

1 **Title:** Whole-body precooling attenuates the extracellular HSP72, IL-6 and IL-10 responses  
2 after an acute bout of running in the heat.

3

4 **Running title:** eHSP72 and inflammatory responses following precooling

5 **Abstract**

6 The impact of whole-body precooling on the extracellular heat shock protein 72 (eHSP72)  
7 and cytokine responses to running in the heat is undefined. The aim of this study was to  
8 determine whether precooling would attenuate post-exercise eHSP72 and cytokine responses.  
9 Eight male recreational runners completed two 90-minute bouts of running at 65%  $\dot{V}O_2\text{max}$   
10 in  $32 \pm 0.9^\circ\text{C}$  and  $47 \pm 6\%$  relative humidity (RH) preceded by either 60-minutes of  
11 precooling in  $20.3 \pm 0.3^\circ\text{C}$  water (COOL) or 60 minutes rest in an air-conditioned laboratory  
12 ( $20.2 \pm 1.7^\circ\text{C}$ ,  $60 \pm 3\%$  RH; CON). eHSP72, TNF- $\alpha$ , IL-6, IL-10 IL-1ra were determined  
13 before and immediately after exercise. The elevation in post-exercise eHSP72 was attenuated  
14 after COOL ( $+0.04 \pm 0.10 \text{ ng}\cdot\text{mL}^{-1}$ ) compared to CON ( $+ 0.29 \pm 0.26 \text{ ng}\cdot\text{mL}^{-1}$ ;  $p < 0.001$ ). No  
15 changes in TNF- $\alpha$  were observed at any stage. COOL reduced the absolute post-exercise  
16 change in IL-6 ( $p = 0.011$ ) and IL-10 ( $p = 0.03$ ) compared to CON. IL-1ra followed this trend  
17 ( $p = 0.063$ ). A precooling-induced attenuation of eHSP72 and proinflammatory cytokines  
18 may aid recovery during multi-day sporting events, but could be counterproductive if a  
19 training response or adaptation to environmental stress is a desired outcome.

20 **Keywords:** Precooling, eHSP72, cytokines, inflammation

21 **Word count:** 4014

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## 1 **Introduction**

2 During prolonged strenuous exercise in warm ( $>30^{\circ}\text{C}$ ) conditions, blood flow is redistributed  
3 to the working muscles and peripheral circulation to enable oxygen supply and enhance heat  
4 loss, leading to splanchnic hypoperfusion and ischemia (Dokladny, Zuhl, & Moseley, 2015;  
5 Ter Steege & Kolkman, 2012; van Wijck et al., 2012). As thermoregulatory strain progresses,  
6 a widening of epithelial tight junction spaces increases intestinal permeability (Lambert,  
7 2009; van Wijck et al., 2012), leading to endotoxaemia and an over-exaggerated systemic  
8 cytokine response (Camus et al., 1998). An exercise-induced increase in core body  
9 temperature  $> 1^{\circ}\text{C}$  results in elevated levels of classic pro-inflammatory (TNF- $\alpha$ , IL-6, IL-  
10  $1\beta$ ) and a compensatory anti-inflammatory (IL-10, IL-1ra) cascade observed when compared  
11 to conditions in which body temperature remains normothermic (Cosio-Lima, Desai, Schuler,  
12 Keck, & Scheeler, 2011; Gill, Hankey, et al., 2015; Gill, Teixeira, et al., 2015; Rhind et al.,  
13 2004; Selkirk, McLellan, Wright, & Rhind, 2009). Exercise in hot environments is also a  
14 potent stimulus for release of heat shock protein 72 (HSP72) into the extracellular space  
15 (eHSP72) compared to exercise without hyperthermia (Fehrenbach, Niess, Veith, Dickhuth,  
16 & Northoff, 2001; Gibson et al., 2014; Moran et al., 2006; Whitham, Laing, Jackson,  
17 Maassen, & Walsh, 2007).

18  
19 Elevated eHSP72 has been suggested to act as an immunological signal for later cytokine and  
20 inflammatory responses and increased levels of endotoxemia and the associated cytokine  
21 cascade has been implicated in heat illness and heat stroke (Asea, Kabingu, Ann Stevenson,  
22 & Calderwood, 2000; Campisi, Leem, & Fleshner, 2003; Leon & Helwig, 2010; Lim &  
23 Mackinnon, 2006). A reduction in systemic inflammation after exercise in the heat could  
24 have implications for enhancing recovery after single and multi-day sporting events in hot  
25 climates, as well as occupational or military exercises conducted under extreme heat

1 conditions. Concomitantly, infection risk and the incidence of gastrointestinal discomfort  
2 during and after exercise-heat stress may be reduced (Gill et al., 2015).

3

4 Precooling is a simple and practical method that can be completed prior to exercise in the  
5 heat, and has been shown to be effective in reducing physiological strain, prolonging  
6 performance and improving perceptual tolerance during exercise-heat stress (Cuddy, Hailes,  
7 & Ruby, 2014). Current evidence indicates that cold water immersion (CWI) may be the  
8 most effective method of precooling for enhancing endurance performance in hot conditions  
9 (Jones, Barton, Morrissey, Maffulli, & Hemmings, 2012). In the 2015 IAAF World Athletics  
10 Championships approximately 10.8% of middle distance runners, and 8.5% of long distance  
11 runners employed CWI prior to competition (Périard et al., 2016), highlighting it's use in  
12 applied settings. However, there is limited information regarding the impact of precooling on  
13 both the eHSP72 and systemic inflammatory response post endurance exercise. Data using a  
14 range of precooling methods applied to intermittent sprint activity show no effect on IL-6  
15 (Duffield, Steinbacher, & Fairchild, 2009), eHSP72 (Castle, Mackenzie, Maxwell, Webborn,  
16 & Watt, 2011) or CRP (Minett, Duffield, Marino, & Portus, 2011, 2012) with a blunting of  
17 creatine kinase (CK) noted in some investigations (Minett et al., 2011, 2012).

