Title: Reproducibility of cardiorespiratory and cellular responses to steady state exercise in hypoxia and preloaded cycling time trial performance reliability in normoxia and acute hypoxia.

Running title: Reproducibility of physiological performance and cellular measures during a hypoxic exercise task.

Key Words: Cycling, normobaric hypoxia, reliability

Authors: Lee BJ¹² and Thake CD²

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

2: Centre for Applied Biological and Exercise Sciences,
Coventry University,
Priory Street,
Coventry,
UK.

Correspondence.

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. The purpose of this study was to assess the reproducibility of cardiorespiratory and cellular (monocyte heat shock protein 70; mHSP70) responses to a fixed load hypoxic stress test (HST) and the reliability of a pre-loaded 16.1 km cycling time trial (pTT) conducted under both normoxic and hypoxic conditions. Methods. Eighteen participants (age, 22 ± 4 years; height, 1.77 ± 0.04 meters; body mass, 76.8 kg; estimated body fat and VO2peak = 3.50 ± 0.60 L.min⁻¹) were divided into three groups. Reliability of responses (HR, SpO2, VO2, CO2, VE and RER) to the HST (FI02 0.14; 15 minutes rest, 60 minutes cycling at 50% normoxic VO2peak) was assessed across 3 repeat trials (HST 1, 2 and 3, n = 6); mHSP70 was measured via flow cytometry before and after each HST (n = 5); resting HSP was also quantified on 4 separate occasions (n=5). Reliability of the pTT (15 min rest, 40 minutes cycling at 50% normoxic VO2peak) was assessed across 3 repeat normoxic (N; FI02 ≈ 0.21; n=6) and 3 repeat hypoxic (FI02 ≈ 0.14; n = 6) trials. All exercise trials were undertaken at the same time of day, following exercise and dietary controls, 7 days apart.

Results. Intra-class correlation coefficients (ICC’s) for mean and peak HR, SpO2, VE, VO2, VO2 and BLA within each trial were improved from HST1 to HST2 (mean data: 0.99, 0.95, 0.75, 0.62, 0.70, 0.90; peak data: 0.98, 0.96, 0.64, 0.69, 0.74, 0.75) to HST2 and HST3 (ICC = 0.99, 0.97, 0.82, 0.85, 0.87 and 0.96 respectively). mHSP70 was a reproducible at rest without (ICC > 0.95) and with HSTs conducted in the previous 7 days (ICC > 0.95), with no difference in pre to post increases in mHSP70 observed between tests. The reliability for time to pTT completion was improved following one trial, and the CV (test 2 vs. 3) was similar under normoxic (CV = 0.62) and hypoxic conditions (CV = 0.63). Conclusion. Cardiorespiratory and cellular responses to the HST were reproducible and the pTT performance time reliable in both N and H. Since the reproducibility of the measurements in HST trials and reliability of pTT improved between the second and third trials one familiarization visit is recommended prior to employing these protocols in future studies.

Introduction

Many modern sporting scenarios and military operations involve exposure to extreme environmental factors (e.g. altitude or heat) that increase physiological strain and reduce physical work capacity and performance (Gore et al., 1996; 1997). Knowledge of the extent that specific environmental conditions impact on these response variables can inform the
preparation and management of exposed individuals and groups. Therefore it is important to consider the utility of tests used to measure the magnitude of response to defined environmental stressors. Accordingly a reasonable degree of confidence that the changes in test measures are due to manipulation of environmental conditions in an acute setting or chronic acclimation and/or training adaptation with repeat or continuous exposure rather than measurement error (Atkinson and Nevill, 1998) or inherent high-test variability (Che Jusoh et al., 2015) is important.

