Short-term reliability of inflammatory mediators in response to exercise in the heat

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Abstract

Prospective application of serum cytokines, lipopolysaccharide, and heat shock proteins requires reliable measurement of these biomarkers of 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. Venous blood was analysed for the serum 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 the mean change 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). 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. This effect is exacerbated by exercise in the heat and, as a consequence, it is important to reliably evaluate the extent of endotoxin leakage into circulation, and measure biochemical indices which accurately reflect whether or not individuals are able to respond or adapt effectively to such exercise challenges that occur in the heat.

Exercise-induced endotoxemia has been attributed primarily to lipopolysaccharide (LPS) translocated 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 extra cellular 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 oxidative stress and effectiveness of anti-LPS mechanisms (Pilch et al., 2014; Pyne et al. 2014; Yeh, Law, and Lim, 2013). Therefore, to accurately determine whether or not individuals are susceptible to heat stress, or have effectively adapted over time to hot conditions through heat acclimation or acclimatisation, it is important to quantify reliable, relevant, and objective outcome measures of the immune and inflammatory responses.

Several studies have used blood biomarkers to quantify the magnitude of adaptation to hot environmental conditions, although a comparison of short-term variability in heat-induced biomarkers has not yet been conducted. This is surprising as there is considerable variation in the magnitude of heat-induced change to markers such as interleukin (IL)-6, C-reactive protein, LPS and eHSP72 following a bout of exercise in hot conditions (Hailes, Slivka, Cuddy and Ruby 2011; Lim et al., 2009; Marshal, Campbell, Roberts, and Nimmo, 2007; Rhind et al., 2004; Wright et al., 2013). As a common length for a short-term heat acclimation protocol for athletes is seven days (Garrett, Rehrer, and Patterson, 2011) further investigation into the variation of these biomarkers is warranted. The utility of individual biomarkers may depend on typical variation (noise) under normal conditions, and the magnitude of the response to exercise in the heat (signal). Another consideration is whether the noise is sufficiently small so as to not mask biologically and/or clinically important changes or differences. It is also plausible that while some biomarkers may demonstrate considerable short-term variability, they could still be useful if the presentation of a heat stimulus produces a sufficiently large signal (response). This is a point often overlooked in the study of reliability of biomarkers. Consequently, evaluating reliability among heat-relevant biomarkers should account for variation, and, importantly, whether the expected

change in biomarker activity on presentation to a large heat and exercise stimulus is greater than that of short-term variation.

The aim of this study was to quantify the reliability (short term test re-test reliability) in the concentration of common inflammatory (blood) biomarkers at rest (twice over seven days, Part A). A second aim was to examine the acute response of those biomarkers to an exercise 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. Blood was drawn 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.

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 sampled for acute response to exercise in the heat was drawn 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.

Subjects

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 for 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

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^{o}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 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, Unites 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 fluid loss normalised for body weight 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 1 and day 7 and presented as mean %CV ± 95% confidence limits (CL). 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 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 were log-transformed where appropriate 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 1 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 1). A comparison of the within-subject variability for each biomarker with an expected reference change is detailed in Table 1. 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, and therefore the biomarkers were categorised as having unclear or poor reliability (Table 1).

TABLE 1 ABOUT HERE

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

Blood biomarkers with the largest pre- to post-exercise change were IL-6 and CRP. The blood biomarkers least sensitive to change following the exercise in the heat exposure were IgM, LPS and eHSP72. The exhaustive nature of the exercise task was confirmed with high levels of physiological and perceptual stress: peak T_c; 38.9 ± 0.2 ^{o}C, peak heart rate; 187 ± 5 bpm, reduction in body mass; 1.7% ± 0.3%, and end point RPE; 17 ± 1 units. Changes in mean blood biomarker concentration in addition to effect sizes pre-to-post exercise in the heat are presented in Figure 2.

FIGURE. 2 ABOUT HERE

Discussion

The biomarker IL-6 exhibited the smallest within-subject short-term variation (19%) and the greatest acute pre- to post-exercise change in the heat (4.5 fold change). For the other biomarkers, the short-term resting variation was similar to that of pre- to post-exercise evaluations in the heat, indicating minimal alteration to an acute bout of exercise. It appears only some biomarkers are potentially useful for the purpose of reliably quantifying acute physiological responses in healthy active individuals to hot environmental conditions that elicit modest rises in T_c. Chronic effects on biomarker activity evaluated over a longer period may reveal further insights, such as adaptations to the heat and possible immunosuppression.

Even in a resting state, considerable weekly variation was evident for each variable. The cytokine IL-6 exhibited the least within-subject variability of 19% although other biomarkers such as CRP varied by 38%. The magnitude of this variation is considered concurrently with the expected change in response to an exercise challenge or a period of training, and can be used to inform the decision making process on effects of heat stress (Table 1). Quantifying variation is an inherent part of studying biological systems and can yield important

information when seeking to determine whether or not intervention-induced change in a 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 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 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 and the inflammatory cascade 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, 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 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 systemic inflammation associated with 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 subjects 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).