18 During prolonged endurance exercise it is plausible that precooling may delay the attainment  
19 of the critical temperature thresholds required for eHSP72 release and preserve perfusion to  
20 the splanchnic region. The preservation of splanchnic perfusion may serve to reduce the  
21 magnitude of systemic cytokine increase following prolonged exercise in the heat (Dokladny,  
22 Zuhl, & Moseley, 2015). Therefore, the aim of this study was to determine whether a period  
23 of precooling prior to an acute bout of endurance running exercise in the heat would attenuate  
24 post exercise eHSP72 concentrations and markers of the systemic cytokine response (TNF- $\alpha$ ,  
25 IL-6, IL-10, IL-1ra). It was hypothesized that precooling preceding prolonged endurance

1 exercise in the heat would reduce eHSP72 and plasma cytokines TNF- $\alpha$ , IL-6, IL-10 and IL-  
2 1ra.

### 3 **Methods**

#### 4 **Participants**

5 Following ethical approval from the Coventry University Ethics Committee, eight male  
6 recreational runners (mean  $\pm$  SD age:  $28 \pm 6$  years; height:  $1.76 \pm 0.08$  m; body mass:  $72.6 \pm$   
7  $12.5$  kg; body surface area:  $1.88 \pm 0.19$  m<sup>2</sup>; absolute maximal oxygen uptake ( $\dot{V}O_{2\max}$ ):  $3.82 \pm$   
8  $0.57$  L·min<sup>-1</sup>; relative maximal oxygen uptake:  $53 \pm 6$  mL·kg<sup>-1</sup>·min<sup>-1</sup>) provided both written  
9 and verbal informed consent prior to completing the study. Each participant completed 90  
10 minutes of treadmill running at 65%  $\dot{V}O_{2\max}$  in the heat ( $32.4 \pm 0.9^\circ\text{C}$  and  $47 \pm 6\%$  relative  
11 humidity) on two occasions separated by at least 1 week in a randomised, counterbalanced  
12 crossover design.

#### 13 **Pre-experimental standardization**

14 To minimise the known confounds on eHSP72, participants were non-smokers who abstained  
15 from caffeine and alcohol consumption for 48 hours before each trial. General  
16 supplementation, prolonged thermal exposures, hypoxic exposures and hyperbaric exposures  
17 were also avoided for the duration of the study (Anbarasi, Kathirvel, Vani, Jayaraman, &  
18 Devi, 2006; Lu, Lai, & Chan, 2008; Hillman et al., 2011; Selkirk et al., 2009; Taylor et al.,  
19 2011; Taylor, Midgley, Sandstrom, Christmas, & McNaughton, 2012). Participants abstained  
20 from physical activity in the 48 hours preceding each trial and recorded their dietary intake  
21 throughout this period. The diet was then replicated in preparation for the second  
22 experimental trial.

1 Participants attended the laboratory after an overnight fast and ingested a volume of  $5 \text{ mL} \cdot \text{kg}^{-1}$   
2  $\text{body mass}$ ;  $363 \pm 63 \text{ mL}$ ) of room temperature ( $\sim 18^\circ\text{C}$ ) water 2 h before the trial, and an  
3 additional  $7 \text{ mL} \cdot \text{kg}^{-1}$  body mass ( $508 \pm 89 \text{ mL}$ ) of  $18^\circ\text{C}$  water ( $\sim 181.7 \text{ mL} \cdot \text{kg}^{-1}$  body mass) *ad*  
4 *libitum* during exercise. All trials were performed at the same time of day to minimise the  
5 circadian variation in internal body temperature.

6

## 7 **Procedures**

8 All procedures were conducted in accordance with the Declaration of Helsinki (2013  
9 Edition). On arrival at the laboratory nude body mass (NBM) was recorded, a flexible  
10 translucent PVC rectal probe (Grant Instruments, UK) was inserted, and stainless steel  
11 mounted skin thermistors (EUS-U-VS5-0, Grant Instruments, UK) attached to the belly of the  
12 *pectoralis*, *triceps brachii*, *vastus lateralis*, and *gastrocnemius* on the right side of the body.  
13 Rectal and skin temperatures were recorded continuously using a data logger (Squirrel  
14 SQ2040, Grant Instruments, UK). Mean skin temperature ( $T_{\text{skin}}$ ) was subsequently calculated  
15 (Ramanathan, 1964). A heart rate monitor (Polar RS400, Polar Electro Oyo, Kempele,  
16 Finland) was worn around the chest.

17 Following instrumentation participants began either a 60-minute period of precooling  
18 (COOL) by means of water immersion up to the sternal notch ( $20.3 \pm 0.3^\circ\text{C}$ ; (Hasegawa,  
19 Takatori, Komura, & Yamasaki, 2006), or 60 minutes of seated rest in an air conditioned  
20 laboratory ( $20.2 \pm 1.7^\circ\text{C}$  and  $60.2 \pm 2.5\% \text{ RH}$ ) wearing shorts. On leaving the water  
21 participants were towel dried and donned a t-shirt (within 10 minutes of the end of  
22 precooling, standardised between the first and second trial) before commencing 90 minutes of  
23 treadmill running at  $65\% \dot{V}\text{O}_{2\text{max}}$  on a 1% gradient. Rectal and skin temperatures were  
24 monitored throughout the resting/cooling period and recorded at 30-minute intervals. During

1 the 90-minute exercise bout HR,  $T_{\text{rectal}}$ , and  $T_{\text{skin}}$  were recorded, and 6-20-point rating of  
2 perceived exertion (Borg, 1976) and 7-point thermal sensation (TS -3 (cold) to +3 point (hot);  
3 (ASHRAE, 2004) scores were sought at 15 minute intervals. The Physiological Strain Index  
4 (PSI), that quantifies physiological strain between 0 (no strain) and 10 units (very high strain)  
5 where 0 represents no strain and 10 represents very high strain, was calculated from heart rate  
6 and  $T_{\text{rectal}}$  (PSI; Moran et al., 1998). Sweat rate was calculated from the change in NBM (pre  
7 to immediately post-exercise) with the volume of fluid ingested and fluid voided as urine  
8 over the course of the trial added to the final value. The  $T_{\text{rectal}}$  area under the curve (AUC)  
9 was calculated using the modified trapezium rule (Hubbard et al., 1977) when  $T_{\text{rectal}}$  exceeded  
10  $38.5^{\circ}\text{C}$  (Cheuvront et al., 2008). AUC for  $T_{\text{rectal}} > 38.5^{\circ}\text{C}$  was calculated as:

