-specific response that could be indicative of infection, illness or other metabolic factors not associated with a heat stimulus. A recent study (Hailes et al., 2011) that measured CRP in serum following 5 consecutive days of exercise in hot and dry conditions (38^{o} C and 40% RH) reported high variability between participants and a standard deviation approximately twice that of the mean after both an acute and ongoing exposure to heat. As the presence of IL-6 is likely to cause an increase in serum levels of CRP (Petersen and Pedersen, 2005), it is likely that the exercise stimulus, and not necessarily the heat load presented to the participants was sufficient to stimulate the release of CRP from the liver. Although both IL-6 and CRP may play important roles in determining the degree of stress placed upon individuals competing or training in more extreme (hot and/or humid) conditions, although it seems unlikely this measure would present useful information in terms of responses or adaptations to the heat specifically.

The low within-subject variability of LPS (CV; 23%) was encouraging for the practical application of this biomarker for evaluating responses to hot environmental conditions. The low concentrations of LPS observed in this study indicate the participants had the capacity to tolerate the heat load with minimal gut leakage (Pyne et al., 2014). As LPS is the primary endotoxin translocated to circulation under heat load (Yeh et al., 2013), its concentration and regulation is a primary consideration in study of responses to the heat. The outcomes of this study indicate that LPS evaluation in circulating blood should yield reliable results provided the participants are well rested or are capable of completing a demanding exercise task. Nevertheless, measurement of LPS alone merely indicates the extent of susceptibility to

endotoxemia and not the responses of the immune system initiated by this challenge, which can be investigated using other measures such as intestinal fatty acid-binding protein (Morrison, Cheung, and Cotter, 2013), tight junction proteins that indicate increased intestinal permeability (Yeh at al. 2013) or soluble CD14 (Stuempfle, Valentino, Hew-Butler, Hecht, & Hoffman., 2015). Therefore, to facilitate a comprehensive view of both the underlying endotoxin threat, and compensatory biochemical mechanisms addressing this challenge, it is worthwhile to consider the utility of other viable biomarkers such as IgM and eHSP72.

The responsiveness of the immune system to release endotoxin is a primary consideration in defence against heat shock. As IgM is a key antibody in neutralising LPS (Camus et al., 1998), its concentration in circulating blood can reflect the body's response to endotoxin accumulation, and the likelihood of protective capacity to further challenges. In this study the observed weekly variability of IgM concentration was 28%. The pre- to post-exercise change was -15%, with 13 of the 20 participants exhibiting a negative change. To our knowledge only one other study has investigated the response of non-specific IgM following exercise in hot and humid conditions (Hailes et al., 2011). However, the reference change reported by Hailes and colleagues (2011) pre- to post-exercise in the heat (CV; 16%) is smaller than the within-subject variability (noise) reported here (CV; 29%). It appears that IgM has shortcomings as a viable biomarker for quantifying the anti-LPS response, and this is possibly related to the capability of the participants to tolerate the heat load placed upon them, although these data suggest that this response could result in either an increase or decrease in circulating concentrations. Future research is needed to clarify why some individuals respond in this manner.

Inducible eHSP72 exhibited high short-term variability (37%), however, the pre- to post-exercise change was trivial. In this study the heat load was seemingly not sufficient to induce a significant change in serum concentration of eHSP72. The usefulness of this variable must also be considered against the intended heat load and it may only be useful to quantify the magnitude of response and adaptations to hot environmental conditions, provided the heat stimulus is large enough (Ogura et al., 2008). This may be achieved through longer duration or core temperature clamping protocols and it seems likely that heat loads that cause an increase in core temperature >39^{o}C are needed to evoke LPS translocation and induction of eHSP72 (Pyne et al., 2014).

Between-subject variation also provides useful information for researchers interested in the utility of different measurements. Low within-subject variation indicates that an individual could be expected to provide a similar result on repeated occasions under constant conditions. Therefore, on an individual basis this increases the likelihood that resting or post-exercise measurements could be useful. Conversely, low between-subject variation indicates that all individuals in a cohort exhibit similar concentrations and/or regulate the variable at a similar level. For example, the participants in this study regulated IL-6 at very low and consistent levels. The observation of large between-subject variation for biomarkers such CRP may necessitate the recruitment of more participants to compress the variation between individuals. However, this type of approach may also limit the interpretation of results and doesn't permit (easy) determination of an individual's response to heat acclimation (Racinais et al., 2013).

Furthermore, as the intra-assay CV was better than the manufacturer stated CV of <10% for all assays (Table 1), it is likely that the changes and variation observed in blood biomarker concentrations were indicative of the biological variation at rest, or in response to the exercise task. Although methods such as repeat quality control of samples could be used if

possible, however due to plate availability limitations it was not possible to do so for all samples in this study. The use of duplicate measure in assays is a standard procedure, although triplicate measures (where possible) can aid in the compression of within-sample variation.

Although this study employed the use of an exercise task in the heat, it has been discussed that exercise in temperate environments can also result in large changes to immune biomarkers such as IL-6 and IgM, and future studies may choose to include an exercise matched task in a temperate environment to quantify the degree of change following exercise in those conditions. The user of an exercise task in the heat in this study was chosen to place a large load on the participants, both from the physical demands of the exercise task, and the demands of thermoregulation in a hot and humid environment. Future studies should also examine whether highly-trained athletes respond differently to moderately-trained individuals, the differential effects of exercise in the heat as well as temperate conditions, and the influence of a prior history of heat acclimation or acclimatisation on concentrations of inflammatory mediators.

A limitation of this study was the differing level of aerobic fitness of the subjects in Parts A (VO_2 max 52 ml.kg.min^{-1}) and B (43 ml.kg.min^{-1}), participants were convenience sampled from a local university and sporting club population, with those unable to commit to the full 14 day period protocol (Group A) allocated to Group B, due to sporting commitments that would likely interfere with resting levels of the blood biomarkers. Although the participants in each group had differing fitness levels as indicated by their VO_2 max this is more likely due to the protocol modality. Participants in Group A underwent their VO_2 max on a treadmill and participants in Group B underwent their VO_2 max on a cycle ergometer, as the

vast majority of participants partook in either running or team sports such as football (soccer), this would likely account for the differences in VO_2 max, as differences of ~11% have been reported between cycling and running protocols in running athletes (Basset and Boulay, 2000). The decision to use a cycle ergometer for Group B was to a) Limit the trips to the laboratory for each participant by using a single test for both VO_2 max and to calculate individual loads for the subsequent HST, although future studies may choose to use more consistent protocols

Conclusion

Quantifying the inherent variation of biological systems affected by exercise in hot and humid environment can help informs the choice of inflammatory biomarkers. The utility of the selected biomarkers IL-6 and CRP appears useful to quantify the inflammatory responses to exercise, even when presented with a high (but tolerable) exercise load in the heat. However, the short-term variability of other biomarkers such as eHSP72, LPS and IgM overshadows the observed change following 65 mins of exercise and exposure to a hot environment. The within-subject analysis also indicates that individuals consistently regulate the concentration of these biomarkers within homeostatic limits when measured seven days apart. However, the relatively high between-subject variation indicates that it is not possible to establish a standardised concentration of each biomarker suitable for all individuals. It appears that a substantial heat and exercise stimulus (i.e. $T_c > 39^oC$) is needed to evoke further responses associated with heat stress and the inflammatory cascade.

Conflict of Interest No conflict of interest, financial or otherwise is declared by the authors.

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