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# A comparison of two commercially available ELISA methods for the quantification of human plasma heat shock protein 70 during rest and exercise stress

**Background.** This study compared resting and exercise heat/hypoxic-stress induced levels of plasma eHSP70 in humans using two commercially available ELISA kits.

**Methods.** EDTA plasma samples were collected from 21 males during two separate investigations. Participants in Part A completed a 60 min treadmill run in the heat (HOT70;  $33.0 \pm 0.1$  °C,  $28.7 \pm 0.8\%$ ,  $n = 6$ ) at  $70\% \text{VO}_{2\text{max}}$ . Participants in Part B completed 60 minutes of cycling exercise at  $50\% \text{VO}_{2\text{max}}$  in either hot (HOT50;  $40.5^\circ\text{C}$ ,  $25.4 \text{RH}\%$ ,  $n = 7$ ) or hypoxic (HYP50;  $\text{F}_{\text{I}}\text{O}_2 = 0.14$ ,  $21^\circ\text{C}$ ,  $35\% \text{RH}$ ,  $n = 8$ ) conditions. Samples were collected prior to and immediately upon termination of exercise and analysed for eHSP70 using EKS-715 high sensitivity HSP70 ELISA, and new ENZ-KIT-101 AMP'D™ HSP70 high sensitivity ELISA. **Results.** ENZ-KIT was superior in detecting resting eHSP70 ( $1.54 \pm 3.27 \text{ ng}\cdot\text{mL}^{-1}$ ; range  $0.08$  to  $14.01 \text{ ng}\cdot\text{mL}^{-1}$ ), with concentrations obtained from 100% of samples compared to 19% with EKS-715 assay. The ENZ-KIT requires optimisation prior to running samples in order to ensure participants fall within the standard curve, a step not required with EKS-715. Using ENZ-KIT, a 1:4 dilution allowed for quantification of resting HSP70 in 26/32 samples, with a 1:8 ( $n = 3$ ) and 1:16 ( $n = 3$ ) dilution required to determine the remaining samples. After exercise eHSP70 was detected in 6/21 and 21/21 samples using EKS-715 and ENZ-KIT respectively. eHSP70 was increased from rest after HOT70 ( $p < 0.05$ ), but not HOT50 ( $p > 0.05$ ) or HYP50 ( $p > 0.05$ ) when analysed using ENZ-KIT. **Conclusion.** It is recommended that future studies requiring the precise determination of resting plasma eHSP70 use the ENZ-KIT (i.e., HSP70 Amp'd® ELISA) instead of the EKS-715 assay, despite additional assay development time and cost required.

1 **Title:** A comparison of two commercially available ELISA methods for the  
2 quantification of human plasma heat shock protein 70 during rest and exercise stress

3

4 **Running title:** Comparison of two ELISA methods for plasma HSP70 quantification.

5

6 **Key Words:** Heat shock proteins (HSP), acute heat stress, acute hypoxia, human

7

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33

34

35 **Abstract**

36 **Background.** This study compared resting and exercise heat/hypoxic-stress induced  
37 levels of plasma eHSP70 in humans using two commercially available ELISA kits.

38 **Methods.** EDTA plasma samples were collected from 21 males during two separate  
39 investigations. Participants in Part A completed a 60 min treadmill run in the heat  
40 (HOT70;  $33.0 \pm 0.1$  °C,  $28.7 \pm 0.8\%$ ,  $n = 6$ ) at  $70\% \dot{V}O_{2\max}$ . Participants in Part B  
41 completed 60 minutes of cycling exercise at  $50\% \dot{V}O_{2\max}$  in either hot (HOT50;  
42  $40.5^\circ\text{C}$ ,  $25.4$  RH%,  $n = 7$ ) or hypoxic (HYP50;  $F_{iO_2} = 0.14$ ,  $21^\circ\text{C}$ ,  $35\%$  RH,  $n = 8$ )  
43 conditions. Samples were collected prior to and immediately upon termination of  
44 exercise and analysed for eHSP70 using EKS-715 high sensitivity HSP70 ELISA, and  
45 new ENZ-KIT-101 AMP'D™ HSP70 high sensitivity ELISA.

46 **Results.** ENZ-KIT was superior in detecting resting eHSP70 ( $1.54 \pm 3.27$  ng.mL<sup>-1</sup>;  
47 range  $0.08$  to  $14.01$  ng.mL<sup>-1</sup>), with concentrations obtained from 100% of samples  
48 compared to 19% with EKS-715 assay. The ENZ-KIT requires optimisation prior to  
49 running samples in order to ensure participants fall within the standard curve, a step  
50 not required with EKS-715. Using ENZ-KIT, a 1:4 dilution allowed for  
51 quantification of resting HSP70 in 26/32 samples, with a 1:8 ( $n = 3$ ) and 1:16 ( $n = 3$ )  
52 dilution required to determine the remaining samples. After exercise eHSP70 was  
53 detected in 6/21 and 21/21 samples using EKS-715 and ENZ-KIT respectively.  
54 eHSP70 was increased from rest after HOT70 ( $p < 0.05$ ), but not HOT50 ( $p > 0.05$ ) or  
55 HYP50 ( $p > 0.05$ ) when analysed using ENZ-KIT.

56 **Conclusion.** It is recommended that future studies requiring the precise determination  
57 of resting plasma eHSP70 use the ENZ-KIT (i.e., HSP70 Amp'd® ELISA) instead of  
58 the EKS-715 assay, despite additional assay development time and cost required.

59

60 **Introduction**

61 Heat shock proteins (HSPs) are an evolutionarily conserved family of proteins, with  
62 individual members named according to their molecular weight. Intracellular HSPs  
63 are expressed both constitutively and accumulate after exposure to a wide array of  
64 physiological and psychological stressors (Mosely 1996; Kregal 2002; Horowitz  
65 2007). The 70 kilodalton (kDa) HSP (HSP70/HSPA1A, Kampinga et al. 2009)  
66 remains the most widely studied member of the HSP family due to its multiple  
67 functions related to de novo protein folding (Fink 1999), refolding (Hartl 1996),  
68 degradation (Garrido et al. 2001) and intracellular anti-inflammatory action observed

69 following induction (Ianaro et al. 2001) – all important factors in the maintenance of  
70 protein homeostasis.

71

72 In addition to its intracellular HSP70 (iHSP70) function, HSP70 has been detected in  
73 the circulation (i.e., plasma; Pockley et al. 1998), where it is described as an  
74 extracellular HSP (eHSP, Fleshner et al. 2003). Extracellular HSP70 stimulates  
75 neutrophil microbicidal activity (Ortega et al. 2006), chemotaxis (Ortega et al.  
76 2009), and induces cytokine production via a CD14 mediated pathway (Asea et al.  
77 2000), thereby promoting innate immune activation (Krause et al. 2015). Elevated  
78 resting concentrations of eHSP70 has been positively correlated with insulin  
79 resistance (Krause et al. 2014), and disease progression in auto-immune (Luo et al.  
80 2008) and inflammatory diseases (Schick et al. 2004; Najafizadeh et al. 2015).

