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

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

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

Authors: Lee BJ[1,2], Sukri, NM[1] Ogden H[1], Vine C[1], Thake CD[2], Turner JE[1], and Bilzon JLJ[1].

1: Department for Health,
University of Bath,
Claverton Down,
Bath,
UK.

2: Department of Biomolecular and Sport Sciences,
Coventry University,
Priory Street,
Coventry,
UK.

Correspondance.
Ben Lee, Ph.D
The University of Bath
Department of Health
Claverton Down
Bath
BA2 7AY

Telephone: +44 (0)1225 383029
Email: B.J.Lee@bath.ac.uk

Abstract

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 ± 0.1 °C, 28.7 ± 0.8%, *n* = 6) at 70% $\dot{V}O_{2max}$. Participants in Part B completed 60 minutes of cycling exercise at 50% $\dot{V}O_{2max}$ in either hot (HOT50; 40.5°C, 25.4 RH%, *n* = 7) or hypoxic (HYP50; F_IO_2 = 0.14, 21°C, 35% 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 ± 3.27 $ng.mL^{-1}$; range 0.08 to 14.01 $ng.mL^{-1}$), 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.

Introduction

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

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

In addition to its intracellular HSP70 (iHSP70) function, HSP70 has been detected in the circulation (i.e., plasma; Pockley et al., 1998), where it is described as an extracellular HSP (eHSP, Fleshner et al., 2003). Extracellular HSP70 stimulates neutrophil microbicidal activity (Ortega et al., 2006), chemotaxis (Ortega et al., 2009), and induces cytokine production via a CD14 mediated pathway (Asea et al., 2000), thereby promoting innate immune activation (Krause et al., 2015). Elevated resting concentrations of eHSP70 has been positively correlated with insulin resistance (Krause et al., 2014), and disease progression in auto-immune (Luo et al., 2008) and inflammatory diseases (Schick et al., 2004; Najafizadeh et al., 2015). Therefore eHSP70 may be a useful biomarker when monitoring the progression of diseases in which low grade inflammation plays a role (Krause et al., 2012), such as sarcopenia (Ogawa et al., 2012), rheumatoid arthritis (Najafizadeh et al., 2015), diabetes (Krause et al., 2014), and obesity (Chung et al., 2008). The balance between the anti-inflammatory action of iHSP70, and pro-inflammatory action eHSP70, may determine the outcome (induction or attenuation of inflammation) during disease progression, in response to a treatment, or following an exercise bout (Krause et al., 2015). Therefore understanding the eHSP70 response and its actions is of importance in clinical situations.

In many studies the determination of basal eHSP70 has proven to be problematic (Ogawa et al, 2012; Lee et al., 2014; Najafizadech et al., 2015) with no consensus normative data for resting eHSP70 reported to date. Sample handling considerations (e.g., blood collection tubes temporarily stored on ice *versus* room temperature, using Heparin *versus* EDTA as an anticoagulant) have been suggested as explanations underlying the variability in eHSP70 values reported in the literature. For example, serum appears to yield lower basal values than those obtained by plasma (Whitham and Fortes, 2006), which may be an artifact of eHSP70 binding to proteins involved in the clotting process, such as fibrin and fibrinogen (Whitham and Fortes, 2006). Additionally, EDTA is recommended for use over heparin as higher resting concentrations are derived (Whitham and Fortes, 2006). Despite the recommendation to use EDTA plasma for eHSP70 determination (Whitham and Fortes, 2006), multiple

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

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

A new ELISA has become available (ENZ-KIT-101-001 HSP70 Amp'd® ELISA), sensitive to 0.007 $ng \cdot mL^{-1}$ with a working range of 0.039-5.00 $ng \cdot mL^{-1}$. The increased sensitivity of this kit is mediated by an alkaline-phosphotase (AP) conjugate binding to a signal amplification substrate, which enhances colour production at lower analyte concentrations. The increased sensitivity therefore affords the potential to determine normative resting eHSP70 values for a range of individuals with a variety of conditions and could allow for a more sensitive determination of stress-induced changes in eHSP70.

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

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

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

Materials and Methods

Participants

Twenty-one recreationally active healthy males provided signed informed consent prior to participation in this study, which was granted approval by the NHS South-West Research Ethics Committee (Reference ID. 14/SW/0098, Part A) and Coventry University local ethics committee (Part B). All procedures were conducted in accordance with the principles outlined in the *Declaration of Helsinki*. Data reported in this investigation were collected from two larger experimental trials (Lee, 2014). The collected data represents a convenience sample of similarly characterised individuals providing EDTA treated plasma before and after a 60-minute bout of exercise under conditions of environmental stress.

