Title: Effective microorganism – x attenuates circulating superoxide dismutase following an acute bout of intermittent running in hot, humid conditions.

Running title: Effective microorganism x attenuates repeated sprint induced disturbances to redox balance.

Key Words: Heat shock proteins (HSP), oxidative stress, redox balance, repeated sprint, exercise, HIT, human**.**

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Abstract

This study determined the effectiveness of antioxidant supplementation on high intensity exercise-heat stress.

Six males completed a high intensity running protocol twice in temperate conditions (TEMP; 20.4°C), and twice in hot conditions (HOT; 34.7°C). Trials were completed following seven days supplementation with 70mL.day^{-1} effective microorganism-x (EM-X; $TEMP_{EMX}$ or HOT_{EMX}) or placebo ($TEMP_{PLA}$ or HOT_{PLA}). Plasma extracellular Hsp72 (eHsp72) and superoxide dismutase (SOD) were measured by ELISA.

eHsp72 and SOD increased pre-post exercise (p<0.001), with greater eHsp72 (p<0.001) increases observed in HOT (+1.5ng.mL^{-1}) compared to TEMP (+0.8ng.mL^{-1}). EM-X did not influence eHsp72 (p>0.05). Greater (p<0.001) SOD increases were observed in HOT (+0.22U.mL^{-1}) vs. TEMP (+0.10U.mL^{-1}) with SOD reduced in HOT_{EMX} vs. HOT_{PLA} (p=0.001). Physiological and perceptual responses were all greater (p<0.001) in HOT vs. TEMP conditions, with no difference followed EM-X (p>0.05).

EM-X supplementation attenuated the SOD increases following HOT, potentiating its application as an ergogenic aid to ameliorate oxidative stress.

Introduction

Increased extracellular heat shock protein 72 (eHsp72) concentrations are evident in response to exercise (Yamada, Amorim, Moseley, & Schneider, 2008) with the greater increases during exercise-heat stress predicted by the magnitude of change in rectal temperature (Gibson et al., 2014) and exercise intensity (Periard, Ruell, Caillaud, & Thompson, 2012).

Reactive oxygen species generation and/or disturbances to redox balance are essential components of adaption (Powers, Duarte, Kavazis, & Talbert, 2010). Conversely, their presence can induce performance decrements when repeated demanding exercise is required with insufficient recovery (Cobley, McGlory, Morton, & Close, 2011) i.e. during tournament competition or within consecutive day, multiday events. Oxidative stress can inhibit the onset of tissue repair (Ascensao et al., 2008) with chronic oxidative stress coinciding with skeletal muscle atrophy (Powers, et al., 2010). Amelioration of short-term negative effects of oxidative stress on demanding exercise with limited recovery time could be advantageous, thus this paradigm (exercise-heat stress; redox balance; Hsp72) requires exploration. N-Acetylcysteine supplementation can achieve this goal, though nausea and gastrointestinal discomfort can present (Cobley, et al., 2011), reducing its practicality. At present, there is no literature directly assessing the interplay between oxidative stress/redox balance disturbances and eHsp72 expression in vivo, though redox disturbances preceding increases in intracellular Hsp72 (iHsp72) are well reported (Taylor et al., 2012).

Antioxidant supplementation may hypothetically blunt the exercise induced eHsp72 response in a similar manner to that seen with hypoxic preconditioning and iHsp72 (Taylor, et al., 2012), in addition to antioxidant mediated ergogenicity specific to high-intensity interval training (HIT) type exercise (Cobley, et al., 2011). Effective Microorganism X (EM-X) has potent antioxidant effects (Deiana et al., 2002; Do, Seo, Hwang, Kim, & Nam, 2007). The antioxidant cocktail (EM-X) is derived from effective microorganisms of lactic acid bacteria, yeast and photosynthetic bacteria (Aruoma et al., 2002) and presents no mutagenic effects

under chronic or acute supplementation (Ke, Liang, Zhong, Higa, & Aruoma, 2005). EM-X administration increases serum dismutase, decreases malondialdehyde (Deiana, et al., 2002), whilst, resisting acute severe oxidative stress mediated damage in the kidney and liver of rats (Aruoma, et al., 2002), and conveying an anti-inflammatory influence at a cellular level, independent of antioxidant activity (Do, et al., 2007). Given the potent stimuli that exercise (Yamada, et al., 2008), exercise-heat stress (Gibson, et al., 2014) and disturbances to redox (Taylor, et al., 2012) represent for HSP induction, supplementation of EM-X may affect exercise induced disturbances to redox balance, thus the HSP exercise response. HIT specific exercise-heat stress induced fatigue, as shown elsewhere with N-Acetylcysteine (Cobley, et al., 2011), may be attenuated with EM-X supplementation by reducing disturbances to redox balance (Aruoma, et al., 2002; Aruoma et al., 2003; Deiana, et al., 2002) and pro-inflammatory cascades (Do, et al., 2007).

The aims of the present study are to investigate; i) the influence of HIT on eHsp72 concentration and plasma superoxide dismutase (SOD) activity; ii) the influence of ambient temperature (thermoneutral/hyperthermic) on any HIT induced alterations in basal eHsp72 concentration and plasma SOD activity; iii) the influence of EM-X supplementation on HIT induced eHsp72 concentration and plasma SOD activity within both environmental conditions (thermoneutral/hyperthermic).

Methods

Subjects and general experimental controls/methods:

Six male subjects (mean ± SD: age 22.0 ± 1.3 years; height 181.0 ± 4.19 cm; mass 73.5 ± 3.1 kg; maximum oxygen uptake (VO_{2max}) 51.7 ± 7 $mL \cdot kg^{-1} \cdot min^{-1}$) volunteered to participate within the present study. Subjects attended the laboratory at the same time of day to minimise circadian variation on performance (Reilly et al., 2007; Winget, Deroshia, & Holley, 1985).

