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23

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, $34.5^{o}C$ 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.

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).

4

94   **Materials and methods**

95   *Experimental Design*

96

97   Part A: Short-term reliability of serum biomarkers.

98   This phase of the study was designed to examine the weekly variation in venous blood of

99   selected biomarkers in a non-exercise context and was conducted over 14 days (Figure 1).

100   The seven days preceding the first test day were used as a "lead-in" period and participants

101   were instructed to abstain from partaking in moderate -high intensity physical activity for the

102   duration of the study period. Participants then had venous blood drawn on two occasions

103   seven days apart. Venous blood was drawn in a seated position prior to and immediately

104   following the heat stress test. Blood was sampled approximately 2 h post-prandial at a similar

105   time of day (morning) to limit diurnal variation. At the beginning of the lead-in period all

106   participants undertook a baseline evaluation of maximum oxygen uptake ($\dot{V}O_{2max}$) using an

107   incremental treadmill running test to exhaustion. A seven day controlled lead-in or baseline

108   period was used to ensure that the participants were not suffering from any residual

109   inflammatory effects of exercise or illness prior to taking part in this study. Participants were

110   instructed to maintain a similar dietary intake and (light) activity levels for 24 h preceding

111   each venous blood sample.
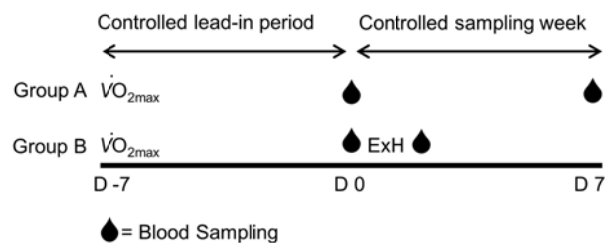
112

113   **Figure 1.** Schematic illustration of the experimental procedures showing that blood was
114   sampled at D 1 (day one, Part A and Part B) and D 7 (Day seven, Part A). ExH; Exercise in
115   the heat intervention (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 $\dot{V}O_{2max}$ 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 $\dot{V}O_{2max}$. 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±4.1 years, $\dot{V}O_{2max}$ 52.0±2.7 ml.kg.min^{-1}, height

132 1.78±0.09 m, mass 73.9±8.5 kg, mean ±SD). Part B participants (acute response to exercise

133 in the heat intervention) comprised twenty males (age 24.6±3.7 years, $\dot{V}O_{2max}$ 43.2±5.4

134 ml.kg.min^{-1}, height 1.78±0.07 m, mass 83.5±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

6

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.

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.

158

159

Exercise in the heat protocol (Part B)

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

Statistical Analysis

The concentration of each biomarker is presented as mean ± SD. Biomarker reliability was calculated as a coefficient of variation (CV) both within- and -between subjects at day o and day 7 and presented as mean %CV ± 95% confidence limits (CL). Day 0 to day seven and pre- to post-exercise changes in biomarker concentrations were analysed with paired t-tests 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

9

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

|		Noise			Signal	Signal to Noise	
Biomarker	Concentration	Within-subject	Between-	Within-subject	Pre to Post	Ratio Score	Inference
	Day 0	CV	subject CV	E.S	E.S		
		Day 0 to Day 7	Day 0				
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.
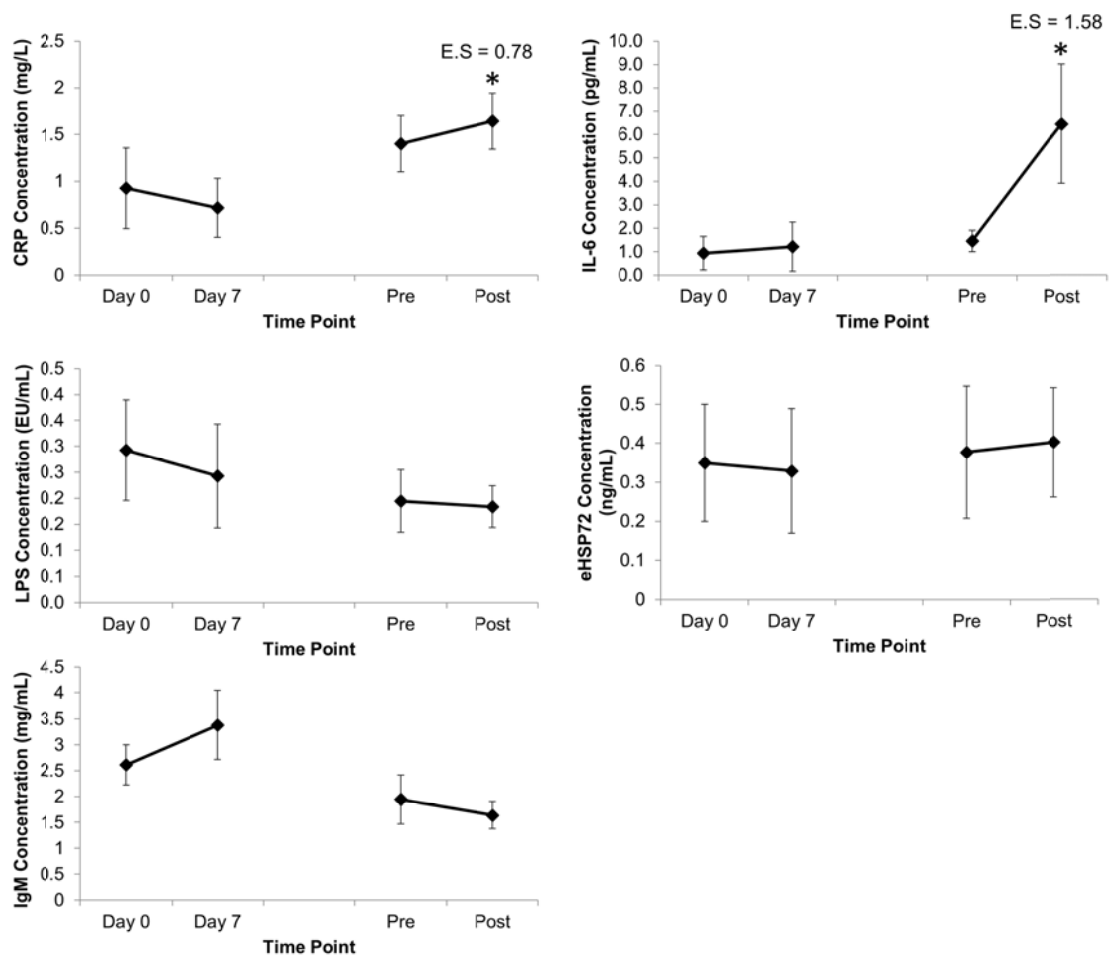
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222   **Figure 2.** Serum biomarker concentrations presented as mean ± SD from Part A (Short-term;
223   Day 1 and Day 7) and Part B (Exercise in the heat; Pre and Post). * = significantly different
224   from pre concentration. CRP; C-reactive protein. 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.

13

244   The exercise presented to the participants resulted in a mean core temperature rise of $1.5^{\circ}C$

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 ($38^{\circ}C$ 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 at 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 $>39^{o}C$ 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

17

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 (VO$_2$ max 52 ml.kg.min^{-1}) and B (43 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 VO$_2$ max this is more likely

365 due to the protocol modality. Participants in Group A underwent their VO$_2$ max on a

366 treadmill and participants in Group B underwent their VO$_2$ 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_2 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_2 max and to calculate

372 individual loads for the subsequent HST, although future studies may choose to use more

373 consistent protocols

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. $T_c > 39^oC$) 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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