

1 Manuscript Type: Original Investigation

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

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

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24 Word count:

25 Abstract: 192 words.

26 Manuscript: 4083 words.

27 **Abstract**

28 Prospective application of serum cytokines, lipopolysaccharide, and heat shock proteins  
29 requires reliable measurement of these biomarkers that can signify exercise-induced heat  
30 stress in hot conditions. To accomplish this, both short-term (seven day) reliability (at rest,  
31 n=12) and the acute responsiveness of each biomarker to exercise in the heat (pre and post 60  
32 min cycling, 34.5°C and 70% RH, n=20) were evaluated. Serum was analysed for the  
33 concentration of C-reactive protein (CRP), interleukin (IL-6), heat shock protein 72  
34 (eHSP72), immunoglobulin M (IgM) and lipopolysaccharide (LPS). Test-retest reliability  
35 was determined as the coefficient of variation (CV). Biomarkers with the least short-term  
36 within-subject variation were IL-6 (19%, ± 20%; CV, ± 95% confidence limits) and LPS  
37 (23%, ± 13%). Greater variability was observed for IgM, eHSP72 and CRP (CV range 28-  
38 38%). IL-6 exhibited the largest increase in response to acute exercise (95%, ± 11%, p =  
39 <0.001) and although CRP had a modest CV (12%, ± 7%) it increased substantially post-  
40 exercise (p = 0.02, ES; 0.78). In contrast, eHSP72 and LPS exhibited trivial changes post-  
41 exercise. It appears variation of common inflammatory markers after exercise in the heat is  
42 not always discernible from short-term (weekly) variation.

43 **Keywords** Lipopolysaccharide, heat shock proteins, inflammatory cytokines, heat  
44 tolerance.

45 **Introduction**

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

54  
55 Exercise-induced endotoxemia has been attributed primarily to lipopolysaccharide (LPS)  
56 translocation from the gut into the circulation (Lim, et al., 2009). An abundance of circulating  
57 LPS can evoke an inflammatory response, leading to heat shock and overwhelming of anti-  
58 LPS mechanisms including the antibody, immunoglobulin M (IgM), (Cohen, Block, Green,  
59 Lowell, and Ofek, 1989), and cytokines such as interleukin-6 (IL-6) operating in an anti-  
60 inflammatory role (Abbasi et al., 2013). Therefore, when the anti-LPS mechanisms and rate  
61 of LPS clearance are inadequate to counter the heat-induced increase of LPS, endotoxemia  
62 may ensue.

63  
64 A rise in extracellular heat shock protein (eHSP) concentration is a consequence of an innate  
65 immune response to whole body hyperthermia (Ahlers et al., 2005). In this scenario an acute  
66 phase immune response is evoked to counteract heat-induced oxidative stress leading to an  
67 increase in leukocyte and eHSP concentrations (Mestre-Alfaro et al., 2012). Numerous  
68 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).



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

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

128

### 129 *Participants*

130 Participants in Part A of this study (short-term variation) comprised twelve healthy  
131 moderately-trained males (age  $24.3 \pm 4.1$  years,  $\dot{V}O_{2\max}$   $52.0 \pm 2.7$  ml.kg.min<sup>-1</sup>, height  
132  $1.78 \pm 0.09$  m, mass  $73.9 \pm 8.5$  kg, mean  $\pm$ SD). Part B participants (acute response to exercise  
133 in the heat intervention) comprised twenty males (age  $24.6 \pm 3.7$  years,  $\dot{V}O_{2\max}$   $43.2 \pm 5.4$   
134 ml.kg.min<sup>-1</sup>, height  $1.78 \pm 0.07$  m, mass  $83.5 \pm 11.0$  kg). All participants completed a pre-  
135 screening medical questionnaire the screened for the use of immunomodulating medications  
136 (none were present). After explanation of the study procedures, benefits and risks,  
137 participants provided written informed consent before inclusion in the project. This study was  
138 approved by the James Cook University Human Research Ethics Committee and conformed  
139 to the guidelines set forth by the Helsinki Declaration. Participants in Part A were also  
140 required to complete a daily physical activity diary for the duration of the study so that any

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

143

#### 144 *Blood collection*

145 For both Parts A and B, blood was drawn via a 22g needle from a prominent superficial  
146 forearm vein located at the antecubital fossa, and drained directly into an 8.5 ml sterile serum  
147 separator Vacutainer tube containing a clot activator and gel for serum separation (Beckton  
148 and Dickson, USA). Samples were refrigerated at 4°C for 30 min to allow clotting and then  
149 centrifuged at 1000 x g at 6°C for 10 min (Rotina 420R, Hettich, Germany). Serum was  
150 removed and stored in 400 µl aliquots frozen immediately for a maximum of three months at  
151 -80°C for later analysis. Levels of IL-6 (Quantikine HS600B, R&D Systems, United States),  
152 inducible eHSP72 (HSP72;ADI-EKS-715, Enzo Life Sciences, United States), IgM  
153 (AB137982, Abcam PLC, United Kingdom), CRP (hsCRP Immunoassay kit 11190, Oxis  
154 International, United States), and LPS (HIT302, Hycult, Biotechnology, Netherlands) were  
155 analysed in duplicate by ELISA according to the manufacturer's instructions. The  
156 manufacturer stated intra-assay precision was <10% for all assays. Additionally, the in-house  
157 intra- and inter-assay coefficient of variations were calculated and are provided in Table 1.