11  $\text{AUC } T_{\text{rectal}} > 38.5^{\circ}\text{C} (^{\circ}\text{C min}^{-1}) = \sum \text{time interval (min)} \times 0.5 (^{\circ}\text{C} > 38.5^{\circ}\text{C at start of interval} + ^{\circ}\text{C above } 38.5^{\circ}\text{C}$   
12  $\text{at the end of the time interval}).$

13

#### 14 **Blood sampling**

15 Venous blood samples (7mL) were collected from an antecubital vein immediately before the  
16 60 minute resting period into an EDTA treated vacutainer (Vacurette, Greiner Bio-One,  
17 Stonehouse, UK), and again at the termination of exercise. Haemoglobin and haematocrit  
18 were determined in triplicate via a B-Haemoglobin Photometer (Hemocue Ltd, Angleholm,  
19 Sweden) and centrifuged capillary tubes (Hawksley Micro Haematocrit Centrifuge, Hawksley  
20 and Son, Lancing, UK), measured using a haematocrit reader. Plasma volume changes were  
21 then calculated according to the equations of Dill and Costill (1974). All later analysis for  
22  $\text{TNF-}\alpha$ , IL-6, IL-10, IL-1ra and eHSP72 were corrected for any observed changes in plasma  
23 volume from pre to post exercise. The remaining blood sample was centrifuged for 10  
24 minutes at 3000RPM and plasma aliquoted for storage at  $-80^{\circ}\text{C}$  prior to analysis for plasma

1 lactate, creatine kinase (CK), and C-reactive protein (CRP; Randox Daytona, County Antrim,  
2 Ireland).

3

4 Circulating TNF-a, IL-1ra, IL-6 and IL-10 were determined in duplicate using commercially  
5 available high sensitivity sandwich ELISA kits (R&D systems, Minneapolis, USA) which  
6 were sensitive to 0.19 pg·mL<sup>-1</sup>, 0.18 pg·mL<sup>-1</sup>, 0.11pg·mL<sup>-1</sup> and 0.17pg·mL<sup>-1</sup> respectively. The  
7 inter-assay variability for TNF-a, IL-1ra, IL-6 and IL-10 was 3.1, 4.0, 1.5 and 1.8%  
8 respectively.

9

10 Circulating eHSP72 was assessed using the commercially available Amp'd<sup>®</sup> HSP72 high  
11 sensitivity ELISA kit (ENZ-KIT-101-001) according to the manufacturer's instructions (Enzo  
12 Lifesciences, Lausen, Switzerland). The ENZ-KIT is sensitive to 0.007 ng·mL<sup>-1</sup> with a  
13 working range of 0.039-5.00 ng·mL<sup>-1</sup>. This assay was found to be more sensitive than the  
14 traditionally used EKS-715 high sensitivity kit (Lee et al., 2015). Samples were diluted 1:4  
15 with assay diluent prior to analysis (Lee et al., 2015) and the inter-assay variability was 1.8%.

16

### 17 **Statistical analysis**

18 The primary outcome variable of this study was the eHSP72 and cytokine response following  
19 each bout of treadmill running. The difference in post-exercise change in eHSP72 between  
20 control and cooling after the running period was assessed using a two tailed randomization  
21 test with 1000 resamples of the data set (Colquhoun, 2014). The same approach was used to  
22 assess post-exercise absolute changes in TNF- $\alpha$ , IL1ra, IL-6, and IL-10.

23 In order to control for the false discovery rate and correct for multiple comparisons, two  
24 families of hypotheses were tested according to the method of Benjamini and Hochberg  
25 (1995); 1) the eHSP72 and cytokine responses; 2) physiological and perceptual responses.

1 Physiological data were analyzed using a 2 x 7 repeated measures linear model, with fixed  
2 effects for trial (CON and COOL) and time (0, 30, 60 minutes of rest; 0, 30, 60, 90 minutes  
3 of exercise). Mean and peak exercise data were also compared via two-tailed randomization  
4 tests (Colquhoun, 2014).

5 All data are reported as means  $\pm$  SD for n = 8 for each trial unless otherwise stated. Precise p  
6 values are reported and Cohen's D (with 95% confidence intervals) effect sizes presented to  
7 indicate the magnitude of observed effects for the main outcome variables (Colquhoun,  
8 2014). Effect sizes of 0.2, 0.5 and 0.8 are considered small, medium and large respectively.

9

## 10 **Results**

### 11 **Physiological and perceptual measures**

12 There was no difference between trials in pre-trial body mass (CON = 71.96  $\pm$  12.2kg, COOL  
13 = 72.04  $\pm$  12.15) or fluid ingested during exercise (CON = 510  $\pm$  106 ml; COOL = 461  $\pm$  122  
14 mL). Similarly, there was no difference in post-exercise change in body mass (CON = 1.8  $\pm$   
15 0.6 kg; COOL = 2.1  $\pm$  0.4 kg) or sweat rate (CON = 19.7  $\pm$  6.2 g min<sup>-1</sup>; COOL = 22.9  $\pm$  4.2  
16 g min<sup>-1</sup>). Heart rate (p = 0.92), thermal sensation (p = 0.31) and perceived exertion (p = 0.69)  
17 were not different between trials at any point (Table 1).

18

### 19 **Thermoregulatory responses to precooling**

20 Baseline T<sub>skin</sub> (CON = 30.79  $\pm$  0.70; COOL 30.78  $\pm$  0.85) and T<sub>rectal</sub> (CON = 36.88  $\pm$  0.36°C;  
21 COOL = 36.98  $\pm$  0.37°C) were similar prior to the pre-cooling or rest period commencing.