81 Therefore eHSP70 may be a useful biomarker when monitoring the progression of  
82 diseases in which low grade inflammation plays a role (Krause et al. 2012), such as  
83 sarcopenia (Ogawa et al. 2012), rheumatoid arthritis (Najafizadeh et al. 2015),  
84 diabetes (Krause et al. 2014), and obesity (Chung et al. 2008). The balance between  
85 the anti-inflammatory action of iHSP70, and pro-inflammatory action eHSP70, may  
86 determine the outcome (induction or attenuation of inflammation) during disease  
87 progression, in response to a treatment, or following an exercise bout (Krause et al.  
88 2015). Therefore understanding the eHSP70 response and its actions is of importance  
89 in clinical situations.

90

91 In many studies the determination of basal eHSP70 has proven to be problematic  
92 (Ogawa et al. 2012; Lee et al. 2014; Najafizadeh et al. 2015) with no consensus  
93 normative data for resting eHSP70 reported to date. Sample handling considerations  
94 (e.g., blood collection tubes temporarily stored on ice *versus* room temperature, using  
95 Heparin *versus* EDTA as an anticoagulant) have been suggested as explanations  
96 underlying the variability in eHSP70 values reported in the literature. For example,  
97 serum appears to yield lower basal values than those obtained by plasma (Whitham  
98 and Fortes 2006), which may be an artifact of eHSP70 binding to proteins involved in  
99 the clotting process, such as fibrin and fibrinogen (Whitham and Fortes 2006).

100 Additionally, EDTA is recommended for use over heparin as higher resting  
101 concentrations are derived (Whitham and Fortes, 2006). Despite the recommendation  
102 to use EDTA plasma for eHSP70 determination (Whitham and Fortes 2006), multiple

103 studies have attempted to quantify eHSP70 in serum for a range of conditions, with  
104 varied results (Dulin et al. 2010; Najafizadeh et al. 2015).

105

106 Another factor affecting the detection of eHSP70 in the circulation at rest could be  
107 attributed to the sensitivity and detection capabilities of the most widely cited enzyme  
108 linked immunosorbant assay (ELISA), 'EKS-715 HSP70 high sensitivity ELISA'  
109 (Enzo life sciences, Lausen, Switzerland). This assay, which has been cited by many  
110 papers investigating eHSP70 in humans, has a working range of 0.20 – 12.5 ng·mL<sup>-1</sup>, a  
111 sensitivity of 0.09 ng·mL<sup>-1</sup>, and is recommended by Cell Stress Society International  
112 (2011). In studies investigating eHSP70, resting values are highly variable, with many  
113 papers unable to detect basal eHSP70 in participants (Lee et al. 2014; Gibson et al.  
114 2014), or concentrations reported at the lower portion of a standard curve (< 0.20  
115 ng·mL<sup>-1</sup>, Ogawa et al. 2012; Rodrigues-Krause et al. 2012; Gibson et al. 2014; Lee et  
116 al. 2014; Lee et al. 2015). This issue appears not to be uniform in the literature  
117 however, as some studies report concentrations that are much higher (17.0 ± 2.6  
118 ng·mL<sup>-1</sup>; Ruell et al. 2006).

119

120 A new ELISA has become available (ENZ-KIT-101-001 HSP70 Amp'd® ELISA),  
121 sensitive to 0.007 ng·mL<sup>-1</sup> with a working range of 0.039-5.00 ng·mL<sup>-1</sup>. The increased  
122 sensitivity of this kit is mediated by an alkaline-phosphatase (AP) conjugate binding  
123 to a signal amplification substrate, which enhances colour production at lower analyte  
124 concentrations. The increased sensitivity therefore affords the potential to determine  
125 normative resting eHSP70 values for a range of individuals with a variety of  
126 conditions and could allow for a more sensitive determination of stress-induced  
127 changes in eHSP70.

128

129 Exercise stress can be used as a tool to study the eHSP70 response. Following  
130 exercise, both with and without a thermal component, eHSP70 is elevated in the  
131 circulation in a duration and intensity dependent manner (Whitham et al. 2007;  
132 Selkirk et al. 2009; Periard et al. 2013; Gibson et al. 2014; Lee et al. 2015). The  
133 magnitude of the post-exercise eHSP70 response has been related to a minimum  
134 endogenous requirement, which suggests that thresholds of core temperature, rate of  
135 core temperature change, and parasympathetic/sympathetic drive all play as of yet  
136 undetermined roles in the magnitude of this response (Gibson et al. 2014). A better

137 understanding of the criteria required to increase or decrease circulating eHSP70 may  
138 provide researchers with a useful biomarker for assessing therapeutic approaches to  
139 inflammation-related diseases, as well as improve understanding regarding eHSP70  
140 function following acute exercise, and repeated periods of exercise, adaptation, and  
141 acclimation to extreme environments.

142

143 The aim of this investigation was to compare resting and exercise induced levels of  
144 EDTA plasma eHSP70 in Humans using the EKS-715 high sensitivity HSP70 ELISA  
145 and ENZ-KIT-101 Amp'd® HSP70 High Sensitivity ELISA methods. It was  
146 hypothesised that the use of the AP conjugate and signal amplification step would  
147 allow a more sensitive determination of basal eHSP70 within different cohorts of  
148 participants at rest while also showing greater sensitivity to different levels of “stress”  
149 induced by exercise undertaken in different environmental conditions.

150

## 151 **Materials and Methods**

### 152 **Participants**

153 Twenty-one recreationally active healthy males provided signed informed consent  
154 prior to participation in this study, which was granted approval by the NHS South-  
155 West Research Ethics Committee (Reference ID. 14/SW/0098, Part A) and Coventry  
156 University local ethics committee (Part B). All procedures were conducted in  
157 accordance with the principles outlined in the *Declaration of Helsinki*. Data reported  
158 in this investigation were collected from two larger experimental trials (Lee, 2014).

159 The collected data represents a convenience sample of similarly characterised  
160 individuals providing EDTA treated plasma before and after a 60-minute bout of  
161 exercise under conditions of environmental stress.

162 All participants described themselves to be physically active, non-smokers with no  
163 prior history of cardiorespiratory illness. Participants were requested to abstain from  
164 caffeine (Lu et al. 2008) and alcohol consumption, as well as prolonged thermal  
165 exposures (baths, saunas, steam rooms, and tanning devices) for 72 hours prior to  
166 each laboratory visit, which were scheduled at similar times (08:30-09:30) between  
167 participants and trials. Participants adhered to an overnight fast prior to each trial and  
168 did not eat until after the final blood withdrawal.