The confounding variables of hypoxic, thermal and hyperbaric exposures, and smoking, glutamine, caffeine, alcohol and generic supplementation were all controlled in line with previous HSP/redox balance exercise projects within the field (Taylor, et al., 2012).

Prior to reporting to the laboratory, subjects were instructed to drink 500 mL of water 2 hrs prior to all laboratory visits, in accordance with the ACSM position stand (Sawka et al., 2007). Upon arrival at the laboratory the subjects provided a urine sample for assessment of specific gravity to determine hydration status using dip test strips (Combur10-test, Roche Diagnostics, Mannheim, Germany). Nude body mass was assessed (Tanita BWB-800, Tokyo, Japan), a rectal temperature probe was inserted at a depth of 10 cm past the anal sphincter (Libra Medical, Reading, UK) and a HR monitor was affixed to the chest (Polar Sports Tester, Polar Electro Oy, Kempele, Finland).

High intensity interval running protocol (HIT_{RP}): The protocol consisted of 20 fast high intensity runs 10 seconds in duration at a velocity corresponding to the final running speed achieved during the Maximal Anaerobic Running Test (MART) test (23.0 ± 1.8 $km.h^{-1}$). with 80 seconds of active recovery at a velocity corresponding to 35% VO_{2max} (6.7 ± 2.2 $km.h^{-1}$) and lasted approximately 40 minutes (37.3 ± 3.5 minutes). The high intensity running speed was supramaximal in relation to the VO_{2max} test. Heart rate (HR) was recorded following each 10 second run and 60 seconds into recovery. Ratings of perceived exertion (RPE; (Borg, Ljunggren, & Ceci, 1985)), thermal sensation (TS; (Gagge, Stolwijk, & Saltin, 1969)) and core temperature (T_c) were recorded 60 seconds into recovery. Venous whole blood samples were obtained at rest before commencement of the sprint protocol and immediately upon completion of the sprint protocol, descriptions of collection and analysis is detailed in *Venous blood sampling, eHsp72 and SOD measurement* below.

Venous blood sampling, eHsp72 and SOD measurement: In line with previous work in the field (Gibson, et al., 2014) a 10 mL whole venous blood sample was drawn from the antecubital fossa. Each sample was divided equally into 5 mL tubes (Starstedt, Germany)

136 containing EDTA (Vacuette®, Greiner BIO-one, UK). Whole blood samples were
137 centrifuged (Eppendorf 5804 R Centrifuge) at 4,500 rpm for 15 min to separate plasma.
138 Plasma was pipetted (Eppendorf Research/Research Pro) into 1.5 mL microtubes (Eppendorf)
139 and stored at -86°C (Sanyo Ultra Low, VIP Series) until analysis.

140 eHsp72 analysis utilised a commercially available pre-prepared Enzyme-Linked
141 Immunosorbent Assay (ELISA) kit in line with manufacturer's instructions (Stress Express
142 HSP70 High sensitivity ELISA kit, EKS-715, Stressgen Bioreagents, Victoria, Canada)
143 utilising a plate reader (ELx800, Bio-Tech Instruments, Inc. Winoski, USA) and read at an
144 absorption of 450 nm. The sensitivity of the ELISA kit was 0.09 ng/mL and both inter- and
145 intra-assay coefficient of variation was 3.2%, in line with previous work in the field (Gibson,
146 et al., 2014).

147 SOD was analysed with a commercially available ELISA kit (Cayman Chemical, Ann Arbor,
148 Michigan, USA) in line with manufacturer's instructions utilising a plate reader and read at
149 an absorption of 450 nm. The dynamic range of the SOD assay was 0.025-0.25 units/mL
150 SOD, and the inter- and intra-assay coefficients of variance were less than 3.7% (Cayman
151 Chemicals SOD ELISA kit, Cayman Chemical, USA).

152 *Plasma Osmolality*: Approximately 20µL of plasma was used to determine if changes in
153 plasma osmolality (Micro Osmometer Model 3300, Advanced Instruments, Inc., USA)
154 between conditions affected the final concentrations of eHsp72 and SOD. Changes in whole
155 venous plasma volume were quantified using established methods in triplicate (~50 µL) in
156 line with Gibson et al (2014). Plasma volume was not significantly different (p=0.05)
157 between any conditions.

158 *Supplementation:* Subjects consumed one pre-prepared 250mL bottle (70mL EM-X
159 (Effective Microorganisms UK) mixed with 180mL water) of EM-X drink, or, volume, taste
160 and odour matched 250mL bottle of placebo drink for seven consecutive days prior to

laboratory attendance. The EM-X drink was prepared in accordance with the manufacturer's recommended dosage (personal communication).

Subjects reported to the laboratory on six occasions having fasted for 2 hours and replicating food intake and activity levels (habitual exercise only) prior to each experimental visit. Compliance for all the aforementioned experimental controls was at 100% in all subjects. The protocol was approved by the institutional Ethics Committee and all subjects signed informed consent following the principles outlined in the Declaration of Helsinki.

Experimental design – Visit 1

Anthropometric measures: During the initial visit subject's height and body mass were obtained to the nearest 0.1cm/kg in the Frankfurt plane (Harpenden Instruments, West Sussex, UK).