22 The reduction in T<sub>skin</sub> and T<sub>rectal</sub> during the pre-exercise period was greater during COOL than  
23 CON (Figure 1, time x trial interaction, p < 0.01 for both) such that there was a between trial  
24 difference at the onset of exercise. The reduction in T<sub>rectal</sub> persisted until 30 min of exercise  
25 was completed, however due to a greater rate of rise in rectal temperature (Figure 2A), there

1 was no difference between conditions at the conclusion of the exercise bout. A similar  
2 response was observed for T<sub>skin</sub> (Figure 2B). Mean exercise T<sub>rectal</sub> was lower in COOL  
3 compared to CON (p = 0.03, d = -1.0, 95% CI = -2.0 to -0.1), however the AUC and total  
4 time >38.5°C was not different between CON and COOL (AUC: p = 0.30, d = -0.2, 95% CI -  
5 1.2 to 0.7; Time > 38.5°C; p = 0.21, d = -0.4, 95% CI = -1.4 to 0.6). PSI was lower in COOL  
6 until 45 minutes of exercise, with no differences between conditions in the remaining 45  
7 minutes of exercise (Figure 2B, d = -0.6, 95% CI -1.6 to 0.45). Post-exercise plasma blood  
8 lactate concentrations were no different between CON (2.65 ± 0.51 mmol<sup>-1</sup>) and COOL (2.35  
9 ± 0.51 mmol<sup>-1</sup>; p = 0.061).

10

### 11 **Extracellular HSP72 and circulating cytokines**

12 Resting eHSP72 was similar between trials (CON = 0.32 ± 0.38 ng·mL<sup>-1</sup>; COOL = 0.31 ±  
13 0.33 ng·mL<sup>-1</sup>). Exercise led to an increase in eHSP72 concentrations post-exercise in CON  
14 (0.77 ± 0.58 ng·mL<sup>-1</sup>; d = 0.7, 95% CI = -0.4 to 1.6) but not COOL (0.35 ± 0.29 ng·mL<sup>-1</sup>; d =  
15 0.1, 95% CI = -0.9 to 1.1, Figure 3A). Post-exercise concentrations in eHSP72 were related to  
16 final T<sub>rectal</sub> temperatures in both CON (r = 0.56, p = 0.15) and COOL (r = 0.63, p = 0.09). The  
17 absolute change in eHSP72 was lower following COOL compared to CON (p < 0.001; d = -  
18 1.1, 95% CI = -2.5 to -0.3; Figure 3B).

19

20 No main effects or interactions were observed for TNF- α (Table 2), however IL-6 (f = 59.4,  
21 p < 0.001), IL-10 (f = 41.2, p < 0.001) and IL-1ra (f = 6.4, p = 0.02) concentrations were  
22 increased following exercise (Table 2). A similar post-exercise increase in IL-10 (f = 1.5, p =  
23 0.23) and IL-1ra (f = 0.9, p = 0.4) was seen following both CON and COOL, whereas the  
24 increase in IL-6 was blunted after COOL (f = 4.4, p = 0.045). The absolute change in both  
25 IL-6 (p = 0.015; d = -1.0, 95% CI = -1.9 to 0.1) and IL-10 (p = 0.04; d = -0.8, 95% CI = -1.8

1 to 0.3) were lower after COOL (Figure 4) whereas no differences in absolute change were  
2 observed for TNF- $\alpha$  ( $p = 0.11$ ,  $d = 0.4$ , 95% CI = -0.6 to 1.3) or IL-1ra ( $p = 0.06$ ;  $d = -0.6$ ,  
3 95% CI = -1.6 to 0.4). Post exercise plasma CK and CRP were not different between the  
4 CON (CK  $393 \pm 275$  U·L<sup>-1</sup>; CRP  $0.95 \pm 0.87$  mg·L<sup>-1</sup>) and COOL (CK  $359 \pm 261$  U·L<sup>-1</sup>; CRP  
5  $0.70 \pm 0.68$  mg·L<sup>-1</sup>) trials.

6

## 7 **Discussion**

8 The aim of this study was to determine whether whole-body precooling would affect the  
9 magnitude of circulating eHSP72 and systemic cytokine responses (TNF- $\alpha$ , IL-6 IL-10, and  
10 IL-1ra) following prolonged running in the heat. We hypothesized that cooling-induced  
11 reductions in heat strain would attenuate post-exercise eHSP72 and plasma cytokine  
12 concentrations. We are the first to show that a 60-minute period of precooling, which  
13 successfully reduced resting rectal and skin temperature for the initial 30 minutes of exercise  
14 in the heat, ameliorated the magnitude of post-exercise eHSP72 increase. Our data also  
15 indicate that the cooling-mediated reduction in thermal stress during the initial phase of  
16 exercise may have some benefits in reducing the post exercise pro-inflammatory responses.  
17 Moderate effect sizes were observed in support for a reduced absolute increase in IL-6 which  
18 was mirrored by the attenuated increases in anti-inflammatory IL-10 after exercise. Data for  
19 TNF- $\alpha$  and IL-1ra were less clear. The blunted increase in post exercise eHSP72 following  
20 precooling occurred despite no marked differences in mean exercise heart rate and final rectal  
21 temperatures between trials. However, a large effect size for the observed reduction in mean-  
22 exercise  $T_{\text{rectal}}$  indicates that a reduced overall temperature signal was experienced following  
23 precooling, despite an increased rate of rise and similar peak  $T_{\text{rectal}}$  observed between trials.  
24 The results of this study add support to the notion that increases in eHSP72 following

1 exercise is not mediated solely by a rise in body temperature, and could be a product of the  
2 overall physiological strain experienced during an exercise challenge.  
3  
4 Our data show that precooling substantially diminished the post-exercise release of eHSP72  
5 (Figure 3A, 3B) despite each condition ending with similar peak  $T_{\text{rectal}}$ , suggesting that  
6 whole-body precooling may have an indirect effect on eHSP72 release via reductions or  
7 alterations in the temperature signal required for eHSP72 release. Such a reduction may be  
8 achieved by reducing the total time spent above a critical ‘threshold’ of thermal load or  
9 physiological strain. It should however be noted that the AUC and time spent above the  
10 hypothesized temperature threshold of 38.5°C for eHSP72 release was not different between  
11 conditions, with the small to moderate effect sizes observed for each variable compounded by  
12 wide confidence intervals. However, a period of precooling effectively imparted two different  
13 levels of physiological strain between trials, with  $T_{\text{rectal}}$  and mean physiological strain being  
14 lower following COOL (Table 1). The rate of  $T_{\text{rectal}}$  increase was higher in COOL (Figure  
15 2A) as a result of the greater thermal gradient at the start of exercise, with rate of temperature  
16 change proportional to the size of the existing thermal gradient (Taylor, Tipton, & Kenny,  
17 2014). In addition, the precooling induced reduction in  $T_{\text{rectal}}$  prior to the onset of exercise  
18 delays the vasodilatory and sweat threshold responses, leading to a greater heat storage until  
19 later in the exercise bout when rate of  $T_{\text{rectal}}$  change matched CON (Figure 1; Marino, 2002).  
20 It should be noted that  $T_{\text{rectal}}$  measurements are not a dynamic measure of core temperature,  
21 with a phase delay in response often observed, therefore the true rate of body core  
22 temperature change may have been masked by this measurement artifact (Taylor, Tipton and  
23 Kenny, 2014).