169

### 170 **Preliminary measurements**

171 Participants in each part of the study were assessed for height, body mass and  
172 percentage body fat in accordance with the International Society for the Advancement  
173 of Kinanthropometry (ISAK) guidelines (Marfell-Jones et al. 2006).

174 Participants in Part A (n = 6) of the investigation completed a continuous incremental  
175 running test to volitional exhaustion on a motorised treadmill (Woodway ELG70,  
176 Weiss, Germany). The test protocol was modified from that of Taylor et al. (1955)  
177 and was performed in thermoneutral conditions ( $19.7 \pm 0.7$  °C,  $46.3 \pm 4.0\%$  RH).

178 After a five-minute warm-up at  $6 \text{ km}\cdot\text{h}^{-1}$ , the test began at a speed of  $10 \text{ km}\cdot\text{h}^{-1}$  on a  
179 1% inclination. Speed was then increased by  $1 \text{ km}\cdot\text{h}^{-1}$  every three minutes until  
180 reaching  $13 \text{ km}\cdot\text{h}^{-1}$ , when inclination was increased by 2% every two minutes.

181 Participants were instructed to run for as long as possible and signal when they felt  
182 they could only complete one more minute to allow for a final set of recordings. Peak  
183 oxygen consumption was determined for participants in Part B using an incremental  
184 exercise test to volitional exhaustion on calibrated SRM cycle ergometer (n = 15,  
185 Table 1) Schoberer Rad Meßtechnik, Welldorf, Germany). Resting blood lactate  
186 (Biosen C-Line analyser, EKF Diagnostics, Germany) was determined from a finger  
187 capillary whole blood sample following a 10-minute seated rest period. The test began  
188 at a workload of 70W for 4-minutes and was then increased by 35W every 4 minutes  
189 until a blood lactate value of  $> 4 \text{ mmol}\cdot\text{L}^{-1}$  was reached. Thereafter, workload  
190 increased 35W every 2 minutes until volitional exhaustion. A cadence of  $70 \text{ rev}\cdot\text{min}^{-1}$   
191 was maintained throughout. In both Part A and Part B, expired gases were collected  
192 using 200L Douglas bags (Cranlea & Co, Birmingham, UK) during the final minute  
193 of each stage. Heart rate (Polar FT1, Polar Electro OY, Kempele, Finland) and  
194 perceived exertion (Borg 1976) were measured at the end of each gas collection.  
195 Respiratory gas analysis was completed as previously described (Lee et al., 2014, Lee  
196 et al., 2015). Peak oxygen consumption was considered to be achieved if two of the  
197 following criteria were met: i) a respiratory exchange ratio of  $>1.1$ , ii) a heart rate  
198 greater than 95% of age predicted maximum ( $220 - \text{age}$ ) and iii) a final blood lactate  
199 value in excess of  $8 \text{ mmol}\cdot\text{mL}^{-1}$ .

## 200 **Experimental design**



201 Samples for eHSP70 analysis were obtained from two separate experiments which  
202 both involved a resting measure of eHSP70 and a measurement collected immediately  
203 after a 60-minute bout of exercise.

204

#### 205 **Part A**

206 Samples were obtained before and immediately after a 60 minute treadmill run at a  
207 speed equivalent of 70%  $\dot{V}O_{2\max}$  (HOT70) from 6 healthy males (mean  $\pm$  SD; age 20  
208  $\pm$  2 years; height  $1.79 \pm 0.04$  meters; body mass  $71.8 \pm 2.7$  kg; % body fat,  $11.8 \pm$   
209  $3.3\%$ ;  $\dot{V}O_{2\max}$   $57.9 \pm 9.7$  mL.kg<sup>-1</sup>.min<sup>-1</sup>). All trials were performed in an environmental  
210 chamber that was regulated at a dry bulb temperature of  $33.0 \pm 0.1^\circ\text{C}$  and relative  
211 humidity (RH) of  $28.7 \pm 0.8\%$ , with blood samples obtained at rest, upon termination  
212 of exercise. Participants (n = 6) returned to the lab on two more occasions each  
213 separated by 14 days, to provide 2 further resting samples, which formed part of a  
214 larger experiment. These resting samples were included in the present analysis of  
215 resting data.

216

#### 217 **Part B**

218 Samples were obtained before and immediately after a 60 minutes of cycling at a  
219 power output equivalent of 50%  $\dot{V}O_{2\max}$  in either hot (HOT50;  $40.5^\circ\text{C}$ , 25.4 RH%) or  
220 hypoxic (HYP50;  $F_{I}O_2$  of  $\sim 0.14$ ,  $21^\circ\text{C}$ , 35% RH) conditions. The hypoxic  
221 environment was generated by an oxygen filtration device (Hypoxico HYP-123  
222 hypoxicator, New York, NY, USA) set to produce the desired  $F_{I}O_2$ . Participant  
223 characteristics for HOT50 (n = 7) were (mean  $\pm$  SD): age =  $22 \pm 5$  years; height  $1.76$   
224  $\pm 0.05$  meters; body mass  $70.9 \pm 5.7$  kg; % body fat  $13.2 \pm 4.0\%$ ,  $\dot{V}O_{2\max}$   $54.9 \pm 3.2$   
225 mL.kg<sup>-1</sup>.min<sup>-1</sup>. Participant characteristics for HYP50 were:  $23.4 \pm 4$  years; height  $1.80$   
226  $\pm 0.08$  meters; body mass  $70.0 \pm 9.1$  kg; % body fat  $12.6 \pm 3.7\%$ ;  $\dot{V}O_{2\max}$   $52.2 \pm 3.3$   
227 mL.kg<sup>-1</sup>.min<sup>-1</sup>.

#### 228 **Participant Preparation**

229 Participant preparation and physiological measurements were completed in the same  
230 manner and at the same time intervals for both Part A and B. Prior to each visit,  
231 participants adhered to an overnight fast (Febbraio et al. 2002) and consumed 500 ml  
232 of plain water one hour before in accordance with the American College of Sports  
233 Medicine position stance on hydration (Sawka et al. 2007). Upon arrival, participants

234 began by voiding their bladder to provide a sample for hydration assessment via urine  
235 specific gravity (USG; Atago Refractometer, Jencons Pls, Leighton Buzzard, UK) and  
236 urine osmolarity ( $U_{osmo}$ ; Advanced 3300 Micro-Osmometer, Advanced Inc,  
237 Massachusetts, USA). Euhydration was assumed for urine specific gravity values of  $\leq$   
238  $1.020 \text{ g}\cdot\text{ml}^{-1}$  and osmolarity values of  $\leq 700 \text{ mOsm}\cdot\text{kg}^{-1}$  (Armstrong et al. 1994). This  
239 control was not violated by any participant during any trial. Following this,  
240 participants measured their own nude body mass (Seca 880, Seca, Hamburg,  
241 Germany), inserted a calibrated rectal thermistor probe (Grant Squirrel 2020, Grant  
242 Instruments, Shepreth, UK) to a depth of 10 cm, and fitted a telemetric heart rate  
243 monitor around their chest (Polar FT1, Polar Electro OY, Kempele, Finland). An  
244 indwelling cannula (BD Insyte-W, Becton Dickinson, Utah, USA) was then inserted  
245 2.5 cm into an antecubital vein of the participants left arm. After a 20 minute  
246 stabilisation period with the participant lying supine, a baseline 10 ml blood sample  
247 was then drawn, with patency of the cannula being maintained with saline (0.9%  
248 sodium chloride, Braun, Melsungen, Germany).