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Table 1. Intra- and inter-assay variability

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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

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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.

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158

159

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°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  $\pm$  SD. Biomarker reliability was  
181 calculated as a coefficient of variation (CV) both within- and -between subjects at day 0 and  
182 day 7 and presented as mean %CV  $\pm$  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



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

187 The criteria to interpret the magnitude of ES were: trivial (0–0.19), small (0.20–0.49),  
188 medium (0.50–0.79) and large (0.80 and greater) (Cohen, 1992). The signal to noise ratio  
189 score was determined by dividing the reference effect size (signal) by the within-subject test-  
190 retest reliability (noise). The utility of a biomarker was considered ‘good’ if the expected  
191 signal was greater than the noise, or ‘unclear’ where the signal was less than the noise. A  
192 minimum of eight participants was deemed sufficient to detect the smallest worthwhile  
193 change between means assuming the reference change was approximately twice the  
194 magnitude of the typical error of measurement, with a Type I error of 5% and Type II error of  
195 20%. Biomarker concentrations and curve fit was performed using GraphPad Prism Version  
196 6.03 (GraphPad Software Inc, United States) according to the manufacturer instructions.  
197 Statistical analyses were performed in IBM SPSS Statistics Version 20 (IBM, United States).

## 198 **Results**

### 199 *Part A: Short-term biomarker reliability*

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

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

211

**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

Biomarker	Concentration Day 0	Noise		Within-subject E.S	Signal	Signal to Noise	
		Within-subject CV Day 0 to Day 7	Between- subject CV Day 0		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

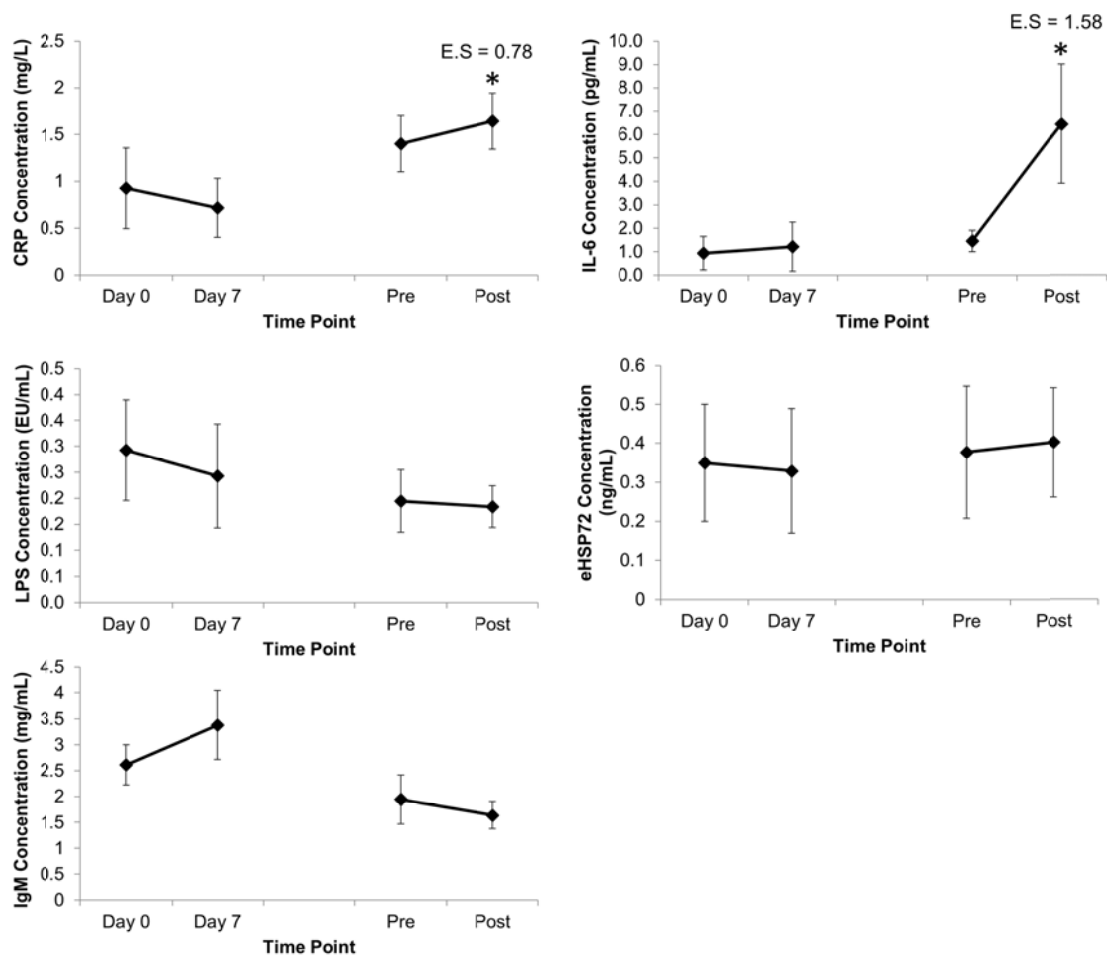
Biomarker concentrations are presented as mean ± SD, within-subject coefficient of variation (CV) is presented as mean, ± 95% CI. E.S; Effect size (Cohen's d), trivial (0–0.19), small (0.20–0.49), medium (0.50–0.79) and large (0.80 and greater). Within-subject effect size was calculated from the typical 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.

