Whole body precooling attenuates the extracellular HSP72, IL-6 and IL-10 responses after an acute bout of running in the heat

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

Running title: eHSP72 and inflammatory responses following precooling

Abstract

The impact of whole-body precooling on the extracellular heat shock protein 72 (eHSP72) and cytokine responses to running in the heat is undefined. The aim of this study was to determine whether precooling would attenuate post-exercise eHSP72 and cytokine responses. Eight male recreational runners completed two 90-minute bouts of running at 65% $\dot{V}O_2max$ in 32 ± 0.9°C and 47 ± 6 % relative humidity (RH) preceded by either 60-minutes of precooling in 20.3 ± 0.3°C water (COOL) or 60 minutes rest in an air-conditioned laboratory (20.2 ± 1.7°C, 60 ± 3% RH; CON). eHSP72, TNF-α, IL-6, IL-10 IL-1ra were determined before and immediately after exercise. The elevation in post-exercise eHSP72 was attenuated after COOL (+0.04 ± 0.10 $ng{\cdot}mL^{-1}$) compared to CON (+ 0.29 ± 0.26 $ng.mL^{-1}$; $p < 0.001$). No changes in TNF-α were observed at any stage. COOL reduced the absolute post-exercise change in IL-6 ($p = 0.011$) and IL-10 ($p = 0.03$) compared to CON. IL-1ra followed this trend ($p = 0.063$). A precooling-induced attenuation of eHSP72 and proinflammatory cytokines may aid recovery during multi-day sporting events, but could be counterproductive if a training response or adaptation to environmental stress is a desired outcome.

Keywords: Precooling, eHSP72, cytokines, inflammation

Word count: 4014

Introduction

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

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

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

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

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

exercise in the heat would reduce eHSP72 and plasma cytokines TNF-α, IL-6, IL-10 and IL-1ra.

Methods

Participants

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

Pre-experimental standardization

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

Participants attended the laboratory after an overnight fast and ingested a volume of 5 mL·kg^{-1} body mass; 363 ± 63 mL) of room temperature (~18°C) water 2 h before the trial, and an additional 7 mL·kg^{1} body mass (508 ± 89 mL) of 18^{o}C water (~18l 7 mL·kg^{-1} body mass) *ad libitum* during exercise. All trials were performed at the same time of day to minimise the circadian variation in internal body temperature.

Procedures

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

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

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

AUC T_{rectal} >38.5°C (°C min^{-1}) = ∑ time interval (min) x 0.5 (°C > 38.5°C at start of interval + °C above 38.5°C at the end of the time interval).

Blood sampling

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

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

Circulating TNF-a, IL-1ra, IL-6 and IL-10 were determined in duplicate using commercially available high sensitivity sandwich ELISA kits (R&D systems, Minneapolis, USA) which were sensitive to 0.19 $pg \cdot mL^{-1}$, 0.18 $pg \cdot mL^{-1}$ 0.11$pg \cdot mL^{-1}$ and 0.17$pg \cdot mL^{-1}$ respectively. The inter-assay variability for TNF-a, IL-1ra, IL-6 and IL-10 was 3.1, 4.0, 1.5 and 1.8% respectively.

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

Statistical analysis

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

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

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

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

Results

Physiological and perceptual measures

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

Thermoregulatory responses to precooling

Baseline T_{skin} (CON = 30.79 ± 0.70; COOL 30.78 ± 0.85) and T_{rectal} (CON = 36.88 ± 0.36°C; COOL = 36.98 ± 0.37°C) were similar prior to the pre-cooling or rest period commencing. The reduction in T_{skin} and T_{rectal} during the pre-exercise period was greater during COOL than CON (Figure 1, time x trial interaction, $p < 0.01$ for both) such that there was a between trial difference at the onset of exercise. The reduction in T_{rectal} persisted until 30 min of exercise was completed, however due to a greater rate of rise in rectal temperature (Figure 2A), there

was no difference between conditions at the conclusion of the exercise bout. A similar response was observed for Tskin (Figure 2B). Mean exercise T_{rectal} was lower in COOL compared to CON (p = 0.03, d = -1.0, 95% CI = -2.0 to -0.1), however the AUC and total time >38.5°C was not different between CON and COOL (AUC: p = 0.30, d = -0.2, 95% CI -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 until 45 minutes of exercise, with no differences between conditions in the remaining 45 minutes of exercise (Figure 2B, d = -0.6, 95% CI -1.6 to 0.45). Post-exercise plasma blood lactate concentrations were no different between CON (2.65 ± 0.51 $mmol^{-1}$) and COOL (2.35 ± 0.51 $mmol^{-1}$; p = 0.061).