#### 249 **Physiological Measurements**

250 Participants entered the regulated environmental chamber at 09:30. The exercise bout  
251 began with a standardised five minute warm-up, running on a motorised treadmill at a  
252 speed calculated to elicit a work rate of  $50\% \dot{V}O_{2max}$  on a fixed 1% inclination (Jones  
253 & Drust 1996) in Part A, or a 15 minute seated wash-in for the hypoxic gas in group  
254 B. Upon completion of the warm-up/wash-in period, participants in Part A began a 60  
255 minute run at a work rate of  $70\% \dot{V}O_{2max}$  and participants in Part B began 60 minutes  
256 of cycling exercise at  $50\% \dot{V}O_{2max}$  in the prescribed environmental conditions  
257 (HOT50 or HYP50).

258 During exercise, heart rate (HR), rectal temperature ( $T_{rectal}$ ), ratings of perceived  
259 exertion (RPE) and thermal sensation (ISO, 1995, Part A, Part B) were all recorded at  
260 ten-minute intervals. The Physiological Strain Index (PSI) was subsequently  
261 calculated at each time point using heart rate and rectal temperature data as described  
262 by Moran et al. (1998). The  $T_{rectal}$  area under curve was calculated using a  
263 modification of the trapezium rule (Hubbard et al. 1977) when  $T_{rectal}$  exceeded  $38.5^{\circ}\text{C}$   
264 (Cheuvront et al. 2008) and  $39.0^{\circ}\text{C}$ . A  $T_{rectal}$  of  $38.5^{\circ}\text{C}$  was selected as a possible  
265 threshold for eHSP70 appearance (Gibson et al. 2014). In instances where participants

266 did not complete the full 60 minute run/cycle, termination time was recorded and all  
267 aforementioned measures taken in the final minute before cessation.

#### 268 **Determination of extracellular HSP70**

269 Circulating eHSP70 was assessed using two commercially available ELISAs, EKS-  
270 715 high sensitivity HSP70 kit  
271 ([http://static.enzolifesciences.com/fileadmin/files/manual/ADI-EKS-715\\_insert.pdf](http://static.enzolifesciences.com/fileadmin/files/manual/ADI-EKS-715_insert.pdf);  
272 hereafter referred to as EKS-715) and ENZ-KIT-101-001 Amp'd® HSP70 high  
273 sensitivity ELISA kit ([http://static.enzolifesciences.com/fileadmin/files/manual/ENZ-  
274 KIT-101\\_insert.pdf](http://static.enzolifesciences.com/fileadmin/files/manual/ENZ-KIT-101_insert.pdf), hereafter referred to as ENZ-KIT) according to the  
275 manufacturer's instructions (Enzo Lifesciences, Lausen, Switzerland).

276  
277 The ENZ-KIT is designed to replace traditional alkaline phosphatase substrates, such  
278 as pNPP (p-Nitrophenyl phosphate), with a combination substrate and amplifier  
279 system that results in greater sensitivity when compared to a classic substrate ELISA.  
280 In the ENZ-KIT, bound AP converts a substrate that is utilized in a second enzyme  
281 reaction system which is initiated by addition of the amplifier reagent. Figure 1  
282 shows typical standard curves (n = 4) prepared on the same 96 well plate (HSP70  
283 Clear Miotiter plate, catalogue number: 80-1581), using the same HSP70 high  
284 sensitivity standard (Cat no: 80-1776). HRP conjugate (Cat no: 80-1778) was added  
285 to HSP70 high sensitivity antibody (Cat no: 80-1777) and a TMB substrate (Cat no:  
286 80-0350) used to develop the EKS-715. For ENZ-KIT, an AP conjugate (Cat no: 80-  
287 2600) was added to the HSP70 high sensitivity antibody (Cat no: 80-1777) and  
288 incubated with signal amplification substrate (Cat no: 80-2596) containing NADPH  
289 prior to a final amplification step (Cat no: 80-2598). The amplification step allows for  
290 greater (amplified) colour production at lower analyte concentrations resulting in an  
291 increased assay sensitivity (Figure 1).

292  
293 The EKS-715 kit has a sensitivity of  $0.090 \text{ ng mL}^{-1}$  and a working range of 0.20 to  
294  $12.5 \text{ ng mL}^{-1}$ . The ENZ-KIT assay is sensitive to  $0.007 \text{ ng mL}^{-1}$  with a working range  
295 of  $0.039\text{-}5.00 \text{ ng mL}^{-1}$ . Following an initial analysis of samples (one ENZ-KIT assay),  
296 it became apparent that the minimum recommended dilution of 1:4 was not sufficient  
297 in all cases, with some samples containing more HSP70 than the top standard. Thus a  
298 further analysis using 1:4, 1:8 and 1:16 dilution step with assay diluent (sodium

299 carbonate) was necessary to determine the optimal dilution for each sample, with  
300 results multiplied by the this dilution factor in order to give eHSP70 values in ng mL<sup>-1</sup>.  
301 <sup>1</sup>. Once the optimal sample dilution was determined for each participant on the ENZ-  
302 KIT, pre and post exercise samples were analysed in duplicate.

303

#### 304 **Statistical analysis**

305 A total of 32 resting blood samples from 21 individuals (11 repeat samples) were  
306 analysed for basal eHSP70. The between-assay co-efficient of variation was  
307 determined using standard concentration curves of 4 separate kits run using both  
308 EKS-715 and ENZ-KIT. The reliability of the ENZ-KIT assay was further assessed  
309 by comparing resting data obtained during a serial dilution test to the data obtained  
310 from a further assay on these samples measuring both pre and post exercise data.  
311 Where each ELISA method provided eHSP70 for paired samples, or samples assayed  
312 on separate occasions, Pearson correlations determined the relationship between each  
313 measurement.