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

Preliminary measurements

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

Participants in Part A (n = 6) of the investigation completed a continuous incremental running test to volitional exhaustion on a motorised treadmill (Woodway ELG70, Weiss, Germany). The test protocol was modified from that of Taylor et al. (1955) and was performed in thermoneutral conditions (19.7 ± 0.7 °C, 46.3 ± 4.0% RH). After a five-minute warm-up at 6 $km \cdot h^{-1}$, the test began at a speed of 10 $km \cdot h^{-1}$ on a 1% inclination. Speed was then increased by 1 $km \cdot h^{-1}$ every three minutes until reaching 13 $km \cdot h^{-1}$, when inclination was increased by 2% every two minutes. Participants were instructed to run for as long as possible and signal when they felt they could only complete one more minute to allow for a final set of recordings. Peak oxygen consumption was determined for participants in Part B using an incremental exercise test to volitional exhaustion on calibrated SRM cycle ergometer (n = 15, Table 1) Schoberer Rad Meßtechnik, Welldorf, Germany). Resting blood lactate (Biosen C-Line analyser, EKF Diagnostics, Germany) was determined from a finger capillary whole blood sample following a 10-minute seated rest period. The test began at a workload of 70W for 4-minutes and was then increased by 35W every 4 until a blood lactate value of > 4$mmol.L^{-1}$ was reached. Thereafter, workload increased 35W every 2 minutes until volitional exhaustion. A cadence of 70 $rev.min^{-1}$ was maintained throughout. In both Part A and Part B, expired gases were collected using 200L Douglas bags (Cranlea & Co, Birmingham, UK) during the final minute of each stage. Heart rate (Polar FT1, Polar Electro OY, Kempele, Finland) and perceived exertion (Borg, 1976) were measured at the end of each gas collection. Respiratory gas analysis was completed as previously described (Lee et al., 2014, Lee et al., 2015). Peak oxygen consumption was considered to be achieved if two of the following criteria were met: i) a respiratory exchange ratio of >1.1, ii) a heart rate greater than 95% of age predicted maximum (220−age) and iii) a final blood lactate value in excess of 8 $mmol.mL^{-1}$.

Experimental design

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

Part A

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

Part B

Samples were obtained before and immediately after a 60 minutes of cycling at a power output equivalent of 50% $\dot{V}O_{2max}$ in either hot (HOT50; 40.5°C, 25.4 RH%) or hypoxic (HYP50; F_IO_2 of ~0.14, 21°C, 35% RH) conditions. The hypoxic environment was generated by an oxygen filtration device (Hypoxico HYP-123 hypoxicator, New York, NY, USA) set to produce the desired F_IO_2. Participant characteristics for HOT50 (n = 7) were (mean ± SD): age = 22 ± 5 years; height 1.76 ± 0.05 meters; body mass 70.9 ± 5.7 kg; % body fat 13.2 ± 4.0%, $\dot{V}O_{2max}$ 54.9 ± 3.2 $mL.kg^{-1}.min^{-1}$. Participant characteristics for HYP50 were: 23.4 ± 4 years; height 1.80 ± 0.08 meters; body mass 70.0 ± 9.1 kg; % body fat 12.6 ± 3.7 %; $\dot{V}O_{2max}$ 52.2 ± 3.3 $mL.kg^{-1}.min^{-1}$.

Participant Preparation

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

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

Physiological Measurements

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

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

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

Determination of extracellular HSP70

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

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

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

carbonate) was necessary to determine the optimal dilution for each sample, with results multiplied by the this dilution factor in order to give eHSP70 values in ng·mL^{-1}. Once the optimal sample dilution was determined for each participant on the ENZ-KIT, pre and post exercise samples were analysed in duplicate.

Statistical analysis

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

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

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

Results

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

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

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

Extracellular HSP70 at rest

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

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

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

Physiological and thermoregulatory responses to each stressor

The duration of exercise undertaken at 70% $\dot{V}O_2max$ (i.e., HOT70, 54.0 ± 9.4 minutes) was shorter than the duration of exercise undertaken at 50% $\dot{V}O_2max$ (i.e.,

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

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

The exercise-induced eHSP70 response

In accordance with the resting data, EKS-715 only detected post exercise eHSP70 in the 6 samples that had detectable eHSP70 at rest, with 5 samples obtained in HYP50 and 1 sample from the HOT50. Post exercise eHSP70 obtained from the EKS-715 kit ($n = 6$, 3.92 ± 4.34 $ng.mL^{-1}$) had a good relationship to those obtained with the ENZ-KIT ($n = 6$, 3.37 ± 5.38 $ng.mL^{-1}$, $r = 0.84$).