Fixed workload protocols are often used to provide ‘steady-state’ data in response to a stimulus e.g. the hypoxic stress test used by Lee and colleagues (2015) to evaluate the impact of short term heat acclimation on responses to exercise in hypoxia. It is generally assumed that such protocols yield reproducible physiological data under defined conditions although the test-retest variation in these measures is seldom reported. In addition to physiological markers, aspects of the cellular response to stressors are also now often examined. For example it has recently been demonstrated that steady state fixed workload exercise in both acute heat and acute hypoxia increases the stress responsive monocyte heat shock protein 70 (mHSP70, Lee et al., 2014; Lee et al., 2015) concentration and that acclimation to both heat and hypoxia elevates basal mHSP70 (McClung et al., 2008; Taylor et al., 2012; Lee et al., 2015). Many studies have used flow cytometry to quantify HSP70 in different peripheral blood mononuclear cell (PBMC) populations, such as monocytes (Taylor et al., 2010, 2011, 2012; Vince et al., 2011; Peart et al., 2013 Lee et al., 2014, 2014a). This technique was shown to be a rapid, easy and sensitive technique for intracellular HSP70 quantification (Bachelet et al., 1998). However, despite its wide spread use (Taylor et al., 2010; 2012; Lee et al., 2014; 2015), no study has reported data on the test-retest reproducibility of HSP70 following environmental stress.

In order to provide an indicator of performance potential steady-state fixed workload protocols may be extended by continuing the exercise bout until exhaustion. However this approach often suffers from high test – retest variability and low ecological validity. For example, trained cyclists had a test-retest coefficient of variation (CV) of ~27% when cycling to exhaustion at 75% of maximal workload ($W_{\text{max}}$; Jeukendrup et al., 1996). Whereas, a performance test (a self-paced 60-minute bout of cycling), conducted with the same participants, returned a test-retest CV of 3.4% (Jeukendrup et al., 1996). This is typical of self-paced tests with a defined end-point (i.e. time trials) that by definition provide more ecologically valid data (Mee et al., 2015; Che Jusoh et al., 2015). Furthermore under heat
stress a comparable CV (3.6%) was reported by Che Jusoh and colleagues (2015) following a 45 minute cycling preload (cycling at 55%\(\dot{V}O_2\)peak). A similar performance test has been used in hypoxic conditions (Beidleman et al., 2007) although no reliability data was presented for the hypoxic performance test variables *per se*. As hypoxic tolerance before and after an intervention is now often studied, it would be useful to determine the reproducibility of a simple fixed-work protocol hypoxic stress test (HST) for use in physiological steady-state conditions as well as the reliability of a preloaded self-paced cycling time trial cycling test. Therefore, the purpose of this study was to assess the reproducibility of both physiological and cellular mHSP70 responses to a HST (60 minute fixed load period of cycling) and the reliability of a preloaded (40 minute bout of fixed-intensity cycling) 16.1km TT conducted in moderate normobaric hypoxia.

**Methods**

**Participants**

The study received ethical approval from the Coventry University local Ethics Committee (REF P2566/P6420) and was conducted in accordance with the declaration of Helsinki. Eighteen healthy and regularly active males accustomed to cycling exercise provided their informed consent to participate. Three groups of 6 participants were formed. Reproducibility of physiological and cellular (HSP72) measurements to a fixed relative work-rate hypoxic stress test (HST) where made on the HST group (age, 23 ± 3 years; height, 1.77 ± 0.05 m; body mass, 74.7 ± 6.9 kg; \(\dot{V}O_2\)peak, 3.50 ± 0.70 L.min\(^{-1}\)); whereas the reliability of a 16.1 km preloaded time trial (pTT) performance test was evaluated using separate groups under normoxic (\(F_iO_2\approx 0.21\)) conditions (NORM; age, 21 ± 2 years; height, 1.77 ± 0.04 m; body mass, 84.0 ± 17.0; \(\dot{V}O_2\)peak, 3.17 ± 0.40 L.min\(^{-1}\)), and hypoxic (\(F_iO_2\approx 0.14\)) conditions (HYP; age, 27 ± 5 years; height, 1.76 ± 0.02 m; body mass, 72.0 ± 7.6 kg; \(\dot{V}O_2\)peak, 3.82 ± 0.60 L.min\(^{-1}\)).