212

213

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.



221

222 **Figure 2.** Serum biomarker concentrations presented as mean  $\pm$  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 (°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.

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

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

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

269 term, within-subject variability) was less than that of the signal (response to the exercise task)  
270 and there was a medium increase in CRP concentration pre- to post-exercise ( $p = 0.02$ , ES;  
271 0.78). Serum levels of CRP can increase rapidly during the acute phase of an inflammatory  
272 process (Pepys and Hirschfield, 2003), but this is a non-specific response that could be  
273 indicative of infection, illness or other metabolic factors not associated with a heat stimulus.  
274 A recent study (Hailes et al., 2011) that measured CRP in serum following 5 consecutive  
275 days of exercise in hot and dry conditions (38° C and 40% RH) reported high variability  
276 between participants and a standard deviation approximately twice that of the mean after both  
277 an acute and ongoing exposure to heat. As the presence of IL-6 is likely to cause an increase  
278 in serum levels of CRP (Petersen and Pedersen, 2005), it is likely that the exercise stimulus,  
279 and not necessarily the heat load presented to the participants was sufficient to stimulate the  
280 release of CRP from the liver. Although both IL-6 and CRP may play important roles in  
281 determining the degree of stress placed upon individuals competing or training in more  
282 extreme (hot and/or humid) conditions, although it seems unlikely this measure would  
283 present useful information in terms of responses or adaptations to the heat specifically.

284

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

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

302

303 The responsiveness of the immune system to release endotoxin is a primary consideration in  
304 defence against heat shock. As IgM is a key antibody in neutralising LPS (Camus et al.,  
305 1998), its concentration in circulating blood can reflect the body's response to endotoxin  
306 accumulation, and the likelihood of protective capacity to further challenges. In this study the  
307 observed weekly variability of IgM concentration was 28%. The pre- to post-exercise change  
308 was -15%, with 13 of the 20 participants exhibiting a negative change. To our knowledge  
309 only one other study has investigated the response of non-specific IgM following exercise in  
310 hot and humid conditions (Hailes et al., 2011). However, the reference change reported by  
311 Hailes and colleagues (2011) pre- to post-exercise in the heat (CV; 16%) is smaller than the  
312 within-subject variability (noise) reported here (CV; 29%). It appears that IgM has  
313 shortcomings as a viable biomarker for quantifying the anti-LPS response, and this is  
314 possibly related to the capability of the participants to tolerate the heat load placed upon  
315 them, although these data suggest that this response could result in either an increase or  
316 decrease in circulating concentrations. Future research is needed to clarify why some  
317 individuals respond in this manner.



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

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

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

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

347

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

359 A limitation of this study was the differing level of aerobic fitness of the subjects in Parts A  
360 ( $\text{VO}_2 \text{ max } 52 \text{ ml.kg.min}^{-1}$ ) and B ( $43 \text{ ml.kg.min}^{-1}$ ), participants were convenience sampled  
361 from a local university and sporting club population, with those unable to commit to the full  
362 14 day period protocol (Group A) allocated to Group B, due to sporting commitments that  
363 would likely interfere with resting levels of the blood biomarkers. Although the participants  
364 in each group had differing fitness levels as indicated by their  $\text{VO}_2 \text{ max}$  this is more likely  
365 due to the protocol modality. Participants in Group A underwent their  $\text{VO}_2 \text{ max}$  on a  
366 treadmill and participants in Group B underwent their  $\text{VO}_2 \text{ max}$  on a cycle ergometer, as the

367 vast majority of participants partook in either running or team sports such as football  
368 (soccer), this would likely account for the differences in VO<sub>2</sub> max, as differences of ~11%  
369 have been reported between cycling and running protocols in running athletes (Basset and  
370 Boulay, 2000). The decision to use a cycle ergometer for Group B was to a) Limit the trips to  
371 the laboratory for each participant by using a single test for both VO<sub>2</sub> max and to calculate  
372 individual loads for the subsequent HST, although future studies may choose to use more  
373 consistent protocols

#### 374 **Conclusion**

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

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

389

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