Extracellular HSP72 and circulating cytokines

Resting eHSP72 was similar between trials (CON = 0.32 ± 0.38 $ng{\cdot}mL^{-1}$; COOL = 0.31 ± 0.33 $ng{\cdot}mL^{-1}$). Exercise led to an increase in eHSP72 concentrations post-exercise in CON (0.77 ± 0.58 $ng{\cdot}mL^{-1}$; d = 0.7, 95% CI = -0.4 to 1.6) but not COOL (0.35 ± 0.29 $ng{\cdot}mL^{-1}$; d = 0.1, 95% CI = -0.9 to 1.1, Figure 3A). Post-exercise concentrations in eHSP72 were related to final T_{rectal} temperatures in both CON (r = 0.56, p = 0.15) and COOL (r = 0.63, p = 0.09). The absolute change in eHSP72 was lower following COOL compared to CON (p < 0.001; d = -1.1, 95% CI = -2.5 to -0.3; Figure 3B).

No main effects or interactions were observed for TNF- α (Table 2), however IL-6 (f = 59.4, p < 0.001), IL-10 (f = 41.2, p < 0.001) and IL-1ra (f = 6.4, p = 0.02) concentrations were increased following exercise (Table 2). A similar post-exercise increase in IL-10 (f = 1.5, p = 0.23) and IL-1ra (f = 0.9, p = 0.4) was seen following both CON and COOL, whereas the increase in IL-6 was blunted after COOL (f = 4.4, p = 0.045). The absolute change in both 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

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

Discussion

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

exercise is not mediated solely by a rise in body temperature, and could be a product of the overall physiological strain experienced during an exercise challenge.

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

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

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

et al., 2008; Rhind et al., 2004). Additionally, IL-6, which is described as both pro and anti-inflammatory depending on its site of production and release, is also elevated after exercise in hot conditions (Gleeson et al., 2011; Gill, Teixeira, et al., 2015; Rhind et al., 2004). Accordingly, the CON trial resulted in post-exercise increases in IL-6 and compensatory increases in anti-inflammatory IL-10 and IL-1ra similar to that observed by others using a comparable level of heat stress (34°C, 32% RH) and workload (60% $\dot{V}O_2$max; Gill et al., 2015) after 2 hours of running. While we did not include a thermoneutral control condition, others have found no increases in post-exercise cytokine concentration after 2 hours of running at 70% $\dot{V}O_2$max in temperate conditions (Costa et al., 2011), suggesting that heat stress is an important mediator in the cytokine response to exertional stress.

Precooling prior to extended periods of endurance activity in the heat can attenuate the level of systemic cellular stress experienced by individuals. Such countermeasures would be useful to already heat acclimated athletes competing over multiple days in hot environments, where pronounced elevations in pro and anti-inflammatory cytokines have been observed (Gill et al., 2015), and are associated with symptoms of gastrointestinal distress (Jeukendrup et al., 2000; Lambert, 2008; Lambert, 2009). However, cooling interventions aimed at reducing body temperature, and by extension the signal required to initiate the heat shock response, may also diminish the stimuli required for adaptation to either training or the environment. Whether other more practical cooling approaches which focus on cooling the periphery (ice vests, cooling packs; Castle et al., 2006; Hasegawa et al., 2006) rather than the deep body temperatures would have a similar benefit needs investigation. It would also be interesting to determine whether the performance enhancing effects of precooling, and the alterations in pacing associated with such interventions (Marino, 2002), would negate any post exercise reductions in eHSP72 due to the increase in metabolic rate later in an exercise bout.