314 Mean and peak physiological, thermoregulatory and eHSP70 responses were analysed  
315 between groups using a one-way analysis of variance (ANOVA), and Tukey's  
316 honestly different test to explore main effects. All data analysis was performed using  
317 PASW software version 20.0 for Mac (SPSS, Chicago, IL, USA).

318 Stepwise multiple regression analysis was performed using the three dependent  
319 variables (time spent above T<sub>rectal</sub> 38.5°C, rate of change in T<sub>rectal</sub>, and AUC for T<sub>rectal</sub>  
320 38.5°C) that were significantly correlated to post exercise eHSP70 concentrations.  
321 The significance level was set at p < 0.05 for all analysis. Data are reported as means  
322 ± SD unless otherwise stated and individual data shown where possible.

323

#### 324 **Results**

325

##### 326 **pNPP Conjugate (EKS-715) compared with AP conjugate and amplifier** 327 **substrate (ENZ-KIT)**

328 Figure 1 illustrates a typical standard curve when the pNPP conjugate and TMB  
329 substrate is used (closed circles) in comparison to the increased sensitivity obtained  
330 from the AP conjugate and amplifier solution (open circles). The intra-assay  
331 precision, obtained by determining the coefficient of variation between duplicate  
332 samples obtained from the standard curves on 4 separate plates, was 2.6% and 4.1%

333 for EKS-715 and ENZ-KIT respectively. These data are lower than the manufacturer  
334 reported intra-assay precision of between 3.9 and 11.4% for EKS-715, and 7 and 15%  
335 for the ENZ-KIT. Inter-assay precision was also determined from the standard curves  
336 of 4 separate assays performed on 4 separate occasions, and was 4.9% and 6.2% for  
337 EKS-715 and ENZ-KIT respectively. These values were also lower than manufacturer  
338 reported inter-assay variation (EKS-715 = 12.8 – 19.1%; ENZ-KIT = 7.7 – 9.7%).

339

#### 340 **Extracellular HSP70 at rest**

341 The ENZ-KIT was able to detect basal eHSP70 in all 32 resting samples (Figure 2,  
342  $1.54 \pm 3.27 \text{ ng}\cdot\text{mL}^{-1}$ ). In contrast, the EKS-715 assay did not detect eHSP70 in 26 out  
343 of 32 resting samples analyzed (81%). When results were available from both kits ( $n$   
344 = 6), there was a good correlation between values ( $r = 0.86$ ,  $p = 0.0004$ , Figure 2),  
345 with values not significantly different between kits ( $t = 0.35$ ,  $p = 0.72$ ). In 15 of the  
346 samples measured with ENZ-KIT (47%), eHSP70 was below the  $0.20 \text{ ng}\cdot\text{mL}^{-1}$  limit  
347 of EKS-715 standard curve ( $0.15 \pm 0.04 \text{ ng}\cdot\text{mL}^{-1}$ ; 95% CI = 0.13 to  $0.17 \text{ ng}\cdot\text{mL}^{-1}$ ).

348

349 A minimum dilution of 1:4 (sample to assay diluent) is recommended to remove  
350 matrix interference during the ENZ-KIT assay. In the present investigation we found  
351 the 1:4 dilution allowed for determination of basal HSP70 in 26/32 samples studied.  
352 For samples with resting concentrations of eHSP70 above the top standard  
353 concentration ( $5.00 \text{ ng}\cdot\text{mL}^{-1}$ ), a 1:8 dilution ( $n = 3$ ) and 1:16 ( $n = 3$ ) were required to  
354 locate data on the standard curve. No participants exhibited eHSP70 values below the  
355 detection limit of  $0.039 \text{ ng}\cdot\text{mL}^{-1}$  using the ENZ-KIT

356

357 A further determination of ENZ-KIT assay reliability was made by comparing resting  
358 eHSP70 data obtained from the serial dilution plate, to the resting data obtained  
359 during the test run on a separate plate (Part A:  $n = 6$ , Part B  $n = 15$ ;  $r = 0.998$ ,  $p <$   
360  $0.001$ , Figure 2 Panel E and F), indicating good inter-assay reliability (CV = 7.9%).

361

#### 362 **Physiological and thermoregulatory responses to each stressor**

363 The duration of exercise undertaken at 70%  $\dot{V}O_2\text{max}$  (i.e., HOT70,  $54.0 \pm 9.4$   
364 minutes) was shorter than the duration of exercise undertaken at 50%  $\dot{V}O_2\text{max}$  (i.e.,

365 HOT50, HYP50,  $60 \pm 0.0$  minutes;  $f_{(2, 18)} = 4.25$ ,  $p = 0.032$ ). Physiological and  
366 thermoregulatory data are shown in Table 1.

367

368 Although some thermoregulatory responses (peak, delta and rate of  $T_{\text{rectal}}$  change)  
369 were greater in HOT50 compared to HYP50 (Table 1), no other differences in  
370 physiological response (e.g. mean and peak HR and PSI) were observed between  
371 these conditions. In contrast, greater mean and peak exercising HR and  $T_{\text{rectal}}$   
372 responses were observed in HOT70 compared to HOT50 and HYP50. Similarly, the  
373 delta change in  $T_{\text{rectal}}$ , the rate of  $T_{\text{rectal}}$  change, the duration of the exercise bout spent  
374 above both  $38.5^{\circ}\text{C}$  and  $39.0^{\circ}\text{C}$  and AUC for these temperatures were all greater in  
375 HOT70 compared to HOT50 and HYP50 ( $p < 0.05$ ; Table 1). The data therefore  
376 indicate two different levels of physiological strain were achieved (HOT70 versus  
377 HOT50 and HYP50).

378

### 379 **The exercise-induced eHSP70 response**

380 In accordance with the resting data, EKS-715 only detected post exercise eHSP70 in  
381 the 6 samples that had detectable eHSP70 at rest, with 5 samples obtained in HYP50  
382 and 1 sample from the HOT50. Post exercise eHSP70 obtained from the EKS-715 kit  
383 ( $n = 6$ ,  $3.92 \pm 4.34$  ng.mL<sup>-1</sup>) had a good relationship to those obtained with the ENZ-  
384 KIT ( $n = 6$ ,  $3.37 \pm 5.38$  ng.mL<sup>-1</sup>,  $r = 0.84$ ).

385

386 There was a significant group  $\times$  time interaction ( $F_{(2, 17)} = 4.235$ ,  $p = 0.03$ ) for  
387 eHSP70 when analyzed using ENZ-KIT. Resting HSP70 was higher in HOT70 than  
388 HOT50 or HYP50 group ( $p < 0.05$ ; Figure 3).