**Experimental design**

Each participant visited the laboratory on 4 occasions (1 preliminary and 3 experimental) each separated by at least 7 days (range 7 – 11 days). During the preliminary visit participants gave informed consent and measures of height, body mass, skin folds (4 sites), normoxic lactate threshold and \(\dot{V}O_2\)peak were made. Thereafter, on separate visits, participants in the
HST group completed 3 fixed work-rate (50% normoxic $\dot{V}O_2$peak, 60 minutes) exercise trials whilst breathing hypoxia (F$_{2}\text{O}_2 \approx 0.14$), and participants in NORM and HYP completed 3 preloaded time-trials whilst breathing F$_{2}\text{O}_2 \approx 0.21$ or 0.14, respectively.

Preliminary testing

Anthropometric data were collected in accordance with the International Society for the Advancement of Kinanthropometry (ISAK) guidelines (Marfell-Jones et al., 2006). Lactate threshold and peak oxygen consumption ($\dot{V}O_2$peak) were determined via an incremental exercise test to volitional exhaustion on calibrated SRM cycle ergometer (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 minutes until a blood lactate value of $>$ 4mmol.L$^{-1}$ was reached. Thereafter, workload increased 35W every 2 minutes until volitional exhaustion. A cadence of 70 revolutions per minute (RPM) was maintained throughout. Expired gas was collected during the last minute of each exercise stage via a two-way nonrebreathable valve (Harvard Ltd, Eldenbridge, UK) and 1.5 m of 30mm diameter polyvinyl tubing supplying a 200L Douglas bag (Cranlea & Co, Birmingham, UK). Heart rate (Polar FT1, Polar Electro OY, Kempele, Finland) was recorded and perceived exertion (Borg, 1976) sought within the last 10 seconds of each gas collection. Respiratory gas analysis was completed as previously described (Lee et al., 2014, Lee et al., 2015). $\dot{V}O_2$peak 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 trials

Laboratory attendance time was consistent for each participant in order to minimize the effects of circadian variation on performance and the known diurnal variation in performance (Drust et al., 2005) and HSP70 (Taylor et al., 2009; Vince et al., 2010). Participants were requested to abstain from caffeine (Luo et al., 2008) and alcohol consumption for 72 h prior to each laboratory visit and required to maintain a food and activity diary as accurately as possible for 3 days prior to each experimental visit (Morton et al., 2006). Participants refrained from all supplementation (i.e. vitamins, ergogenic aids) throughout the study period and were requested to abstain from prolonged thermal exposures (baths, saunas, steam rooms,
and tanning devices) and vigorous physical activity for seven days prior to each laboratory visit. Persons who had visited or resided at altitudes in excess of 1000 m (Taylor et al., 2010) or climates with ambient temperatures in excess of 30°C (Sandstrom et al., 2009; Selkirk et al., 2009; Lee et al., 2014) or had experienced high pressure environments, for example, hyperbaria (Taylor et al., 2013), within the three months prior to study commencement were excluded during recruitment due to the possible influence of such environments on basal HSP70 expression.

Participants adhered to an overnight fast (Febbraio et al., 2002) and consumed 500 ml of plain water one hour before visiting the laboratory in accordance with the American College of Sports Medicine position stance on hydration (Sawka et al., 2007). Upon arrival (0630 – 0730 hrs), participants voided their bladder to provide a sample for hydration assessment via urine specific gravity (USG; Atago Refractometer, Jencons Pls, Leighton Buzzard, UK) and urine osmolarity (Uosmo; 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) 10 cm past the anal sphincter, and fitted a telemetric heart rate monitor around their chest (Polar FT1, Polar Electro OY, Kempele, Finland).