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

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

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

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

Abbreviations

AUC; Area under the curve. **BLa;** blood lactate. **CK;** Creatine kinase. **CRP;** C- reactive protein. **eHSP72;** extracellular HSP72. **HR;** Heart rate. **HSP;** heat shock protein; **HSP72;** heat shock protein 72; **HSR;** Heat shock response. **IL-1β;** Inerleukin 1β. **IL-1ra;** Inerleukin 1 receptor antagonist. **IL-6;** Interleukin – 6. **IL-10;** Interleukin 10. **iHSP72;** intracellular HSP72. **kDa;** Kilodalton**..** **NBM;** nude body mass. **PSI;** Physiological strain index. **RER;** Respiratory exchange ratio. **RH;** relative humidity. **RPE;** rating of perceived exertion. **T-AUC;** total area under the curve. $\mathbf{T_{body}}$; Mean body temperature; **TLR;** Toll like receptor. **TNF-α**; Tumor necrosis factor – alpha. $\mathbf{T_{rectal}}$; mean rectal temperature. $\mathbf{T_{skin}}$; mean skin temperature. $\mathbf{\dot{V}O_2}$**;** oxygen consumption. $\mathbf{\dot{V}O_2max}$**;** maximal oxygen consumption. $\mathbf{\dot{V}O_2peak}$**;** peak oxygen consumption. $\mathbf{W\ m^{-2}}$**;** Watts per meter squared.

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Table 1. Physiological and perceptual responses to 90 minutes running at 65% VO_2max 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 ($bts \cdot min^{-1}$)	159 ± 12	158 ± 14
Peak HR ($bts \cdot min^{-1}$)	171 ± 15	169 ± 16
Mean exercise T_{rectal} (°C)	38.25 ± 0.34	37.86 ± 0.41*
Mean T_{rectal} final 60 mins (°C)	38.60 ± 0.41	38.47 ± 0.41
Peak T_{rectal} (°C)	38.88 ± 0.53	38.85 ± 0.51
Change in T_{rectal} (°C)	2.19 ± 0.64	3.14 ± 1.01
Rate of T_{rectal} change (90 minutes)	1.56 ± 0.45	2.15 ± 0.72*
AUC T_{rectal} 38.5°C	11.72 ± 11.76	8.80 ± 12.10
Time T_{rectal} 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_{rectal} 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 ($mg \cdot mL^{-1}$)		
CON	0.95 ± 0.86	0.95 ± 0.87
COOL	0.79 ± 0.82	0.70 ± 0.68
Creatine Kinase ($U \cdot L^{-1}$)		
CON	333 ± 275	393 ± 275*
COOL	320 ± 295	359 ± 261*
TNF-α ($pg \cdot mL^{-1}$)		
CON	3.0 ± 1.1	3.3 ± 1.3
COOL	2.7 ± 0.8	3.1 ± 0.9
IL-6 ($pg \cdot mL^{-1}$)		
CON	0.5 ± 0.5	5.6 ± 2.2†
COOL	0.5 ± 0.5	3.5 ± 1.9*
IL-1ra ($pg \cdot mL^{-1}$)		
CON	142.6 ± 27.9	302.9 ± 244.4†
COOL	150.6 ± 20.0	212.9 ± 24.4†
IL-10 ($pg \cdot mL^{-1}$)		
CON	2.1 ± 1.4	13.4 ± 6.1†
COOL	2.2 ± 1.4	9.7 ± 4.9†