389

390 Exercise results in an increase in eHSP70 from  $2.79 \pm 2.59$  ng.mL<sup>-1</sup> (95% CI = 0.074  
391 to  $5.51$  ng.mL<sup>-1</sup>) at rest to  $3.51 \pm 2.90$  ng.mL<sup>-1</sup> (0.47 to  $6.56$  ng.mL<sup>-1</sup>) in the HOT70  
392 group ( $t = 3.82$ ,  $p = 0.012$ ). However, with the HOT50 trial, eHSP70 was unchanged  
393 from  $0.22 \pm 0.13$  ng.mL<sup>-1</sup> (95% CI = 0.084 to  $0.362$  ng.mL<sup>-1</sup>) at rest to  $0.20 \pm 0.16$   
394 ng.mL<sup>-1</sup> (95% CI = 0.034 to  $0.370$  ng.mL<sup>-1</sup>) following exercise ( $t = 0.886$   $p = 0.410$ ).  
395 In addition, in the HYP50 trial, eHSP70 was unchanged from  $2.82 \pm 5.51$  ng.mL<sup>-1</sup>  
396 (95% CI = 2.96 to  $8.60$  ng.mL<sup>-1</sup>) at rest to  $2.85 \pm 5.56$  ng.mL<sup>-1</sup> (95% CI = 2.98 to  $8.69$   
397 ng.mL<sup>-1</sup>) following exercise ( $t = 0.635$ ,  $p = 0.545$ ). Thus only HOT70 induced  
398 changes of eHSP70 above resting values (Figure 3).

399

#### 400 **Relationship between eHSP70 and thermo-physiological measures**

401 Time spent above 38.5°C ( $r = 0.54$ ), rate of change in  $T_{\text{rectal}}$  ( $r = 0.52$ ), and AUC for  
402  $T_{\text{rectal}} > 38.5^\circ\text{C}$  ( $r = 0.47$ ) were entered into a stepwise multiple regression analysis to  
403 assess the association of these variables in post exercise eHSP70 concentration. The  
404 only predictor variable was the duration of exercise above 38.5°C. The adjusted  $R^2$  for  
405 this model was 0.26 with a large standard error of 55.1.

406

#### 407 **Discussion**

408 The aim of this study was to compare two commercially available high sensitivity  
409 ELISAs for the determination of eHSP70 in plasma. The results illustrate that the  
410 ENZ-KIT (Enzo Lifesciences, Lausen, Switzerland) is more sensitive than the EKS-  
411 715 (Enzo Lifesciences, Lausen, Switzerland) when quantifying both resting and post  
412 exercise eHSP70 values in a sample of healthy, moderately trained males. The  
413 increased sensitivity and lower working range, facilitated by the use of amplifier  
414 reagents, significantly improves the ability of the ENZ-KIT assay to detect resting  
415 eHSP70 in plasma thereby supporting our hypothesis.

416

417 In the present investigation only 6 of the 21 samples analysed using the EKS-715 kit  
418 allowed for the quantification of eHSP70 at rest and after exercise, whereas ENZ-KIT  
419 provided data for all resting and all post exercise samples (Figure 2, Panel B). The  
420 increased sensitivity of ENZ-KIT introduces the potential requirement for serial  
421 dilution of samples to ensure results are not above the standard curve, thereby  
422 reducing the reliance on extrapolation. In the current analysis, the manufacturers  
423 recommended minimum sample dilution of 1:4 was sufficient to detect eHSP70 in  
424 26/32 samples. Additional dilutions of 1:8 ( $n = 3$ ) and 1:16 ( $n = 3$ ) were necessary in  
425 the instances when data fell above the standard curve. It may be prudent for  
426 researchers using the ENZ-KIT to conduct a 1:4 and 1:8 serial dilution of all resting  
427 samples prior to full analysis to ensure that all samples can be analysed together,  
428 potentially saving both time and the additional cost of running more assays. The  
429 ENZ-KIT demonstrated excellent reproducibility between individual assay kits, with  
430 duplicate measurements of resting values highly correlated between assays ( $R^2 = 0.99$ ,  
431 Figure 2, Panel F).

432

433 Heat shock proteins play an important role in maintaining cellular protein  
434 homeostasis, with HSP dysfunction implicated in the pathology of Alzheimer's  
435 disease, Parkinson's disease, cardiovascular disease, and sarcopenia (Krause et al.  
436 2015). It is therefore surprising that there is currently no substantial normative data  
437 regarding resting eHSP70 for either healthy individuals or clinical cohorts. Indeed, a  
438 characteristic of eHSP70 research is the large variability both between studies (Ruell  
439 et al. 2006; Whitham and Fortes 2006; Gibson et al. 2014; Lee et al. 2015) and within  
440 studies, likely exacerbated by small samples sizes typically used in exercise studies  
441 (Gibson et al. 2014; Lee et al. 2015). Between-participant variation is evident in the  
442 present investigation, in which three groups of seemingly physiologically-matched  
443 males present significantly different eHSP70 values at rest (Figure 3). Such a large  
444 disparity in resting data will limit the ability to detect changes in eHSP70  
445 concentrations between two or more matched groups. When a repeated measures  
446 design is not feasible it may be appropriate for experimenters to match individuals  
447 based on resting eHSP70 concentrations rather than more common physiological and  
448 anthropometric features (providing that eHSP70 is an important outcome). Doing so  
449 may facilitate a clearer understanding regarding responders, such as the participant  
450 with high post HOTS50 eHSP70 concentration (Figure 3, Panel B; rest =  $0.67 \text{ ng.mL}^{-1}$ ,  
451 post exercise =  $2.29 \text{ ng.mL}^{-1}$ ), and non-responders to thermal or hypoxic stress.

452  
453 The reasons for the observed disparity between similarly matched groups is unclear  
454 and could not be determined in the present work. In order to elucidate the role this  
455 biochemical marker plays in health and disease future studies should aim to  
456 thoroughly characterize an individual's lifestyle factors, normal weekly physical  
457 activity levels, and anthropometric and physiological characteristics. The increased  
458 sensitivity of the ENZ-KIT compared to other commercially available kits allow for a  
459 sensitive quantification of resting eHSP70 across a wide range of populations. Only  
460 once these data have been collected in a sufficiently large sample can eHSP70 be  
461 investigated as a potential biomarker of, for example, sarcopenia (Ogawa et al. 2012).  
462 Some studies have attempted to determine the efficacy of eHSP70 in clinical  
463 scenarios using the EKS-715 assay. Based on the present work, it is likely that prior  
464 results have been influenced by sensitivity. For example Ogawa et al. (2012)  
465 conducted a detailed study in which 652 elderly Japanese males and females were  
466 screened for a range of biochemical and physiological markers related to sarcopenia,



467 such as TNF- $\alpha$ , interleukin-6 (IL-6) and C-reactive protein (CRP). However the  
468 majority of the eHSP70 data reported (436/652) was near to ( $n = 207$ ;  $0.13 - 0.22$   
469  $\text{ng.mL}^{-1}$ ), or below ( $n = 229 < 0.13 \text{ ng.mL}^{-1}$ ) the EKS-715 assay standard curve ( $0.20$   
470  $\text{ng.mL}^{-1}$ ). Thus, although these results are important, the present work highlights that  
471 the data should be interpreted with caution. The data in the present investigation has  
472 demonstrated that, even where eHSP70 values are theoretically within the measurable  
473 range of the EKS-715 assay (e.g. concentrations of over  $0.20 \text{ ng.mL}^{-1}$ ), they are not  
474 always detectable by EKS-715, but were all detected with ENZ-KIT (Figure 2).  
475 Thus, when resting eHSP70 concentrations are required for clinical observations or  
476 for studying the role of eHSP70 in health and disease, the use of the ENZ-KIT is  
477 recommended above EKS-715.