**Fixed work-rate hypoxic stress test (HST)**

Participants in the HST group completed 15 minutes of seated rest in normoxic conditions prior to a resting venous blood sample being drawn into an EDTA vacutainer (n = 5; Vacuette®, Greiner Bio-one, UK) for quantification of mHSP70. A 15 minute resting ‘wash-in’ period of breathing hypoxic gas (FIO₂ = 0.14) was then completed. The gas was delivered to the participant via a mouthpiece and 30 mm diameter connector (Harvard Ltd, Eldenbridge, UK) connected to a two-way non-rebreathable valve (Harvard Ltd, Eldenbridge, UK). Ethylene clear vinyl tubing was used to connect the inspiratory side of the valve to a series of 1000L Douglas bags which were filled via a hypoxicator (Hypoxico HYP123 Hypoxicator, New York, USA). At the end of the wash-in period participants began 60 minutes of cycling exercise at an intensity corresponding to 50% normoxic VO₂peak (145 ± 16 Watts) on an SRM ergometer (Schoberer Rad Meßtechnik, Welldorf, Germany) at a
cadence of 70 RPM. Measures of heart rate (HR), arterial hemoglobin oxygen saturation (SpO2), respiratory variables (VE, VO2, VCO2) via Douglas bag collections, and fingertip capillary blood samples for determination of glucose and lactate concentrations were taken at the start and end of each 15 minute resting period and every 10 minutes throughout exercise. Upon termination of the exercise bout a further 7mL venous blood sample was collected to assay for post exercise mHSP70 concentrations.

Preloaded time trial (pTT)

Participants undertook 30 min of seated rest (NORM FiO2 = 0.21 throughout; HYP 15 min FiO2 = 0.21 followed by 15 min FiO2 = 0.14 ‘wash in’ period) prior to undertaking a 40 minute period of cycling exercise at an intensity corresponding to 50% normoxic VO2peak (144 ± 18 Watts) whilst breathing either FiO2 = 0.21 (NORM) or FiO2 = 0.14 (HYP). Physiological variables were collected every 10 minutes as previously described. At the end of the 40 minute fixed load exercise bout and after 5 min of passive recovery the self-paced 16.1km cycling pTT was commenced. The pTT was controlled within the SRM ergometers open-ended mode, which creates a braking force that has a cubic relationship with speed, mimicking the effect of air resistance on a moving bicycle. Participants were instructed to complete distance as quickly as possible. The only feedback available to participants was the distance completed at any given time. During the time trial only measures of HR and power output were collected in order to avoid providing any external time cues.

Determination of monocyte HSP70

Flow cytometry was used to quantify intracellular HSP70 in peripheral blood mononuclear cells (PBMC’s). Whole blood (100µL) from EDTA tubes was transferred into 2mL of red cell lysing buffer (Erythrolyse, AbD Serotec, UK) and incubated at room temperature for 10 minutes. The sample was centrifuged at 500g for 5 minutes and the supernatant discarded. Cells were then washed by 2mL of phosphate buffer saline (PBS) and centrifuged for 5 minutes at 500g. Cells obtained following red cell lysis were fixed for 15 minutes by adding 100 µL of fixative (Reagent A, Leukoperm, AbD Serotec, UK) at room temperature and then washed with PBS as previously described. Cells were then permeabilised (Reaged B, Leukoperm, AbD Serotec, UK) and an IgG1 negative control (4 µL; FITC, AbD Serotec, UK) or an anti-HSP70 antibody (4 µL; SPA-810, Assay Designs, USA) was added to a final concentration of 100 µg/mL to label 1 x 10^6 cells according to manufactures instructions. The stained cells were then incubated in the dark for the 30 minutes and washed with PBS.
Samples were analysed on a BDFACScaliber (BD Bioscience, UK) with monocytes gated by forward/side scatter properties and further discriminated by CD14 expression. Mean florescence intensity (MFI) was calculated using CELLQuest software (BD Biosciences, UK) with a total of 15,000 cells counted. Figure 1 illustrates a typical forward side scatter plot showing different cell types, and a frequency histogram for cells stained with the negative or HSP70 positive antibody.