24

1 It is possible that the cooling induced reduction in pre-exercise skin and rectal temperatures,  
2 which were maintained until ~30 minutes into the exercise, prolonged the time until the  
3 required thermal signal(s) for eHSP72 release are met. Our results are supportive of previous  
4 data indicating that multifaceted signals are required for eHSP72 release (Gibson et al.,  
5 2014), suggesting that different modes, durations and intensities of exercise elicit a  
6 combination of different thermal and physiological signals for eHSP72 release. The relative  
7 importance of any one signal is difficult to determine and would require investigation specific  
8 to a mode of exercise (e.g. running vs cycling). A measurement of eHSP72 at different stages  
9 of the exercise bout (e.g. 45 minutes) may have provided further information regarding the  
10 precooling dose response. Alternatively, extended periods of cooling during exercise ('Per-  
11 cooling'), such as the application of ice towels or fanning, could further diminish or delay the  
12 signal for eHSP72 release, lessening its role as an 'immune danger signal' following  
13 exertional heat stress (Asea, 2006).

14  
15 The principle sources of eHSP72 release into the circulation are the hepatosplanchnic,  
16 vascular, and brain tissue, and peripheral blood mononuclear cells (Febbraio et al., 2002;  
17 Johnson & Fleshner, 2006; Lancaster & Febbraio, 2005; Lancaster et al., 2004). Exercise-  
18 induced ischemia at the hepatosplanchnic viscera is known to be greater during exercise in  
19 hot conditions compared to thermoneutral conditions due to the preferential redistribution of  
20 blood to the periphery for heat dissipation (Ter Steege & Kolkman, 2012), and may therefore  
21 be one driver of eHSP72 release. Hepatosplanchnic ischemia is also proposed to disturb  
22 intestinal integrity via widening of tight junction spaces (Dokladny et al., 2015), contributing  
23 to the observed increases in classic pro-inflammatory circulating cytokines (e.g. TNF- $\alpha$ ), and  
24 anti-inflammatory IL-1ra and IL-10 following exercise in the heat compared to more  
25 temperate conditions (Cosio-Lima et al., 2011; Costa, Walters, Bilzon, & Walsh, 2011; Peake

1 et al., 2008; Rhind et al., 2004). Additionally, IL-6, which is described as both pro and anti-  
2 inflammatory depending on its site of production and release, is also elevated after exercise in  
3 hot conditions (Gleeson et al., 2011; Gill, Teixeira, et al., 2015; Rhind et al., 2004).  
4 Accordingly, the CON trial resulted in post-exercise increases in IL-6 and compensatory  
5 increases in anti-inflammatory IL-10 and IL-1ra similar to that observed by others using a  
6 comparable level of heat stress (34°C, 32% RH) and workload (60%  $\dot{V}O_{2max}$ ; Gill et al.,  
7 2015) after 2 hours of running. While we did not include a thermoneutral control condition,  
8 others have found no increases in post-exercise cytokine concentration after 2 hours of  
9 running at 70%  $\dot{V}O_{2max}$  in temperate conditions (Costa et al., 2011), suggesting that heat  
10 stress is an important mediator in the cytokine response to exertional stress.

11

12 Precooling prior to extended periods of endurance activity in the heat can attenuate the level  
13 of systemic cellular stress experienced by individuals. Such countermeasures would be useful  
14 to already heat acclimated athletes competing over multiple days in hot environments, where  
15 pronounced elevations in pro and anti-inflammatory cytokines have been observed (Gill et  
16 al., 2015), and are associated with symptoms of gastrointestinal distress (Jeukendrup et al.,  
17 2000; Lambert, 2008; Lambert, 2009). However, cooling interventions aimed at reducing  
18 body temperature, and by extension the signal required to initiate the heat shock response,  
19 may also diminish the stimuli required for adaptation to either training or the environment.

20 Whether other more practical cooling approaches which focus on cooling the periphery (ice  
21 vests, cooling packs; Castle et al., 2006; Hasegawa et al., 2006) rather than the deep body  
22 temperatures would have a similar benefit needs investigation. It would also be interesting to  
23 determine whether the performance enhancing effects of precooling, and the alterations in  
24 pacing associated with such interventions (Marino, 2002), would negate any post exercise  
25 reductions in eHSP72 due to the increase in metabolic rate later in an exercise bout.

1  
2 Whilst the present study did not focus on intestinal epithelial permeability, it is possible that  
3 the whole-body precooling period reduced temperatures at the hepatosplanchnic viscera,  
4 creating a temperature sink between the deep core and peripheral tissues. A precooling  
5 mediated delay to vasodilation and warming of the circulating blood may preserve a lower  
6 tissue temperature for a longer period of time, thus delaying the tissue specific thermal signal  
7 for eHSP72 release and downstream cytokine release. Our data show that post exercise  
8 absolute changes in IL-6 and IL-10 were lower following precooling despite a similar overall  
9 thermal burden between trials. Due to the small sample size in the present investigation (n =  
10 8) a type 2 error cannot be ruled out for our observed cytokine responses, with the commonly  
11 high inter-individual variability in the post exercise cytokine response also making firm  
12 inferences difficult. Despite this, our data suggest that whole body precooling decreased the  
13 absolute change in post exercise IL-6 and IL-10. Further investigation into this area  
14 recommended to confirm these findings.