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

Exercise results in an increase in eHSP70 from 2.79 ± 2.59 $ng·mL^{-1}$ (95% CI = 0.074 to 5.51 $ng·mL^{-1}$) at rest to 3.51 ± 2.90 $ng·mL^{-1}$ (0.47 to 6.56 $ng·mL^{-1}$) in the HOT70 group ($t = 3.82$, $p = 0.012$). However, with the HOT50 trial, eHSP70 was unchanged from 0.22 ± 0.13 $ng·mL^{-1}$ (95% CI = 0.084 to 0.362 $ng·mL^{-1}$) at rest to 0.20 ± 0.16 $ng·mL^{-1}$ (95% CI = 0.034 to 0.370 $ng·mL^{-1}$) following exercise ($t = 0.886$ $p = 0.410$). In addition, in the HYP50 trial, eHSP70 was unchanged from 2.82 ± 5.51 $ng·mL^{-1}$ (95% CI = 2.96 to 8.60 $ng·mL^{-1}$) at rest to 2.85 ± 5.56 $ng·mL^{-1}$ (95% CI = 2.98 to 8.69 $ng·mL^{-1}$) following exercise ($t = 0.635$, $p = 0.545$). Thus only HOT70 induced 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_{rectal} (r = 0.52), and AUC for
402 T_{rectal} 38.5°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
405 for 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 manufactures
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

Heat shock proteins play an important role in maintaining cellular protein homeostasis, with HSP dysfunction implicated in the pathology of Alzheimer's disease, Parkinson's disease, cardiovascular disease, and sarcopenia (Krause et al., 2015). It is therefore surprising that there is currently no substantial normative data regarding resting eHSP70 for either healthy individuals or clinical cohorts. Indeed, a characteristic of eHSP70 research is the large variability both between studies (Ruell et al., 2006; Whitham and Fortes, 2006; Gibson et al., 2014; Lee et al., 2015) and within studies, likely exacerbated by small samples sizes typically used in exercise studies (Gibson et al., 2014; Lee et al., 2015). Between-participant variation is evident in the present investigation, in which three groups of seemingly physiologically-matched males (Table 1) present significantly different eHSP70 values at rest (Figure 3). Such a large disparity in resting data will limit the ability to detect changes in eHSP70 concentrations between two or more matched groups. When a repeated measures design is not feasible it may be appropriate for experimenters to match individuals based on resting eHSP70 concentrations rather than more common physiological and anthropometric features (providing that eHSP70 is an important outcome). Doing so may facilitate a clearer understanding regarding responders, such as the participant with high post HOT50 eHSP70 concentration (Figure 3, Panel B; rest = 0.67 $ng.mL^{-1}$, post exercise = 2.29 $ng.mL^{-1}$), and non-responders to thermal or hypoxic stress.

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

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

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

In addition to the ability of the ENZ-KIT to quantify resting eHSP70, we therefore examined the post-exercise response at 3 levels of physiological strain using two distinctly different modes (running and cycling) of hyperthermic exercise, as well as hypoxic stress (Table 1). The data presented in Tables 1 indicate two levels of physiological strain were achieved, with HOT70 eliciting significantly greater mean and peak HR, T_{rectal} and PSI compared to HOT50 and HYP50. No significant differences between the HOT50 and HYP50 groups were observed, agreeing with

previous work showing exercise at 50%$\dot{V}O_2$max in either 40°C heat or an F_IO_2 of 0.14 to be of a comparable physiological stress up to 40 minutes of exercise (Lee et al., 2014). The 60 minute duration of exercise used herein was therefore insufficient for significant differences in physiological strain between HOT50 and HYP50 to become apparent (Girard and Rancinas, 2014; Lee et al., 2014).