VO_{2max} test: Maximal aerobic capacity was determined using an incremental test to exhaustion on a treadmill (Woodway, Waukesha, WI, USA). Participants began the test at a speed of 9km.h^{-1}, 1% gradient. The test consisted of 2 minute stages, during the second minute of each stage expired air was collected for approximately 60s via open circuit spirometry. HR was monitored throughout the test using a HR monitor (Polar Sports Tester, Polar, Electro Inc, Finland). Treadmill speed was increased by 1 km.h^{-1} at the onset of each new stage. Subjects were instructed to continue for as long as possible with. verbal encouragement throughout. VO_{2max} was taken as the highest VO_2 value obtained in any 10 second period and was taken as having been achieved when meeting end-point criteria in accordance with the guidelines of the British Association of Sport and Exercise Sciences (Bird & Davison, 1997). Expired air was analysed using an infrared and paramagnetic analyser (model 1400, Servomex Controls, Crowbourough, UK). Results of the VO_{2max} test were used to calculate running speeds for the active recovery stage by using the linear regression equation generated from the graph of running speed (km.h^{-1}) and VO_2 (mL.kg^{-1}.min^{-1}).

Visit 2

Maximal Anaerobic Running Test (MART): Two days after the VO_{2max} test, subjects began a standardised warm up on a motorised treadmill (Woodway, Waukesha, WI, USA) and then began the MART (Nummela, Alberts, Rijntjes, Luhtanen, & Rusko, 1996) at a speed of 12 $km.h^{-1}$,10% gradient running for 20 seconds, runs were followed by 100 seconds of passive recovery before beginning a new stage whereby speed was increased by 0.5 $km.h^{-1}$. The test was terminated when the subject could no longer keep pace with the treadmill, the final completed running speed was used to prescribe the running velocity during the intermittent running protocol.

Visits 3 to 6

Subjects completed one high intensity interval running protocol (HIT_{RP}) on each of the four remaining visits in a randomised order utilising a double blind, cross over design, within a purpose built environmental chamber with temperature and humidity controlled using automated computer feedback (WatFlow control system; TISS, Hampshire, UK). Two visits were completed in temperate conditions (TEMP; 20.4 ± 1.7°C, 41, ± 4.2 % RH), and two in hot and humid conditions (HOT; 34.7 ± 2.0°C, 51.7 ± 4.5 % RH). One hot and one temperate trial were completed following a period of supplementation with EM-X ($TEMP_{EMX}$ or HOT_{EMX}) or placebo ($TEMP_{PLA}$ or HOT_{PLA}). The HIT_{RP} were conducted in their entirety for all participants in each of the four experimental trials.

Statistical Methods

All statistical analyses were completed using IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL). The normality of each dependent variable was checked using quantile-quantile (Q-Q) plots and deemed plausible in each instance. The central tendency and dispersion of each dependent variable are therefore reported as the mean (SD). The effects of Ingestion (placebo vs. EM-X) and Temperature (temperate vs. hot) on the response of each dependent variable

over the twenty intervals (or pre- and post-intervention for eHsp72 and SOD) were investigated using linear mixed models. The best fitting covariance structure for each model was identified by minimising the Hurvich and Tsai's criterion. The assumptions of normally distributed residuals around a mean of zero and constant variance were checked using Q-Q plots and scatter plots and deemed plausible. Statistical significance was accepted as $p \leq 0.05$.

Results

Plasma eHsp72 increased from pre- to post-exercise (F=62.1, p=0.001), however, this time effect was moderated by Temperature (F=30.8, p=0.001) see Fig.1. In TEMP eHsp72 increased by 0.8 ng·mL^{-1} (95% CI=0.3 to 1.2 ng·mL^{-1}, p=0.005), compared to 1.5 ng·mL^{-1} in HOT (95% CI=1.1 to 1.9 ng·mL^{-1}, p=0.001). The main effects for Ingestion (F=1.7, p=0.20) and the interaction effect (F=0.3, p=0.59) were not significant. The mean difference in plasma eHsp72 between the placebo and EM-X conditions was not different in TEMP and HOT (F=0.4, p=0.53).

SOD activity increased over time (F=70.1, p=0.001), the effect of which, was moderated by Temperature (F=9.4, p=0.004) see Fig. 2. In TEMP there was a 0.10 U·mL^{-1} mean increase in SOD activity (95% CI=0.047 to 0.16 units, p=0.001), compared to a 0.22 U·mL^{-1} mean increase in HOT (95% CI=0.16 to 0.28 U·mL^{-1}, p=0.001). There was no pre-intervention difference between SOD activity in TEMP and HOT (p=0.74). SOD activity was higher in HOT than TEMP post-intervention (p=0.001). Ingestion type did not influence SOD activity over time, either alone (F=1.7, p=0.20), or as an interaction with Temperature (F=1.4, p=0.24). Mean SOD was lower in the EM-X conditions compared to placebo (F=9.7, p=0.004), but higher in HOT compared to TEMP (F=6.7, p=0.014). A significant interaction was observed (F=4.5, p=0.042). In HOT there was a 0.10 U·mL^{-1} higher mean SOD activity in the placebo condition compared to the EM-X condition (95% CI=0.045 to 0.16 U·mL^{-1}, p=0.001), with no difference between placebo and EM-X in TEMP (mean difference=0.019 U·mL^{-1}, 95% CI=-0.036 to 0.075 U·mL^{-1}, p=0.48).

HR during the relief intervals increased by an average of 17 b·min^{-1} over the period of the 20 intervals (F=491.7, p=0.001), but, like T_c, this effect was not moderated by Ingestion (F=0.08, p=0.78), but was moderated by Temperature (F=10.9, p=0.001) see Fig. 3. In TEMP HR increased at a mean rate of 0.9 b·min^{-1} per interval, compared to 0.7 b·min^{-1} per interval in HOT (mean slope difference=0.2 b·min^{-1}, 95% CI=0.1 to 0.4 b·min^{-1}, p=0.001). This difference was due to an interaction effect, whereby the mean rate of increase in HR decelerated at a mean rate of 0.07 b·min^{-1} per interval in HOT and 0.02 b·min^{-1} per interval in TEMP (mean difference=0.05 b·min^{-1}, 95% CI=0.02 to 0.07 b·min^{-1}, p=0.001). Although the rate of increase in HR during successive relief intervals was higher in TEMP, HR was, on average, 14 b·min^{-1} higher in HOT than TEMP (95% CI=13 to 15 b·min^{-1}, p=0.001). The main effect for Ingestion (F=1.5, p=0.28) and the interaction effect (F=2.5, p=0.11) were not statistically significant.