478

479 Exercise is known to increase concentrations of eHSP70 in an intensity and duration  
480 dependent manner, with a 60 minute run at  $75\% \dot{V}O_{2\text{max}}$  leading to a 175% increase  
481 in eHSP70, compared to a 140% increase when running for 120 minutes at  $60\%$   
482  $\dot{V}O_{2\text{max}}$  (Fehrenbach et al. 2005). The sum exercise stress of cycling is less that of  
483 running due to its low impact and less muscle-damaging nature, thus cycling induced  
484 elevations in eHSP70 concentration are much lower than those observed following  
485 running (Febbraio et al. 2002; Febbraio et al. 2004; Lancaster et al. 2004). Post  
486 exercise increases in eHSP70 have been hypothesized to relate to a minimum  
487 endogenous level of thermal strain, corresponding to a  $T_{\text{rectal}}$  of  $> 38.5^{\circ}\text{C}$  (Amorim et  
488 al. 2008; Gibson et al. 2014). Both the rate of  $T_{\text{rectal}}$  increase, and change in  $T_{\text{rectal}}$  are  
489 also thought to be important factors affecting eHSP70 concentrations after a stressor  
490 (Periard et al. 2012; Gibson et al. 2014).

491

492 In addition to the ability of the ENZ-KIT to quantify resting eHSP70, we therefore  
493 examined the post-exercise response at 3 levels of physiological strain using two  
494 distinctly different modes (running and cycling) of hyperthermic exercise, as well as  
495 hypoxic stress (Table 1). The data presented in Table 1 indicate two levels of  
496 physiological strain were achieved, with HOT70 eliciting significantly greater mean  
497 and peak HR,  $T_{\text{rectal}}$  and PSI compared to HOT50 and HYP50. No significant  
498 differences between the HOT50 and HYP50 groups were observed, agreeing with  
499 previous work showing exercise at  $50\% \dot{V}O_{2\text{max}}$  in either  $40^{\circ}\text{C}$  heat or an  $F_{\text{I}O_2}$  of 0.14

500 to be of a comparable physiological stress up to 40 minutes of exercise (Lee et al.  
501 2014). The 60 minute duration of exercise used herein was therefore insufficient for  
502 significant differences in physiological strain between HOT50 and HYP50 to become  
503 apparent (Girard and Racinais, 2014; Lee et al. 2014).

504

505 As with the resting data, the EKS-715 kit only detected eHSP70 in the 6 samples that  
506 also provided resting data (n = 5 from HYP50, and n = 1 from HOT50). In contrast a  
507 52% increase (Pre,  $2.79 \pm 2.58$ ; Post,  $3.51 \pm 2.90$  ng.mL<sup>-1</sup>) in eHSP70 was observed  
508 immediately after HOT70 when samples were analyzed with ENZ-KIT. This is  
509 comparable with other studies using a similar level of external heat stress at a lower  
510 exercise intensities (e.g. Periard et al. 2012) but for a longer duration (90 minutes,  
511 Gibson et al. 2014), and further illustrates the utility of the ENZ-KIT over EKS-715.

512

513 The higher post exercise eHSP70 concentrations reported in the present investigation  
514 were observed despite the shorter duration of exposure ( $54.0 \pm 9.3$  minutes compared  
515 to  $90.0 \pm 0.0$  minutes in Gibson et al. 2014) and are likely due to the increased  
516 exercise intensity (70% vs 50%  $\dot{V}O_{2max}$  in Gibson et al. 2014), and different exercise  
517 mode (running Vs. cycling) used between investigations. Additionally,  
518 thermoregulatory stress, evidenced by the AUC for a  $T_{rectal}$  of  $>38.5^{\circ}C$  ( $9.21 \pm$   
519  $1.95^{\circ}C \cdot min^{-1}$ ), the duration spent above  $38.5^{\circ}C$  ( $17.5 \pm 10.4$  minutes) and duration  
520 spent above  $39^{\circ}C$  ( $8.9 \pm 1.6$  minutes), mean and peak  $T_{rectal}$ , change in  $T_{rectal}$  and rate  
521 of change in HOT70 were all higher than those reported after cycling exercise at 50%  
522  $\dot{V}O_{2max}$  for 90 minutes at  $30.2^{\circ}C$ , 51% RH (Gibson et al. 2014). Thus the total level  
523 of endogenous strain was greater in the present investigation, and is reflected in the  
524 eHSP70 results.

525

526 In contrast, no post exercise increase was observed following HOT50 or HYP50  
527 (Figure 3). It is likely that while the thermal component of HOT50 was sufficient for  
528 increased eHSP70, the duration of exercise, and therefore overall level of exogenous  
529 strain, was not sufficient to increase eHSP70. Of the suggested endogenous  
530 requirement for post exercise increases in eHSP70 (peak  $T_{rectal}$  of  $>39.2^{\circ}C$ , a mean  
531  $T_{rectal}$  of  $38.6^{\circ}C$  for a period of ~57 minutes, a core temperature change of  $2.2^{\circ}C$  from  
532 baseline at a rate of  $1.6^{\circ}C h^{-1}$  and a mean heart rate of  $153$  bt.min<sup>-1</sup>), the HOT50 trial  
533 only achieved the required heart rate, and the HYP50 group failed to reach any of

534 these potential eHSP70 inducing thresholds. Our data therefore lend support to the  
535 notion of a minimum endogenous criteria required for eHSP70 induction (Amorim et  
536 al. 2008; Gibson et al. 2014). The increased sensitivity afforded by the ENZ-KIT may  
537 allow for a more detailed and nuanced description of minimum eHSP70 inducing  
538 criteria during and after exercise stress in future studies.