Conclusion

Quantifying the inherent variation of biological systems affected by exercise in hot and humid environment informs the choice of inflammatory biomarkers. The utility of the selected biomarkers IL-6 and CRP appears useful even when presented with a high (but tolerable) heat load. 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. Nevertheless, selected biomarkers should be useful for determining the magnitude of responsiveness and stress placed upon individuals training or competing in hot environmental conditions. It appears that a substantial heat 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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Table 1. Coefficient of variation both within (day zero to day seven) and between subjects with inferences to the reliability and usefulness 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	1.7	Unclear
LPS	0.29 ± 0.04 EU/mL	23%, ± 13%	41%	-0.55	-0.06	1.8	Unclear
IL-6	0.94 ± 0.45 pg/mL	19%, ± 20%	153%	0.16	1.58	8.1	Good
IgM	2.56 ± 0.29 mg/mL	28%, ± 17%	261%	0.73	-0.42	9.3	Unclear
CRP	0.93 ± 0.72 mg/mL	38%, ± 21%	93%	-0.28	0.78	2.4	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). 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; reference effect size was calculated from the typical change (pre & post mean & SD) in the measured parameter from selected intervention studies. Ratio score was calculated by dividing the reference effect size by the within-subject effect size. CRP; C-reactive protein. eHSP72; extracellular heat shock protein. IL-6; interleukin-6. LPS; lipopolysaccharide. IgM; immunoglobulin M.

Figure Captions.

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).

Figure 2. Serum biomarker concentrations presented as mean ± SD from Part A (Short-term; Day 1 and Day 7) and Part B (Exercise in the heat; Pre and Post). * = significantly different from pre concentration. CRP; C-reactive protein. eHSP72; extracellular heat shock protein. IL-6; interleukin-6. LPS; lipopolysaccharide. IgM; immunoglobulin M

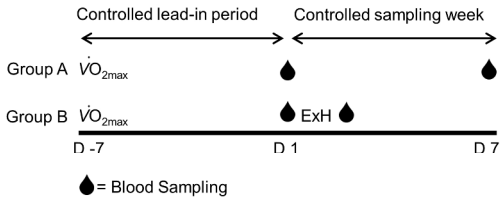

Figure 1. Schematic illustration of the experimental procedures showing that blood was sampled at D 1 (day one, Part A and Part B) and D 7 (Day seven, Part A). ExH; Exercise in the heat intervention (Part B).
254x190mm (300 x 300 DPI)

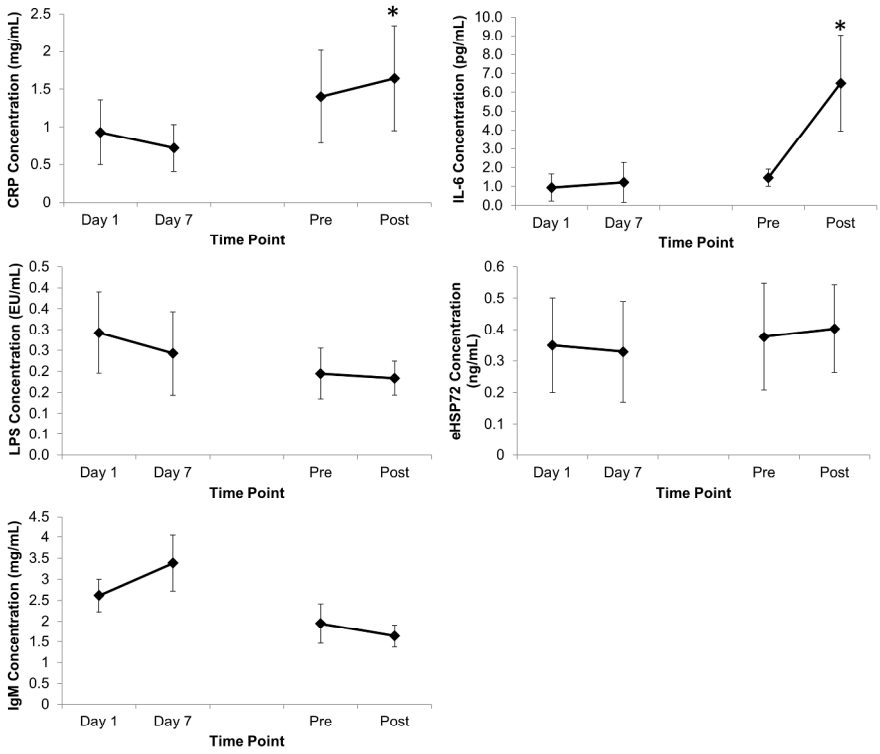

Figure 2. Serum biomarker concentrations presented as mean ± SD from Part A (Short-term; Day 1 and Day 7) and Part B (Exercise in the heat; Pre and Post). * = significantly different from pre concentration. CRP; C-reactive protein. eHSP72; extracellular heat shock protein. IL-6; interleukin-6. LPS; lipopolysaccharide. IgM; immunoglobulin M
287x309mm (252 x 252 DPI)