15  
16 An alternative explanation/mechanism for cooling-mediated reductions in cytokines is the  
17 known influence of stress hormones on both eHSP72 release, and the circulating cytokine  
18 cascade (Rhind et al., 2004). To determine the relative effects circulating stress hormones  
19 (Rhind et al., 2004) and improvements in tight junction integrity impart upon circulating  
20 eHSP72 and cytokines, future research could utilize the lactulose/rhamnose sugars test in  
21 order to determine effects on intestinal permeability alongside measures of stress hormones to  
22 quantify the relative importance of each. A limitation of the present study was that we were  
23 unable to conduct the control trial in thermoneutral water, thereby potentially introducing  
24 some small bias as a result of different hydrostatic pressure between trials. It is unlikely that  
25 any hydrostatic pressure effects would affect our blood-borne markers as a result of the pre

1 and post exercise sample being separated by a 90-minute exercise period. The perturbations  
2 caused by this exercise bout would likely override any small effects caused by different  
3 pressures.

4  
5 In summary, our findings suggest that precooling may limit the exertional-heat related  
6 increases in eHSP72 and reduce the post-exercise pro-inflammatory cytokineamia, which is  
7 associated with manifestations of gastrointestinal symptoms during prolonged exercise in the  
8 heat. A reduction in pro-inflammatory mediators, such as eHSP72, may be beneficial in  
9 limiting the development of exertional-heat related sub-clinical and clinical manifestations  
10 during recovery in multi-day events, but could be counterproductive if a training response or  
11 heat adaptation is a desired outcome.

12

### 13 **Abbreviations**

14 **AUC**; Area under the curve. **BLa**; blood lactate. **CK**; Creatine kinase. **CRP**; C- reactive  
15 protein. **eHSP72**; extracellular HSP72. **HR**; Heart rate. **HSP**; heat shock protein; **HSP72**;  
16 heat shock protein 72; **HSR**; Heat shock response. **IL-1 $\beta$** ; Inerleukin 1 $\beta$ . **IL-1ra**; Inerleukin  
17 1 receptor antagonist. **IL-6**; Interleukin – 6. **IL-10**; Interleukin 10. **iHSP72**; intracellular  
18 HSP72. **kDa**; Kilodalton.. **NBM**; nude body mass. **PSI**; Physiological strain index. **RER**;  
19 Respiratory exchange ratio. **RH**; relative humidity. **RPE**; rating of perceived exertion. **T-**  
20 **AUC**; total area under the curve. **T<sub>body</sub>**; Mean body temperature; **TLR**; Toll like receptor.  
21 **TNF- $\alpha$** ; Tumor necrosis factor – alpha. **T<sub>rectal</sub>**; mean rectal temperature. **T<sub>skin</sub>**; mean skin  
22 temperature.  **$\dot{V}O_2$** ; oxygen consumption.  **$\dot{V}O_{2max}$** ; maximal oxygen consumption.  **$\dot{V}O_{2peak}$** ;  
23 peak oxygen consumption. **W m<sup>-2</sup>**; Watts per meter squared.

24

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**Table 1.** Physiological and perceptual responses to 90 minutes running at 65% VO<sub>2</sub>max in hot conditions without prior precooling (HEAT; n = 8) and after 60 minutes of cold water immersion (COOL; n = 8). Data show the mean ± SD exercise data recorded throughout the exercise bout.

Variable	CON	COOL
Mean HR (bts·min <sup>-1</sup> )	159 ± 12	158 ± 14
Peak HR (bts·min <sup>-1</sup> )	171 ± 15	169 ± 16
Mean exercise T <sub>rectal</sub> (°C)	38.25 ± 0.34	37.86 ± 0.41*
Mean T <sub>rectal</sub> final 60 mins (°C)	38.60 ± 0.41	38.47 ± 0.41
Peak T <sub>rectal</sub> (°C)	38.88 ± 0.53	38.85 ± 0.51
Change in T <sub>rectal</sub> (°C)	2.19 ± 0.64	3.14 ± 1.01
Rate of T <sub>rectal</sub> change (90 minutes)	1.56 ± 0.45	2.15 ± 0.72*
AUC T <sub>rectal</sub> 38.5°C	11.72 ± 11.76	8.80 ± 12.10
Time T <sub>rectal</sub> over 38.5°C (mins:secs)	25:55 ± 23:12	17:30 ± 19:18
Mean PSI (AU)	6.7 ± 1.1	6.1 ± 1.2*
Peak PSI (AU)	8.4 ± 1.6	8.2 ± 1.8
Mean RPE	13 ± 2	13 ± 2
Peak RPE	16 ± 3	16 ± 3
Mean TS	2.6 ± 0.6	2.3 ± 0.8
Peak TS	3.0 ± 1.0	3.0 ± 1.0

Rate of change was calculated from the T<sub>rectal</sub> recorded immediately prior to exercise.

\* p < 0.05

**Table 2.** Circulating C-reactive protein, creatine kinase, and cytokine responses to 90 minutes running at 65%  $\dot{V}O_2$ max in hot conditions without prior precooling (CON; n = 8) and after 60 minutes of cold water immersion (COOL; n = 8).

	Rest	After exercise
C-Reactive protein (mg·mL <sup>-1</sup> )		
CON	0.95 ± 0.86	0.95 ± 0.87
COOL	0.79 ± 0.82	0.70 ± 0.68
Creatine Kinase (U·L <sup>-1</sup> )		
CON	333 ± 275	393 ± 275*
COOL	320 ± 295	359 ± 261*
TNF- $\alpha$ (pg·mL <sup>-1</sup> )		
CON	3.0 ± 1.1	3.3 ± 1.3
COOL	2.7 ± 0.8	3.1 ± 0.9
IL-6 (pg·mL <sup>-1</sup> )		
CON	0.5 ± 0.5	5.6 ± 2.2†
COOL	0.5 ± 0.5	3.5 ± 1.9*
IL-1ra (pg·mL <sup>-1</sup> )		
CON	142.6 ± 27.9	302.9 ± 244.4†
COOL	150.6 ± 20.0	212.9 ± 24.4†
IL-10 (pg·mL <sup>-1</sup> )		
CON	2.1 ± 1.4	13.4 ± 6.1†
COOL	2.2 ± 1.4	9.7 ± 4.9†