T_c increased by ~1.6°C over the period of the 20 intervals (F=163.8, p=0.001). The rate of increase was moderated by Temperature. In TEMP T_c increased at a mean rate of 0.062°C per interval, compared to 0.081°C in HOT (mean slope difference=0.019°C, 95% CI=0.013 to 0.025°C, p=0.001). Mean T_c was, on average, 0.32°C higher in HOT compared to TEMP (95% CI=0.29 to 0.35°C, p=0.001). Although the main effect for Ingestion was not significant (F=2.3, p=0.19), an interaction was observed (F=18.4, p=0.001). In TEMP no difference in T_c was observed between placebo and EM-X (mean difference=0.004°C, 95% CI=-0.10 to 0.11°C, p=0.93), whereas in HOT mean T_c was 0.12°C higher for placebo compared to EM-X (95% CI=0.016 to 0.22°C, p=0.03).

TS increased by around 2 units during the 20 intervals (F=143.9, p=0.001), with the rate of increase moderated by Temperature (Fig. 4). TS increased at a mean rate of 0.12 units per interval in HOT and 0.09 units in TEMP (mean slope difference=0.03 units, 95% CI=0.02 to 0.04 units, p=0.001). There was no significant effect for Ingestion (F=0.2, p=0.70) and the interaction also was not significant (F=2.1, p=0.15). However, an Ingestion-by-Temperature interaction was observed (F=13.3, p=0.001), highlighting that in TEMP TS was 0.4 units

lower in the EM-X condition compared to placebo (95% CI=0.2 to 0.6 units, p=0.006), whereas in HOT the 0.2 unit difference between placebo and EX--X did not reach statistical significance (95% CI=-0.03 to 0.4 units, p=0.08).

RPE increased by around 7 units during the 20 intervals (F=37.7, p=0.002), with the rate of increase moderated by Temperature (Fig. 4). The RPE increased at a rate of 0.35 units per interval in HOT and by 0.28 units in TEMP (mean slope difference=0.06 units, 95% CI=0.04 to 0.09 units, p=0.001). The RPE was, on average, 0.7 units higher in HOT compared to the TEMP (95% CI=0.6 to 0.9 units, p=0.001). The main effect for Ingestion (F=3.6, p=0.12) and the interaction effect (F=2.4, p=0.12) were not statistically significant.

Discussion

eHsp72 and SOD concentrations increased pre to post exercise in HOT and TEMP (see Fig. 1 and Fig. 2). Elevated eHsp72 in HOT (+418%) has been reported elsewhere but not at the magnitude to which our data observed (Whitham et al., 2007 (+200%); Magalhães et al., 2010 (+34%); Periard et al., 2012 (~125%); Gibson et al., 2014 (172%)). Our observed increases in TEMP (+212%) are less frequently observed but comparable with others (Whitham et al., 2006 (+200%); Whitham et al., 2007 (+100%)). A minimum endogenous criteria is required to increase eHsp72, this criteria – sufficient change in the absolute (≥38.5°C ;(Amorim, Yamada, Robergs, Schneider, & Moseley, 2008)) and rate of T_c increase (1.6°C.hr^{-1} (Gibson et al. 2014); 2.0 & 2.5°C.hr^{-1} (Périard et al. 2012)), and significant (≥ 153 beats·min^{-1}) sympathetic activity (Gibson, et al., 2014) can be achieved via exercise, a thermal environment, or a combination of the two. Even in TEMP conditions our mean HR responses of ≥150 beats·min^{-1} and final rectal temperatures of ~38.5°C meet the above required endogenous criteria, and the proposed α-adrenergic stimulation (Johnson & Fleshner, 2006). Greater eHsp72 concentration in HOT are likely a result of either greater magnitude of HR (HOT >170 beats·min^{-1}; TEMP >160 beats·min^{-1}; Fig. 3) and/or T_c (HOT

290 >38.5°C; TEMP >38.0°C; Fig. 3) responses, or the earlier attainment of each stimuli, hence
291 greater duration above the previously stated minimum endogenous threshold.

292 SOD is an established biomarker of oxidative stress. Increased physiological strain (Fig. 3)
293 when exercising under hyperthermia vs normothermia (Lafrenz, Wingo, Ganio, & Cureton,
294 2008) led to greater concentrations in HOT (+14.7%) than TEMP (+6.8%; Fig. 2).
295 Amelioration in SOD increases within the HOT_{EMX} (+11.8%) condition compared to HOT_{PLA}
296 (+17.7%) is likely facilitated by the potent antioxidant capacity of EM-X. Amelioration was
297 not observable in TEMP where the difference between $TEMP_{EMX}$ (+6.7%) and $TEMP_{PLA}$
298 (+6.9%) were negligible due to reduced physiological strain. EM-X composition is diverse
299 containing ~40 minerals and compounds, of which many have antioxidant effects both *in-*
300 *vitro* and *in vivo* (ubiquinone, α-tocopherol (vitamin E), lycopene, saponin and the flavonoid
301 quercetin (Aruoma, et al., 2002). We are unable to determine precisely which antioxidant
302 compound has potentially mediated this amelioration of oxidative stress, however, α-
303 tocopherol, lycopene, ubiquinone (Peternelj & Coombes, 2011) and flavonoids (Kressler,
304 Millard-Stafford, & Warren, 2011) are all known to exert an antioxidant influence *in vivo*
305 within humans. The use of a mixed antioxidant profile, rather than one specific antioxidant
306 compound, to ameliorate the reactive oxygen species response to exercise is supported
307 (Balakrishnan & Anuradha, 1998), giving efficacy to this ergogenic aid. Whilst reactive
308 oxygen species generation and/or disturbances to redox balance are essential components of
309 exercise adaption (Powers, et al., 2010), this is an undesirable response during repeated
310 competition due to the potential performance detriments (Cobley, et al., 2011). Ameliorating
311 redox balance disturbances can attenuate fatigue after repeated bouts of intermittent high-
312 intensity exercise within temperate environments (Cobley, et al., 2011).