Statistical analysis

Data are reported as mean ± SD unless otherwise stated. Physiological data were analysed using trial x time ANOVA, with repeated measures over time. Between-day resting, and between-trial resting mHSP70 was analysed via a one-way repeated measures ANOVA. In order to assess test-retest reproducibility in the mean physiological and cellular measures obtained at rest and during exercise throughout HST, NORM and HYP, the mean difference between trials (change in mean), intra-class correlation coefficient (ICC) and coefficient of variation (CV) was calculated between the 3 repeat conditions in each group (Hopkins, 2000). The technical error of measurement between trials was calculated for all participants by calculating the difference scores and standard deviation of difference scores between trials and dividing the square root of the standard deviation of the difference by 2 (Hopkins, 2000).

Results

All participants adhered here to the activity and nutrition controls specified in the participant information sheet prior to each testing session.

Physiological responses to the HST

The cardiorespiratory responses to each HST can be seen in Figure 2. Based on participants mean oxygen consumption and mean exercising heart rate, participants were exercising at an intensity eliciting 62 ± 11, 58 ± 9 and 61 ± 16% $\dot{V}$O$_2$peak at the end of the HST. No main effect for trial ($p > 0.05$) was observed for heart rate, $\text{SpO}_2$, $\dot{V}$E, $\dot{V}$O$_2$, $\dot{V}$CO$_2$, and RER over the course of the 3 trials. The technical error of measurement, ICC and coefficient of variation for mean exercise measurements are shown in Table 1. Heart rate increased from 62 ± 7, 63 ± 8, and 66 ± 9 beats.min$^{-1}$ at rest to 153 ± 12, 154 ± 14, and 152 ± 17 beats.min$^{-1}$ at the end of the 40 minute exercise period for TT1, TT2 and TT3 respectively. Arterial oxygen saturation decreased from 98 ± 1, 98 ± 0.5, and 98 ± 0.5% at rest to 89 ± 2, 90 ± 2, and 90 ± 4% at the end of rest and 79 ± 4, 79 ± 3, and 80 ± 2% at the end of exercise. Oxygen
consumption increased from 0.32 ± 0.1, 0.36 ± 0.1 and 0.33 ± 0.1 L.min⁻¹ at the end of rest to 2.11 ± 0.26, 1.98 ± 0.22, and 2.06 ± 0.24 L.min⁻¹ at the end of the exercise period.

Monocyte HSP70 Five participants from the HST group attended the laboratory on 4 separate occasions at 10:00am to provide a resting blood sample in the 2 weeks prior to the exercise testing. There was no main effect for time (f = 0.18, p = 0.945) for mHSP70. Table 2 shows the technical error of measurement, ICC and CV between each time point.

Resting mHSP70 was also consistent between HST trials 1 - 3 (T1, 3.85 ± 1.47 AU; T2, 3.75 ± 1.42 AU; T3, 3.69 ± 1.25 AU, p > 0.05) with a CV of 5.6, 3.6 and 5.3% observed between HST1 and HSTT2, HST1 and HST3 and HST2 and HST3 respectively. The post HST response of mHSP70 was also consistent between TT1, TT2, and TT3, increasing post exercise by 31 ± 12%, 29 ± 13% and 25 ± 13% after T1, T2 and T3 (p < 0.05, Figure 3), although post exercise mHSP70 in HST3 was lower than after HST1 (p < 0.05). The post HST mHSP72 response was shown to be inversely related to basal mHSP72 in each HST (Figure; HST1, R² = 0.85 ; HST2, R² = 0.86; HST3, R² = 0.71).