\* p < 0.05 compared to rest

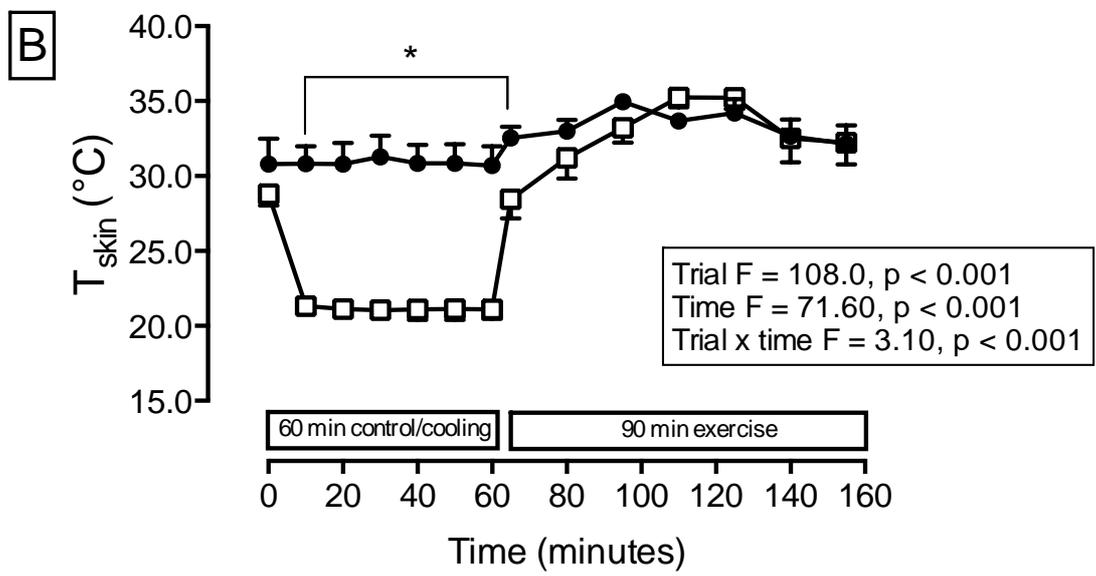
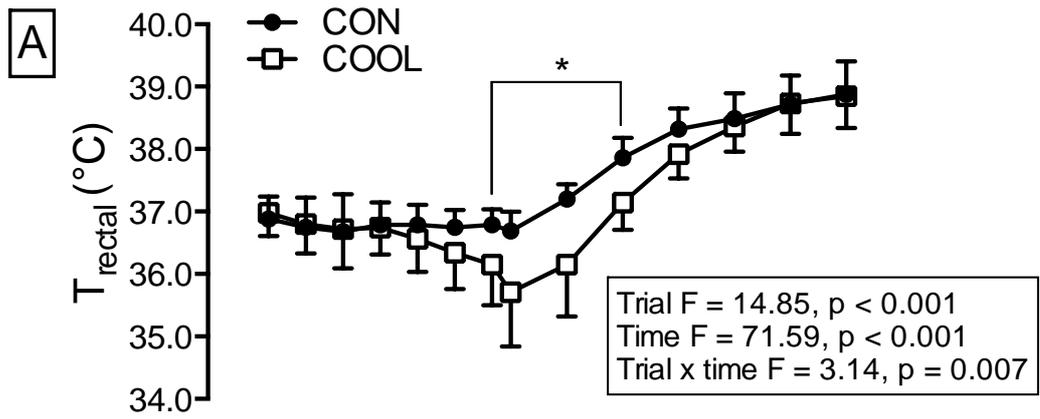
† p < 0.01 compared to rest

**Figure 1. Rectal temperature (A) and skin temperature (B) throughout 60 minutes of precooling and 90 minutes of running at 65%  $\dot{V}O_2\text{max}$  (\*  $p < 0.05$  compared to CON).** Rectal and skin temperatures increased throughout exercise during both conditions (main effect for time:  $T_{\text{rectal}}$ ,  $F = 17.6$ ,  $p < 0.001$ ;  $T_{\text{skin}}$   $F = 71.6$ ,  $p < 0.001$ ) and was lower after a period of precooling (main effect for trial:  $T_{\text{rectal}}$ ,  $F = 14.9$ ,  $p = 0.001$ ;  $T_{\text{skin}}$   $F = 108$ ,  $p < 0.001$ ). Both rectal and skin temperatures were lower at the onset of exercise following precooling, and remained lower than the control trial until 30 minutes of exercise (time x trial interaction:  $T_{\text{rectal}}$ ,  $F = 3.1$ ,  $p = 0.007$ ;  $T_{\text{skin}}$ ,  $F = 3.1$ ,  $p < 0.001$ ).

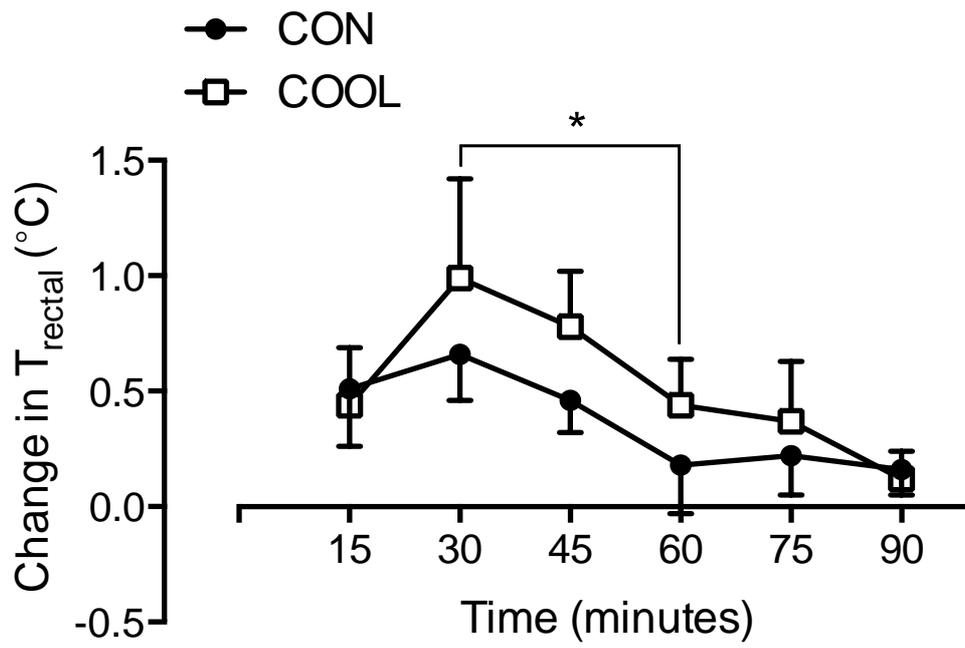
**Figure 2. Change in rectal temperature (A) and physiological strain (B) throughout 60 minutes of precooling and 90 minutes of running at 65%  $\dot{V}O_2\text{max}$  (\*  $p < 0.05$  compared to CON).** During exercise rectal temperatures increased (main effect for time,  $F = 47.9$ ,  $p < 0.001$ ), with the rate of increase higher following precooling (main effect for trial,  $F = 19.8$ ,  $p < 0.001$ ). Precooling increased the rate of change in  $T_{\text{rectal}}$  from 30 – 60 minutes of exercise (time x trial interaction,  $F = 2.9$ ,  $p = 0.011$ ). Physiological strain increased during exercise (main effect for time,  $F = 16.6$ ,  $p = 0.005$ ) and was lower following precooling (main effect trial,  $f = 12.0$ ,  $p = 0.034$ ). Lower physiological strain was observed during the initial 45 minutes of exercise after precooling, after which (trial x time interaction,  $F = 14.4$ ,  $p = 0.026$ ).