313 Antioxidant supplementation during HIT related exercise performance, may be better suited
314 to tournament situations, whereby, fatigue "resistance/recovery from" is paramount and
315 adaptation is a negligible goal (Cobley, et al., 2011). Team sports require repeated sprint/HIT
316 based movement patterns, often in tournament situations in challenging environments, with

recovery time between competition suboptimal. EM-X supplementation elicited no such negative side effects previously observed with N-Acetylcysteine supplementation (Cobley, et al., 2011). Our data suggests the presence of reactive oxygen species, and the established pathways which elicit decrements in repeated sprint exercise performance with limited recovery time (Cobley, et al., 2011) appear to/could be attenuated by the supplementation of EM-X during exercise in the heat.

No direct cause and effect relationship between EM-X supplementation and peripheral mechanisms of exercise fatigue attenuation can be claimed from the present study. Future studies should seek to quantify the potential ergogenic effect of EM-X, across a range of relative dosages, on fatiguing high-intensity interval training exercise, within hot environments akin to the study design of others (Cobley, et al., 2011). The efficacy of EM-X as an ergogenic aid to attenuate oxidative stress resulting from prolonged continuous exercise in the heat could additionally be considered as a means to ameliorate short-term negative physiological effects which contribute to cumulative performance decrements observed during consecutive multiday competition.

Classical physiological responses to exercise in hot vs. temperate conditions were observed, with increased HR and T_c (Galloway & Maughan, 1997; Gibson, et al., 2014; Lafrenz, et al., 2008), and TS and RPE (Gagge, et al., 1969; Galloway & Maughan, 1997; Gibson, et al., 2014). Insufficient heat dissipation in HOT_{EMX} and HOT_{PLA} increased heat storage in comparison with TEMP (1.86°C.hr^{-1} in TEMP vs 2.43°C.hr^{-1} in HOT, see Fig. 3) irrespective of the matched work, VO_2 and metabolic heat production (MHP). Increased physiological (HR HOT >170 beats·min^{-1}; TEMP >160 beats·min^{-1}) and thermal responses (Tc HOT >38.5°C; TEMP >38.0°C) during our HIT in comparison to data reported for continuous exercise of approximately similar average intensity in the heat (Gibson, et al., 2014; Houmard et al., 1990) are not unexpected as intermittent exercise is known to elicit greater thermal and cardiovascular strain than continuous exercise of the same average intensity over a fixed duration (Taylor and Cotter 2006). This difference is further increased when running in

344 comparison to cycling, where increased absolute VO_2 (Kang, Hoffman, Walker, Chaloupka,
345 & Utter, 2003), thus MHP, is also greater at a given % of VO_{2max} (Smoljanic, Morris, Dervis,
346 & Jay, 2014).

347 **Conclusion**

348 High intensity exercise in hot conditions elicited greater eHsp72 and SOD than matched
349 exercise performance in temperate conditions, likely due to the increases in relative exercise
350 intensity, and associated increases in physiological strain induced by hyperthermia.
351 Supplementation of EM-X attenuated the SOD increases in hot conditions, suggesting
352 oxidative stress had been reduced.

353 **Abbreviations**

354 eHsp72; Extracellular heat shock protein 72. EM-X; Effective Microorganism X. HIT; High-
355 intensity interval training. $HIT_{RP;}$ High intensity interval running protocol. HOT; Hot and
356 humid conditions. HOT_{EMX}; Supplementation with EM-X in hot conditions. HOT_{PLA};
357 Supplementation with placebo in hot conditions. HR; Heart rate. iHsp72; Intracellular Hsp72.
358 MART; Maximal Anaerobic Running Test. RPE; Ratings of perceived exertion. SOD;
359 Superoxide dismutase. T_c; Core temperature. TEMP; Temperate conditions. $TEMP_{EMX}$;
360 Supplementation with EM-X in temperate conditions. $TEMP_{PLA}$; Supplementation with
361 placebo in temperate conditions. TS; Thermal sensation.VO_{2max}; Maximum oxygen uptake