Preloaded exercise – physiological responses

Based on participants mean ∆O₂ and HR participants in the NORM group were exercising at an intensity eliciting 51.1 ± 8%, 51.3 ± 11 and 52 ± 10% ∆O₂peak, and participants in the HYP group at 67 ± 6, 65 ± 9 and 65 ± % ± for T1, T2, and T3 respectively. Cardiorespiratory responses are shown in Figure 4. Main effects for time (p < 0.0001) but not trial (p > 0.05) or interaction (p > 0.05) were observed for HR, SpO₂, ∆V₂, ∆E and RER during the 40 minute preload. HR was unchanged during the rest period in NORM (TT1, 72 ± 8 to 75 ± 14; TT2, 73 ± 10 to 76 ± 4; TT3, 73 ± 9 to 78 ± 15 beats.min⁻¹) and increased to 140 ± 12, 142 ± 7 and 140 ± 10 beats.min⁻¹ at the end of the 40 minute preload. HR increased from 66 ± 5, 67 ± 5, and 76 ± 9 beats.min⁻¹ at the beginning of rest to 78 ± 8, 78 ± 9 and 86 ± 10 beats.min⁻¹ at the end of the rest period (p < 0.05), which corresponded to the decrease in SpO₂ at the end of the rest period (98 ± 1 to 90 ± 3% across all trials, p < 0.001). Heart rate was 156 ± 6, 156 ± 9, and 158 ± 4 beats.min⁻¹ at the end of the 40 minute exercise period in HYP, for TT1, TT2 and TT3 respectively, which was greater than that in NORM (p < 0.05).

Time trial performance

Time to complete the 16.1km course was longer in HYP compared to NORM during TT1 (42.3 ± 5.5 versus 39.4 ± 1.1 minutes), TT2 (41.4 ± 5.5 versus 39.1 ± 1.5 minutes) and TT3...
(41.8 ± 5.3 versus 38.8 ± 0.6 minutes), although no TT x condition effect was observed ($F_{(2, 20)}=1.36, p=0.250$). The typical error for completion time, mean power output and mean HR during the 3 time trials are presented in Table 2. Based on mean power output during each TT, the NORM group completed the distance at 63 ± 5%, 63 ± 6% and 66 ± 9% and the HYP group at 60 ± 6%, 62 ± 6% and 61 ± 6% of normoxic $W_{\text{max}}$ for TT1, TT2 and TT3 respectively.

The TEM for completion time between TT1 and TT2 was between 59 and 79 seconds in NORM, and 68 – 92 seconds in HYP. The TEM between TT2 and TT3 was 34 – 46 seconds and 32 – 43 seconds for NORM and HYP respectively, with a CV of 0.6%. The smallest worthwhile change in TT performance is > 46 seconds.

**Discussion**

This study reports that physiological and cellular (mHSP70) responses to a 60 minute steady-state HST are reproducible and that preloaded (40 minutes of steady state cycling) 16.1km TT performance, conducted under both normoxic and acute normobaric hypoxic conditions ($\text{FiO}_2=0.14$), is reliable. Importantly these data demonstrate that the reproducibility of the measurements in HST trials and reliability of pTT performance (under both N and H conditions) improved between the second and third trials. Accordingly when using such protocols in future investigations we would advise conducting at least one familiarisation trial were participants carry out the full protocol under the proposed experimental conditions. Furthermore an awareness of the typical variance of each of these tests under the conditions studied can help provide context to the effect of an intervention on defined outcome measures.