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

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

In contrast, no post exercise increase was observed following HOT50 or HYP50 (Figure 3). It is likely that while the thermal component of HOT50 was sufficient for increased eHSP70, the duration of exercise, and therefore overall level of exogenous strain, was not sufficient to increase eHSP70. Of the suggested endogenous requirement for post exercise increases in eHSP70 (peak T_{rectal} of >39.2°C, a mean T_{rectal} of 38.6°C for a period of ~57 minutes, a core temperature change of 2.2°C from

baseline at a rate of 1.6°C h^{-1} and a mean heart rate of 153 $bt.min^{-1}$), the HOT50 trial only achieved the required heart rate, and the HYP50 group failed to reach any of these potential eHSP70 inducing thresholds. Our data therefore lend support to the notion of a minimum endogenous criteria required for eHSP70 induction (Amorim et al., 2008; Gibson et al., 2014). The increased sensitivity afforded by the ENZ-KIT may allow for a more detailed and nuanced description of minimum eHSP70 inducing criteria during and after exercise stress in future studies.

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

Abbreviations

AUC; Area under the curve. **AP**; Alkaline phosphatase**. ENZ-KIT-101**; Amp'd® HSP70 High Sensitivity ELISA kit. **eHSP70**; Extracellular heat shock protein 70. **EKS-715;** HSP70 high sensitivity ELISA kit. **ELISA**; Enzyme linked immunosorbant assay. **F_IO_2**; fraction of inspired oxygen. **HEAT70;** Heat running trial. **HEAT50**; heat cycling trial **HSP70**; Heat shock protein 72. **HR**; Heart rate. **HYP**; Hypoxic trial. **IL-10**; Interleukin 10. **kDa**; KiloDalton. **pNPP**; p-Nitrophenyl phosphate. **PSI**; Physiological strain index. **RPE**; rating of perceived exertion. **T_{rectal}**; Rectal temperature. **RH**; Relative humidity. **SpO_2**; Arterial oxygen saturation. **TS**; Thermal sensation. **TNF-α**; Tumour necrosis factor alpha. **$\dot{V}O_2max$**; Maximal oxygen consumption.

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567
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Table 1. Mean and peak physiological and thermoregulatory responses to each trial. Data are mean ± SD.

	Experimental group			ANOVA
Variable	**HOT70**	**HOT50**	**HYP50**	**Trial Main effect**
Exercise duration (mins)	54.0 ± 9.4***	60.0 ± 0.0	60.0 ± 0.0	F = 4.25, p = 0.032
Mean HR (beats.min^{-1})	180 ± 8***	153 ± 12	149 ± 13	F = 14.17, p < 0.0001
Peak HR (beats.min^{-1})	190 ± 7***	165 ± 10	157 ± 15	F = 14.61, p < 0.0001
Mean T_{rectal} (°C)	38.4 ± 0.3$^{\#}$	38.0 ± 0.2	37.9 ± 0.4	F = 4.41, p = 0.028
Peak T_{rectal} (°C)	39.3 ± 0.4$^{*\#}$	38.7 ± 0.3$^{\#}$	38.2 ± 0.4	F = 15.62, p < 0.0001
Mean PSI (AU)	7.2 ± 0.6*	5.2 ± 1.0	4.8 ± 1.1	F = 11.81, p = 0.001
Peak PSI (AU)	9.2 ± 0.8*	7.4 ± 1.2	5.9 ± 1.4	F = 13.74, p < 0.0001
Delta change in T_{rectal}	+2.5 ± 0.3***	+1.4 ± 0.4$^{\#}$	+0.8 ± 0.4	F = 34.34, p < 0.0001
Rate of T_{rectal} change (°C h $^{-1}$)	2.9 ± 0.7***	1.4 ± 0.4$^{\#}$	0.8 ± 0.4	F = 31.79, p < 0.0001
AUC for T_{rectal} 38.5°C (°C min^{-1})	7.7 ± 4.1***	1.3 ± 2.9	1.0 ± 2.7	F = 8.95, p = 0.002
AUC for T_{rectal} 39.0°C (°C min^{-1})	1.2 ± 0.9$^{*\#}$	0.0 ± 0.0	0.0 ± 0.0	F = 11.46, p = 0.001
Duration above 38.5°C (mins)	17.3 ± 9.7$^{*\#}$	5.3 ± 7.5	3.1 ± 8.8	F = 5.06, p = 0.018
Duration above 39.0°C (mins)	7.4 ± 3.9***	1.4 ± 3.8	0.0 ± 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 ± 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 ± 2.68 ng·mL^{-1}), whereas ENZ-KIT measured eHSP70 in all 32 resting observations (1.54 ± 3.19 ng·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.