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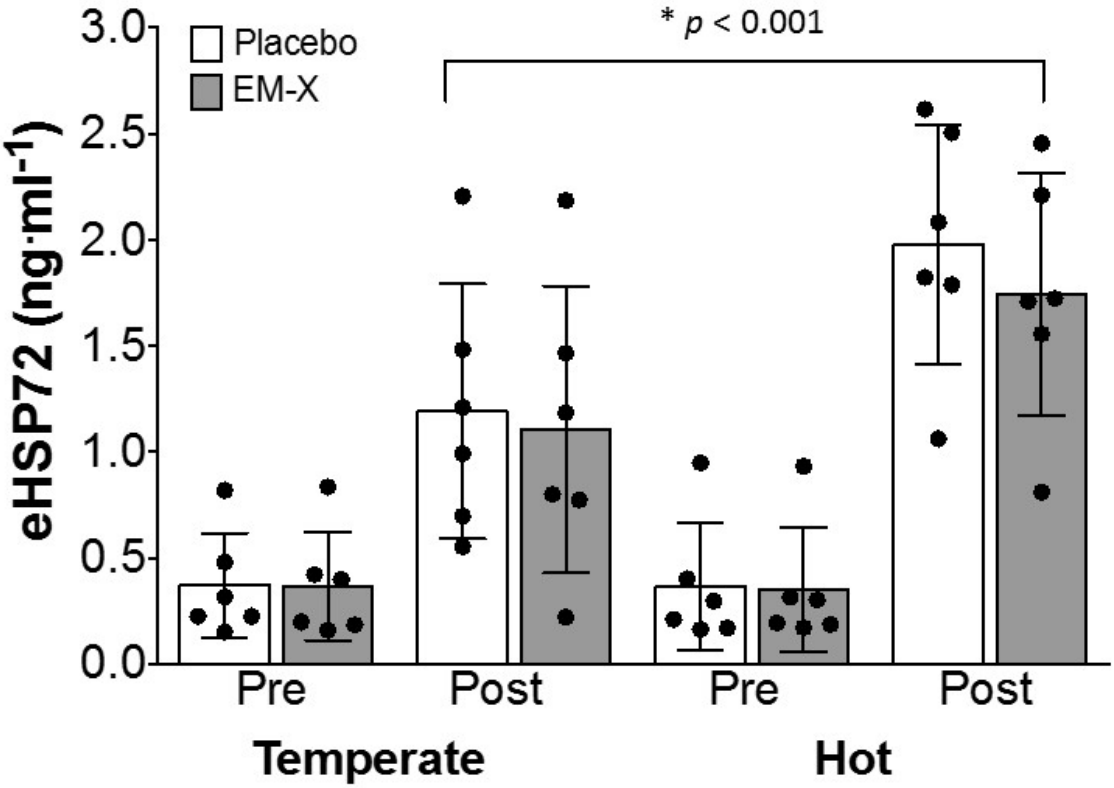

453

454 **Figure 1.** Mean (SD) plasma eHsp72 concentrations at pre- and post-exercise for the four
455 experimental conditions. Spots represent individual subject data * Post-exercise plasma
456 eHsp72 in hot conditions significantly higher than post- exercise plasma eHsp72 in temperate
457 conditions

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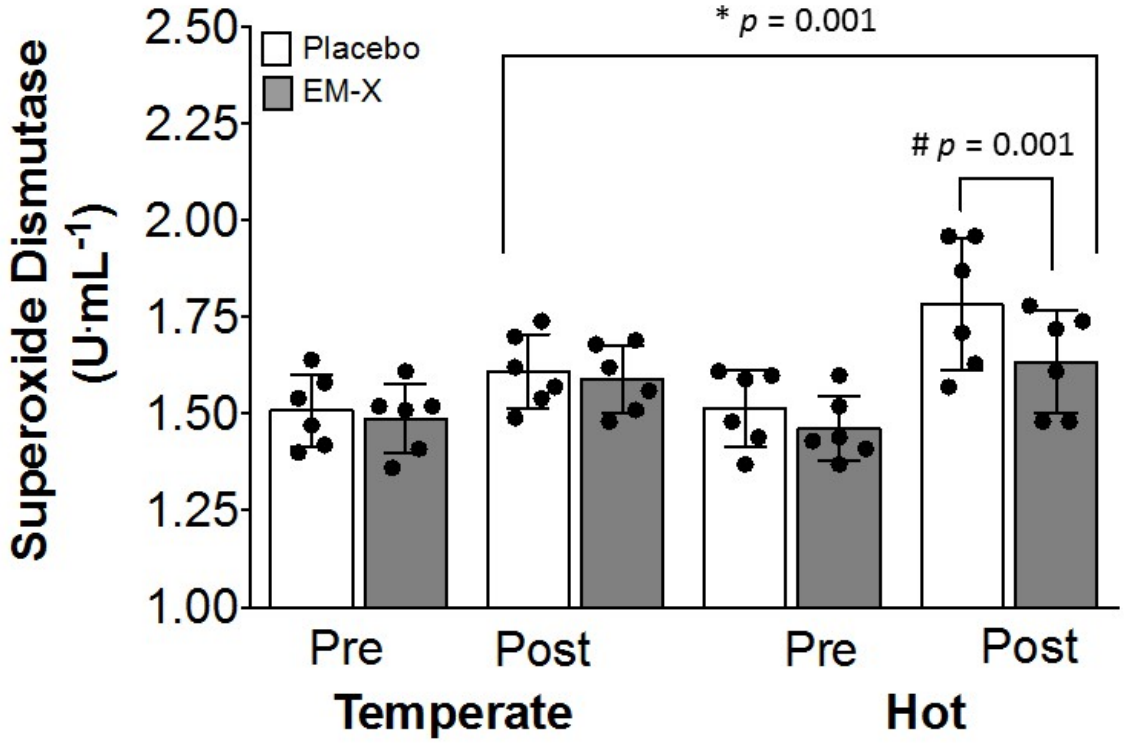

459

460 **Figure 2.** Mean (SD) superoxide dismutase (SOD) concentrations at pre- and post-exercise
461 for the four experimental conditions. Spots represent individual subject data. * Mean post-
462 exercise SOD activity in hot conditions significantly higher than mean post-exercise SOD
463 activity SOD activity in temperate conditions. # Mean post-exercise SOD activity lower
464 following EM-X vs. Placebo in hot conditions.