During each HST trial classic markers of hypoxic physiology (HR, SpO2, $\dot{V}_O2$, $\dot{V}_CO2$, $\dot{V}_E$, RER and blood lactate) were measured. Our data indicate that this simple to conduct fixed-workload test had good agreement for all the physiological variables studied (Table 1) when tests are conducted one week apart, supporting its use in future studies investigating hypoxic tolerance. Typically, a correlation coefficient of over 0.90 is considered high, 0.70-0.80 moderate, and below 0.70 considered too low for a reliable and sensitive physiological test (Vincent, 1995). In the present investigation the ICC for each physiological variable was over 0.80 when HST2 and HST3 were considered. Based on this observation, the whole body physiological measurements of HR, SpO2, $\dot{V}_O2$, $\dot{V}_CO2$, RER and $\dot{V}_E$ all have an acceptable
level of reproducibility between repeated trials in the conditions studied if one familiarisation visit is conducted. With reference to the TEM data we suggest that changes in HR, SpO₂, $\dot{V}_F$ and $\dot{V}_O_2$ of 4 beats.min⁻¹, 2%, 6.3 L.min⁻¹, 0.32 L.min⁻¹ respectively, would constitute a statistically meaningful change in these variables between HST tests conducted before and after an intervention (e.g. an acclimation period).

The reproducibility of a commonly used marker of cellular stress mHSP70 was assessed at rest and following exercise in 5 of the HST participants (Figure 1). Our data show that there is a considerable inter-subject variation in resting mHSP70. However within-subject reproducibility is shown to be high both at rest, and following the HST. Based on these observations we suggest that researchers presenting mHSP70 data (or indeed any continuous data variable) should present data in a way that allows for a visual analysis of data distribution (Weissgerber et al., 2015; Figure 3). In small sample size studies such as this one, and others (Taylor et al., 2010, 2011, 2012), a more appropriate data presentation can allow readers to assess the distribution of the data set visually (Weissgerber et al., 2015). The low CV (< 6%) and high ICC (> 90%) for resting mHSP70 suggests that this measure is sensitive to changes in resting data. It also indicates that a washout period of 7 days allows for the ‘resetting’ of baseline mHSP70 following a hypoxic exercise induced increase. In support of this our data show that the variation in repeated resting measurements, made without any form of exercise stressor in between them, was similar to the repeated measurements obtained prior to all 3 HSTs (Table 2). If any residual effect of the previous weeks HST were still present a higher CV would be expected. In addition, we showed a consistent yet more variable (CV ~8 – 10.3%) post exercise increase in mHSP70 of ~25-30% following each trial. However it is noted that our post hypoxic exercise mHSP70 response is lower than the increase observed following a 75 minute period of hypoxic rest in which mHSP70 was assessed using the same method described herein (Taylor et al., 2011; 2012). Furthermore the well reported inverse relationship between basal HSP72 and its induction post stressor (Vince et al., 2010; Taylor et al., 2010, 2011) was evident with the post HST mHSP72 response being inversely related to basal mHSP72 in each HST. This also occurred in a repeatable manner following the 7 day wash-out period.

The mHSP70 data highlight implications that should be considered when designing and implementing experiments that measure the heat shock response. In order to avoid the known influence of basal values on stressor mediated increases in mHSP72, the blood sample collection and scheduling of interventions require careful planning in order to mitigate the
known effects of circadian variation on basal values (Taylor et al., 2009, Vince et al., 2010).

Thus when attempting to manipulate basal mHSP72 in vivo via an environmental stressor, different interventions must be administered at identical times within the experimental design. This ensures that changes in mHSP72 are due to the intervention and not as a result of circadian variation. Therefore care should be taken to schedule all testing sessions at the same time of day for a given participant, and to allow at least 7 days between the end of one experimental trial and the commencement of the next.