* $p < 0.05$ compared to rest

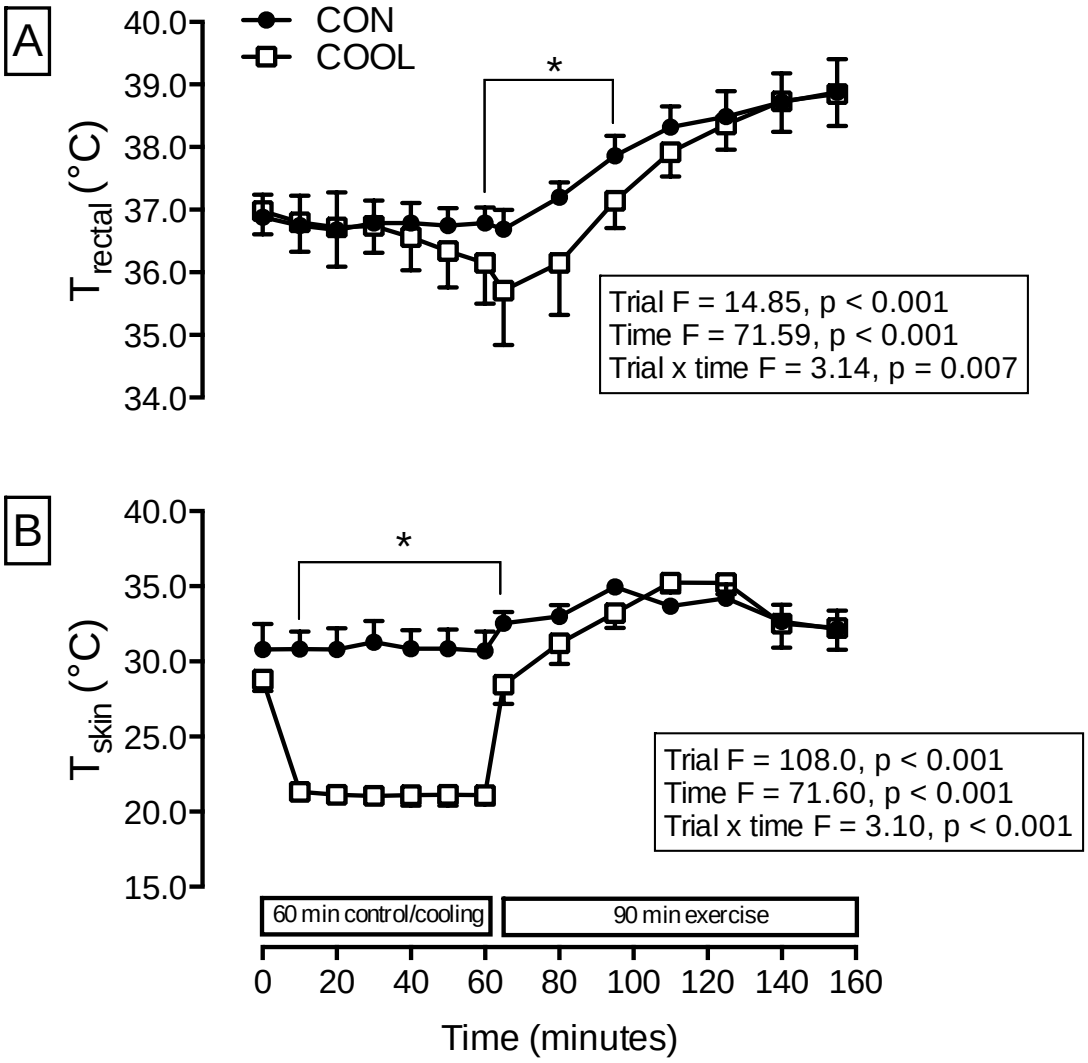
† $p < 0.01$ compared to rest

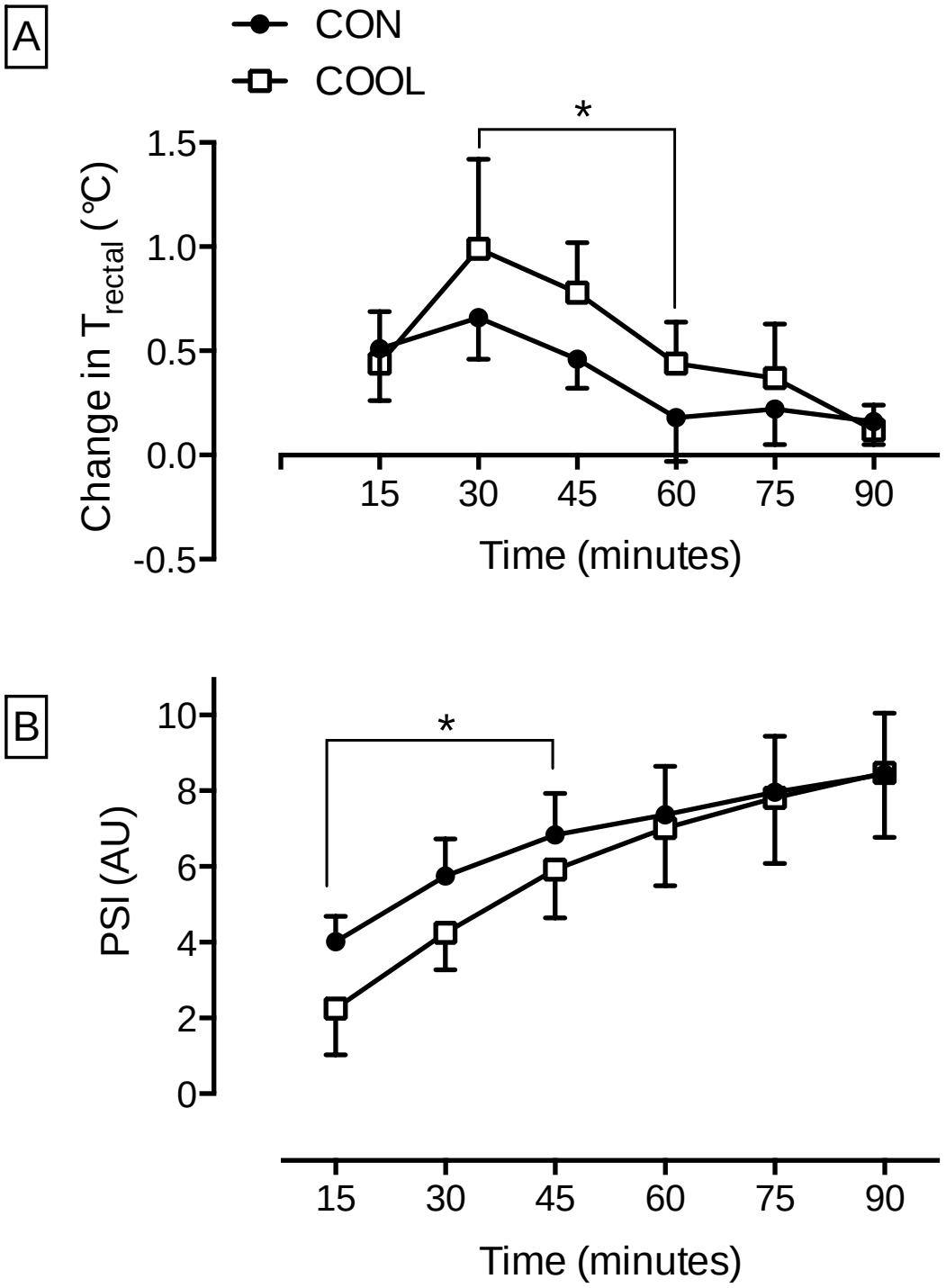
Figure 1. Rectal temperature (A) and skin temperature (B) throughout 60 minutes of precooling and 90 minutes of running at 65% $\dot{V}O_2$max (* p < 0.05 compared to CON). Rectal and skin temperatures increased throughout exercise during both conditions (main effect for time: T_{rectal}, F = 17.6, p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