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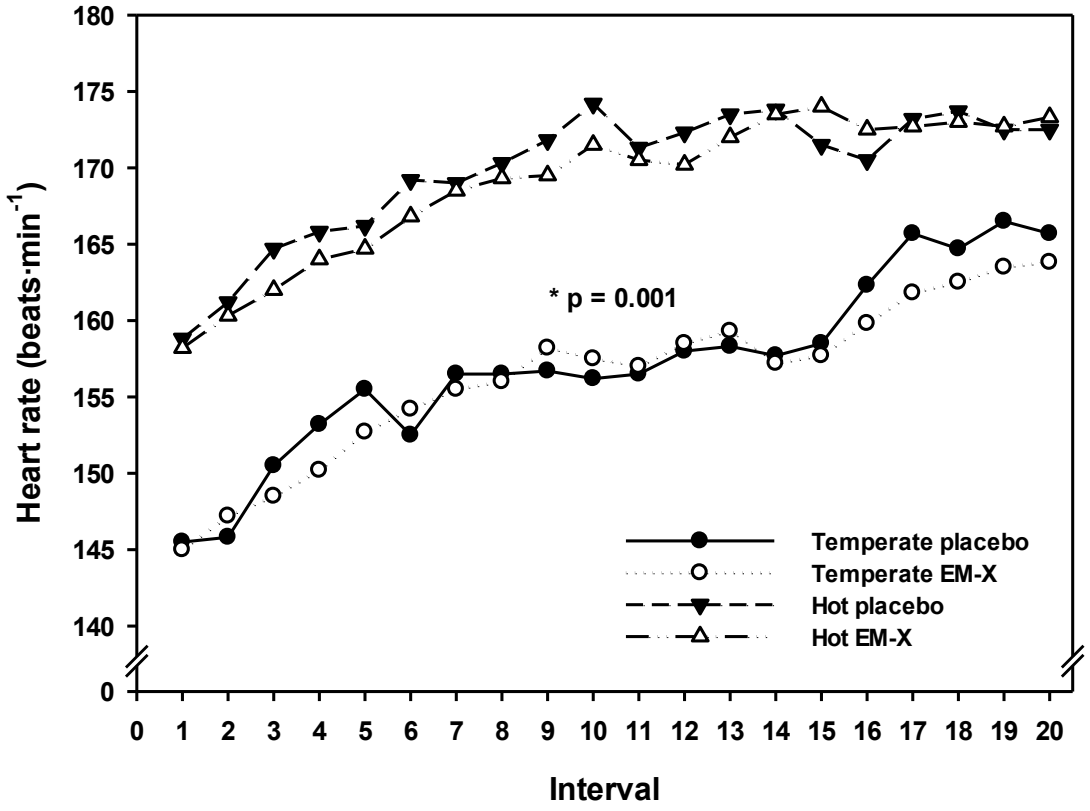

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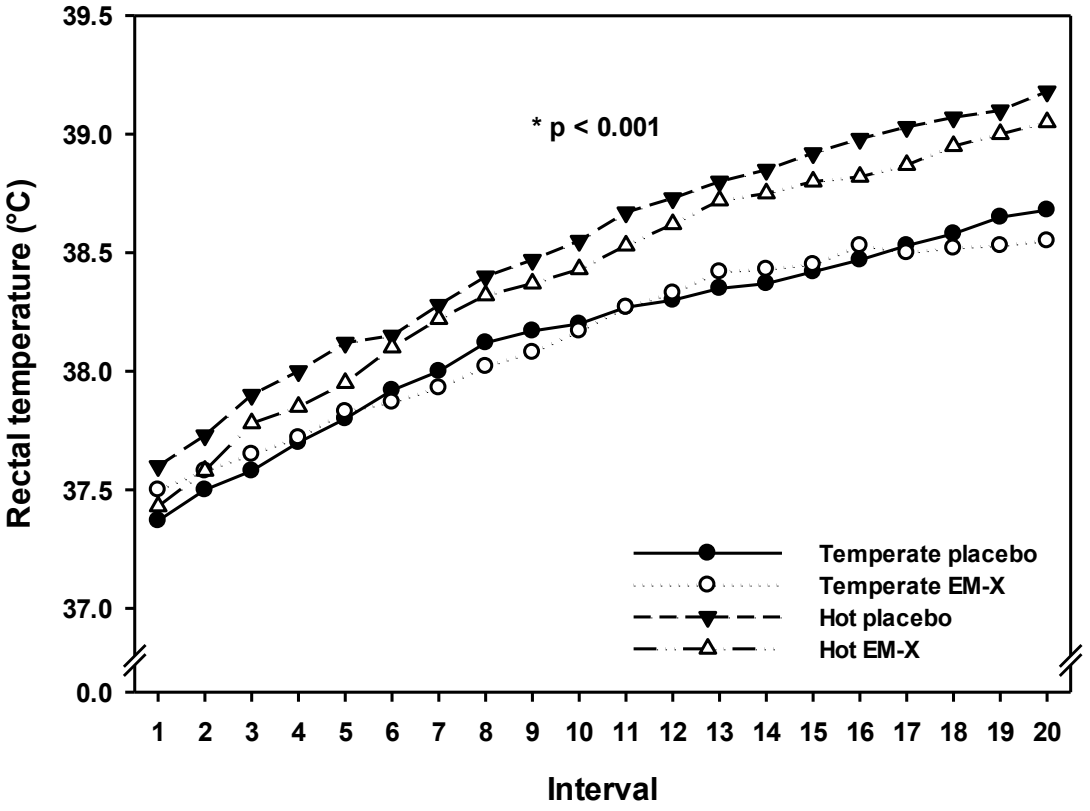
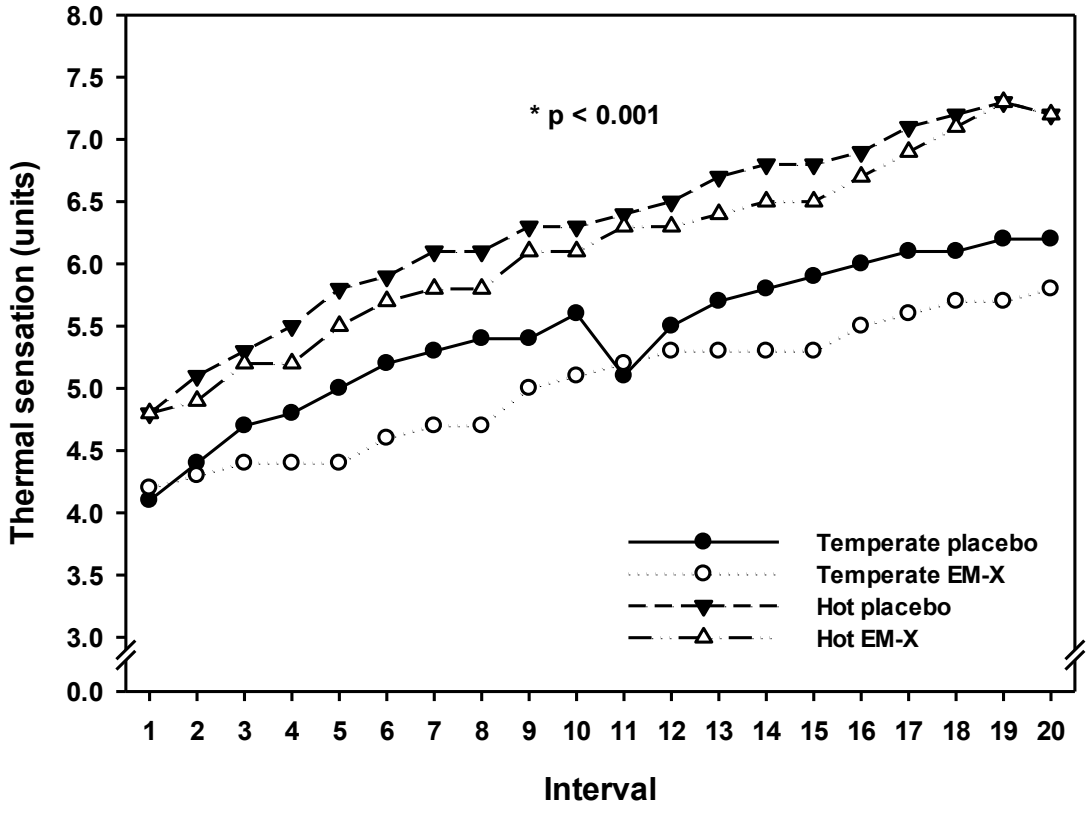