539

540 In summary, this investigation presents preliminary data showing the effectiveness of  
541 the ENZ-KIT assay for detecting and quantifying resting eHSP70 at the low end of  
542 the measurable range in young healthy males. Secondly, our results support the notion  
543 that a minimum endogenous strain threshold needs surpassing in order to increase  
544 systemic HSP70. As a result, it is recommended that all future investigations  
545 requiring accurate resting eHSP70 quantification use the ENZ-KIT assay in place of  
546 EKS-715. The increased sensitivity afforded by this assay could provide a more in  
547 depth understanding of normal and abnormal levels of systemic eHSP70, and provide  
548 novel information regarding the use of eHSP70 as a biomarker of disease.

549

#### 550 **Abbreviations**

551 **AUC**; Area under the curve. **AP**; Alkaline phosphatase. **ENZ-KIT-101**; Amp'd®  
552 HSP70 High Sensitivity ELISA kit. **eHSP70**; Extracellular heat shock protein 70.  
553 **EKS-715**; HSP70 high sensitivity ELISA kit. **ELISA**; Enzyme linked  
554 immunosorbant assay. **FiO<sub>2</sub>**; fraction of inspired oxygen. **HEAT70**; Heat running  
555 trial. **HEAT50**; heat cycling trial **HSP70**; Heat shock protein 72. **HR**; Heart rate.  
556 **HYP**; Hypoxic trial. **IL-10**; Interleukin 10. **kDa**; KiloDalton. **pNPP**; p-Nitrophenyl  
557 phosphate. **PSI**; Physiological strain index. **RPE**; rating of perceived exertion. **T<sub>rectal</sub>**;  
558 Rectal temperature. **RH**; Relative humidity. **SpO<sub>2</sub>**; Arterial oxygen saturation. **TS**;  
559 Thermal sensation. **TNF- $\alpha$** ; Tumour necrosis factor alpha.  **$\dot{V}O_{2max}$** ; Maximal oxygen  
560 consumption.

561

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566

567 **Conflict of Interest**

568 The authors declare no conflict of interests

569

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**Table 1.** Mean and peak physiological and thermoregulatory responses to each trial.

Data are mean  $\pm$  SD.

Variable	Experimental group			ANOVA
	HOT70	HOT50	HYP50	Trial Main effect
Exercise duration (mins)	54.0 $\pm$ 9.4 <sup>***</sup>	60.0 $\pm$ 0.0	60.0 $\pm$ 0.0	F = 4.25, p = 0.032
Mean HR (beats.min <sup>-1</sup> )	180 $\pm$ 8 <sup>***</sup>	153 $\pm$ 12	149 $\pm$ 13	F = 14.17, p < 0.0001
Peak HR (beats.min <sup>-1</sup> )	190 $\pm$ 7 <sup>***</sup>	165 $\pm$ 10	157 $\pm$ 15	F = 14.61, p < 0.0001
Mean T <sub>rectal</sub> (°C)	38.4 $\pm$ 0.3 <sup>#</sup>	38.0 $\pm$ 0.2	37.9 $\pm$ 0.4	F = 4.41, p = 0.028
Peak T <sub>rectal</sub> (°C)	39.3 $\pm$ 0.4 <sup>#</sup>	38.7 $\pm$ 0.3 <sup>#</sup>	38.2 $\pm$ 0.4	F = 15.62, p < 0.0001
Mean PSI (AU)	7.2 $\pm$ 0.6 <sup>*</sup>	5.2 $\pm$ 1.0	4.8 $\pm$ 1.1	F = 11.81, p = 0.001
Peak PSI (AU)	9.2 $\pm$ 0.8 <sup>*</sup>	7.4 $\pm$ 1.2	5.9 $\pm$ 1.4	F = 13.74, p < 0.0001
Delta change in T <sub>rectal</sub>	+2.5 $\pm$ 0.3 <sup>***</sup>	+1.4 $\pm$ 0.4 <sup>#</sup>	+0.8 $\pm$ 0.4	F = 34.34, p < 0.0001
Rate of T <sub>rectal</sub> change (°C h <sup>-1</sup> )	2.9 $\pm$ 0.7 <sup>***</sup>	1.4 $\pm$ 0.4 <sup>#</sup>	0.8 $\pm$ 0.4	F = 31.79, p < 0.0001
AUC for T <sub>rectal</sub> 38.5°C (°C min <sup>-1</sup> )	7.7 $\pm$ 4.1 <sup>***</sup>	1.3 $\pm$ 2.9	1.0 $\pm$ 2.7	F = 8.95, p = 0.002
AUC for T <sub>rectal</sub> 39.0°C (°C min <sup>-1</sup> )	1.2 $\pm$ 0.9 <sup>#</sup>	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	F = 11.46, p = 0.001
Duration above 38.5°C (mins)	17.3 $\pm$ 9.7 <sup>#</sup>	5.3 $\pm$ 7.5	3.1 $\pm$ 8.8	F = 5.06, p = 0.018
Duration above 39.0°C (mins)	7.4 $\pm$ 3.9 <sup>***</sup>	1.4 $\pm$ 3.8	0.0 $\pm$ 0.0	F = 11.29, p = 0.001

Total  $n$  = 21, HOT70  $n$  = 6, HOT50  $n$  = 7; HYP50  $n$  = 8.

AUC = Area under the curve. PSI = Physiological strain index.

\*\*\* denotes significantly different from HOT50 and HYP50 (p < 0.001)

# denotes significantly different from HYP50 group (p < 0.05)

\* denotes significantly different from HOT50 (p < 0.05)

## Figure Legends

**Figure 1.** Standard curves (mean  $\pm$  SD; n = 4) generated in duplicate from the same pre-prepared standards analyzed on the same 96 well plate. The amplification steps produce a clear increase in the assays sensitivity when compared to the EKS-715 kit reagents allowing for determination of low levels of eHSP70 in plasma samples.

**Figure 2.** Panel A and B display the optical density and eHSP70 values obtained from each assay. EKS-715 was able to detect eHSP70 in 6 of the 32 resting observations ( $3.57 \pm 2.68 \text{ ng}\cdot\text{mL}^{-1}$ ), whereas ENZ-KIT measured eHSP70 in all 32 resting observations ( $1.54 \pm 3.19 \text{ ng}\cdot\text{mL}^{-1}$ ). When data for an individual was available from both assays (n = 12, Panel C and D) the ENZ-KIT tended to indicate lower values, though this was not statistically significant ( $p = 0.501$ ;  $R^2 = 0.73$ ). Between test reliability for samples assayed on two different occasions was high (Panel E), with the between test CV 7.86% and a correlation coefficient of 0.99 (Panel F).

**Figure 3.** eHSP70 was detected at all-time points in each trial using the ENZ-KIT, and was only elevated post exercise in HOT70 (Panel A, n = 6), with no post exercise change in eHSP70 expression observed after HOT50 (Panel B, n = 7) and HYP50 (Panel C, n = 8). Individual data are shown with bars representing mean eHSP70.