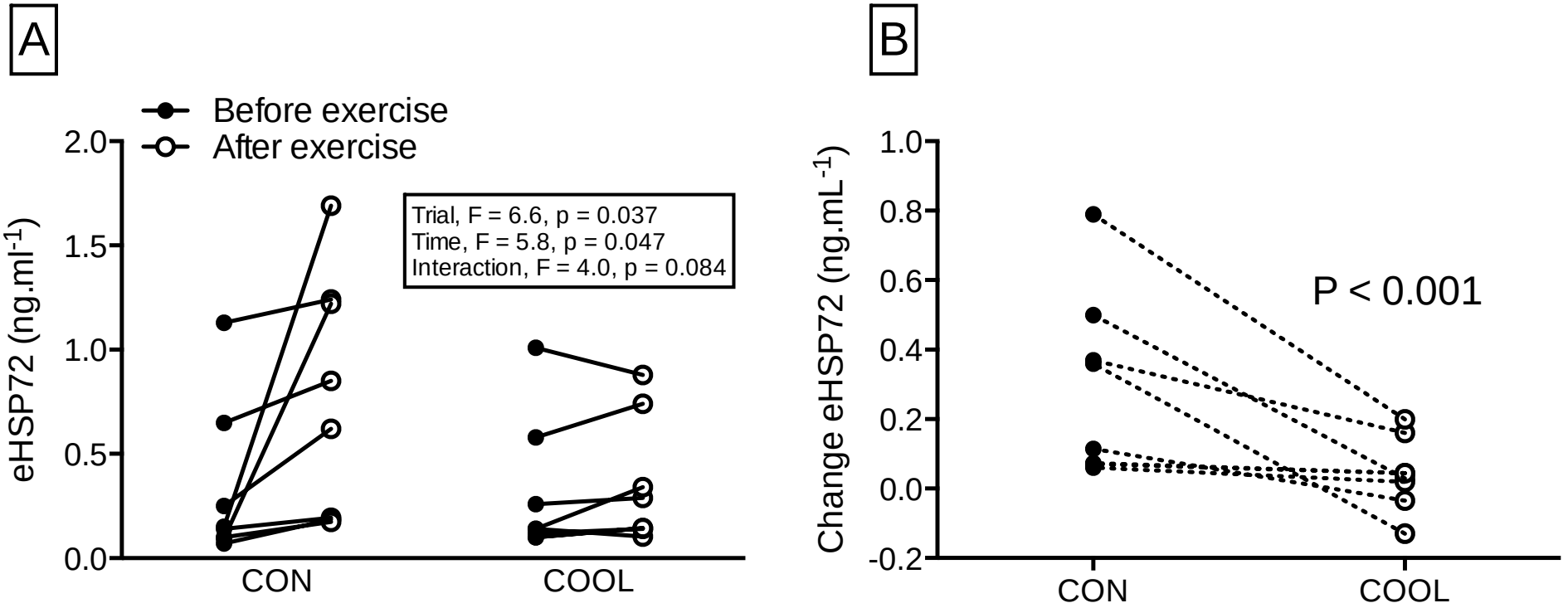
Figure 2. Change in rectal temperature (A) and physiological strain (B) throughout 60 minutes of precooling and 90 minutes of running at 65% $\dot{V}O_2$max (* p < 0.05 compared to CON). During exercise rectal temperatures increased (main effect for time, F = 47.9, p < 0.001), with the rate of increase higher following precooling (main effect for trial, F = 19.8, p < 0.001). Precooling increased the rate of change in T_{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).