467

468 **Figure 3.** Physiological responses (Mean HR; top. T_c; bottom) over the 20 intervals in the
469 four experimental conditions. Error bars have been omitted for clarity. * Slopes for hot
470 conditions significantly higher than for the temperate conditions.



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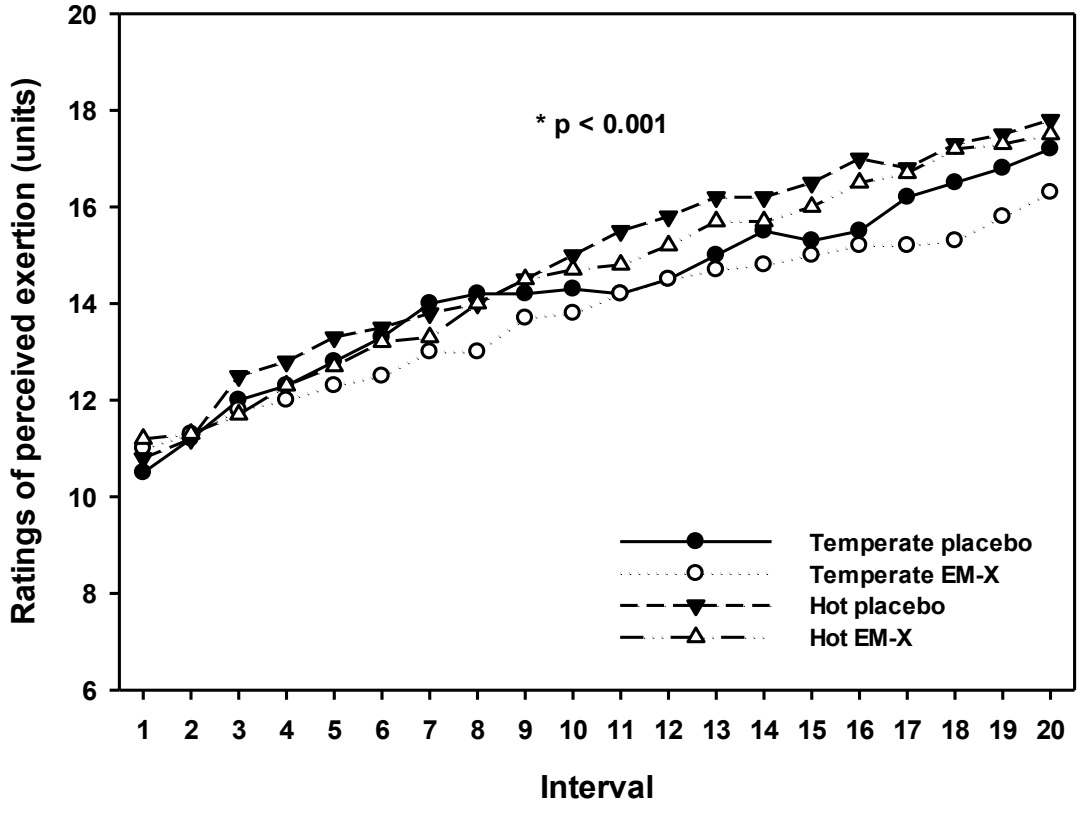

472

473 **Figure 4.** Perceptual responses (Mean TS; top. RPE; bottom) over the 20 intervals in the four
474 experimental conditions. Error bars have been omitted for clarity. * Slopes for hot conditions
475 significantly higher than for the temperate conditions.