**Figure 3. Individual changes in eHSP72 (A) and post-exercise absolute change in eHSP72 (B) following 90 minutes running at 65%  $\dot{V}O_2\text{max}$  in hot conditions.** eHSP72 was increased following exercise (main effect for time,  $F = 5.8$ ,  $p = 0.047$ ), and was attenuated by a period of precooling (main effect for trial,  $f = 6.6$ ,  $p = 0.037$ ), with a weak time x trial interaction observed ( $f = 4.0$ ,  $p = 0.08$ ). The absolute change in post-exercise eHSP72 was attenuated following precooling ( $p < 0.001$ ).

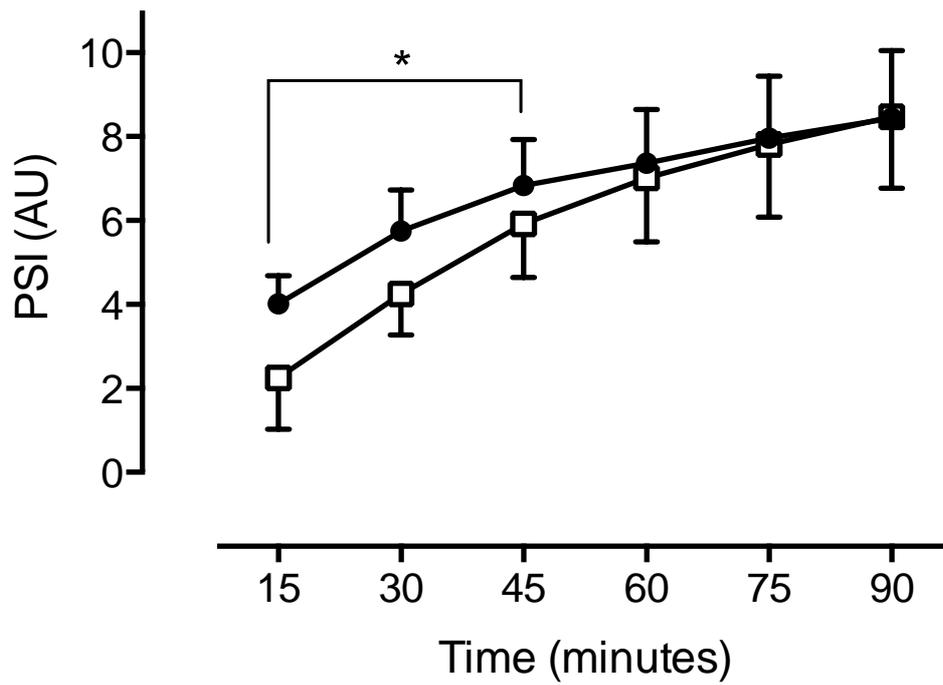
**Figure 4. Individual post-exercise absolute changes in circulatory TNF- $\alpha$  (Panel A), IL-6 (Panel B), IL-10 (Panel C) and IL-1ra (Panel D) following 90 minutes running at 65%  $\dot{V}O_2\text{max}$  in CON and COOL.**



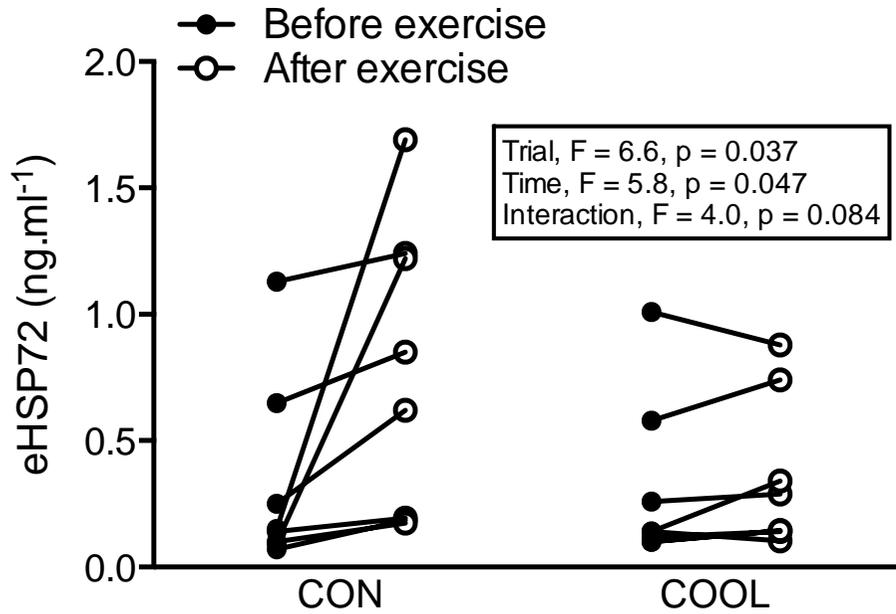
A



B



**A**



**B**