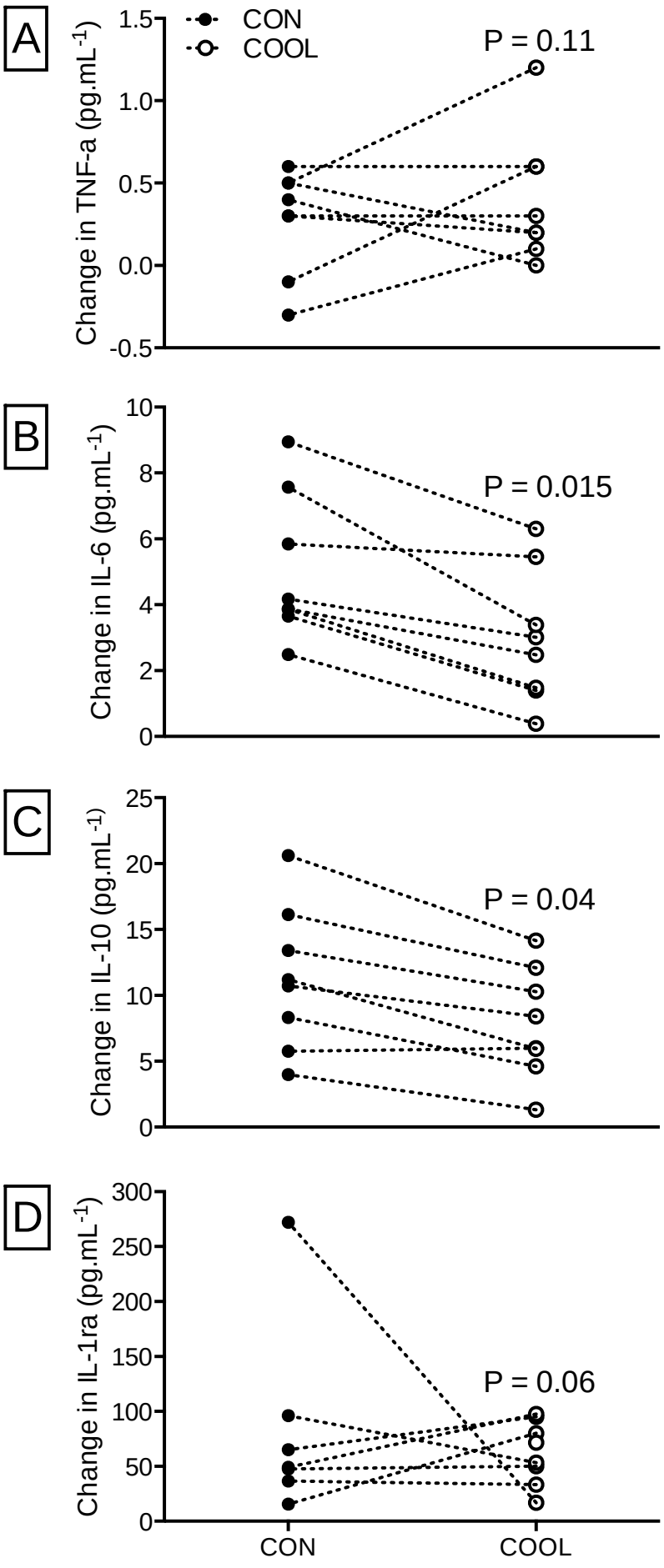
Figure 3. Individual changes in eHSP72 (A) and post-exercise absolute change in eHSP72 (B) following 90 minutes running at 65% $\dot{V}O_2$max in hot conditions. eHSP72 was increased following exercise (main effect for time, F = 5.8, p = 0.047), and was attenuated by a period of precooling (main effect 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-α (Panel A), IL-6 (Panel B), IL-10 (Panel C) and IL-1ra (Panel D) following 90 minutes running at 65% $\dot{V}O_2$max in CON and COOL.


A
CON
COOL
40.0
39.0
38.0
37.0
36.0
35.0
34.0
T rectal (°C)
*
Trial F = 14.85, p < 0.001
Time F = 71.59, p < 0.001
Trial x time F = 3.14, p = 0.007
B
40.0
35.0
30.0
25.0
20.0
15.0
T skin (°C)
*
Trial F = 108.0, p < 0.001
Time F = 71.60, p < 0.001
Trial x time F = 3.10, p < 0.001
60 min control/cooling
90 min exercise
0
20
40
60
80
100
120
140
160
Time (minutes)


A
CON
COOL
*
1.5
1.0
0.5
0.0
-0.5
Change in T_{rectal} (°C)
15
30
45
60
75
90
Time (minutes)
B
*
10
8
6
4
2
0
PSI (AU)
15
30
45
60
75
90
Time (minutes)


A
Before exercise
After exercise
eHSP72 (ng.ml-1)
2.0
1.5
1.0
0.5
0.0
Trial, F = 6.6, p = 0.037
Time, F = 5.8, p = 0.047
Interaction, F = 4.0, p = 0.084
CON
COOL
B
Change eHSP72 (ng.mL-1)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
P < 0.001
CON
COOL


A
CON
COOL
P = 0.11
Change in TNF-a (pg.mL-1)
1.5
1.0
0.5
0.0
-0.5
B
P = 0.015
Change in IL-6 (pg.mL-1)
10
8
6
4
2
0
C
P = 0.04
Change in IL-10 (pg.mL-1)
25
20
15
10
5
0
D
P = 0.06
Change in IL-1ra (pg.mL-1)
300
250
200
150
100
50
0
CON
COOL