In addition to the fixed workload trial we also examined the reliability of a 16.1km TT conducted immediately following a 40-minute, submaximal preload. This type of experimental design allows researchers to assess both steady state responses, and incorporate a measure of physical performance. As the TEM and CV for NORM and HYP are similar this indicates that any familiarisation sessions can be completed in normoxic conditions, thereby reducing the potential for conferred acclamatory effect prior to subsequent hypoxic exposure. It should be noted that, while not trained cyclists, all participants used in the present investigation were not naive to laboratory testing protocols. To avoid further confounds care was taken to remove the influence of diurnal variation, previous activity, and diet, as these may all effect endurance performance (Atkinson et al., 2005; Rauch et al., 2005). Like other time trial procedures, feedback in the form of distance completed was provided (Jeukendrup et al., 1996; Tyler and Sutherland et al., 2008; Biedleman et al., 2014). This increases the external validity of the test and allows participants to use a pacing strategy to a known endpoint (Che Jusoh et al., 2015). One criticism levelled at the use of fixed intensity steady-state preload periods is that by the time participants begin the self-paced performance period they are already near to their physiological limit (Che Jusoh et al., 2015). To minimise this potential confound a workrate of 50% normoxic VO$_2$ max was selected as pilot data indicated this allowed the participants to perform exercise below both the normoxic and hypoxic lactate threshold. At the end of the preload period, HR was 156, 156, and 158 for the HYP group, and 140, 142, and 140 for the NORM group (Figure 4), which would suggest that while this 40 minute period presents a moderate physiological challenge, participants began the time trial with considerable capacity to increase physiological strain, i.e., from 156 beats.min$^{-1}$ to 180 beats.min$^{-1}$ at the end of the TT. The TEM for performance time following one experimental visit (TT1 and TT2) was between 59 – 79 seconds and 68 – 92 seconds for the NORM and HYP time trials respectively. However if one trial is conducted prior to an
experimental trial the TEM is reduced to 34 – 46 seconds (NORM) and 32 – 43 seconds (HYP) indicating that a statistically significant change in performance time induced about by an intervention would need to be > 46 seconds.

Conclusion

Knowledge of the variance in physiological, cellular and performance measures between repeated trials is essential to inform the number of familiarisation trials required prior to exposing participants to an intervention. Without such information confidence that any changes in test measures are due to the manipulation of environmental conditions rather than measurement error and / or inherent high test variability is limited. Such an approach may potentially highlight hitherto identified potential confounders that could potentially influence one or more outcome measures. The current data demonstrate that the reproducibility of the physiological and cellular measurements used in HST trials and reliability of pTT improved between the second and third repeats. Therefore one familiarization visit is recommended prior to employing these protocols in future studies.

Acknowledgements

The authors wish to thank all participants for their time and effort expended completing these trials. We are also grateful to Mr Roy Petticrew and Susie Wilson for their excellent technical assistance throughout data collection.

Conflict of interest

The authors declare no conflicts of interest

References


Kuennen M, Gillum T, Dokladny K et al. (2011) Thermotolerance and heat acclimation may share a common mechanism in humans. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 301:R524-R533


Table 1. Measures of reliability for mean exercise and peak physiological variables during the HST ($n = 6$).

<table>
<thead>
<tr>
<th>Variable</th>
<th>HST1 – HST 2</th>
<th>HST2 – HST 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICC</td>
<td>TEM</td>
</tr>
<tr>
<td>Mean HR (bts min$^{-1}$)</td>
<td>0.99 (0.93 – 0.99)</td>
<td>1.6</td>
</tr>
<tr>
<td>Peak HR (bts min$^{-1}$)</td>
<td>0.98 (0.89 – 0.99)</td>
<td>2.44</td>
</tr>
<tr>
<td>Mean $\text{SpO}_2$</td>
<td>0.95 (0.65 – 0.99)</td>
<td>0.91</td>
</tr>
<tr>
<td>Peak $\text{SpO}_2$</td>
<td>0.96 (0.69 – 0.99)</td>
<td>0.83</td>
</tr>
<tr>
<td>Mean $\dot{V}_E$ BTPS (L min$^{-1}$)</td>
<td>0.75 (0.74 – 0.97)</td>
<td>3.92</td>
</tr>
<tr>
<td>Peak $\dot{V}_E$ BTPS (L min$^{-1}$)</td>
<td>0.64 (-2.7 – 0.95)</td>
<td>6.20</td>
</tr>
<tr>
<td>Mean $\dot{V}_O_2$ (L min$^{-1}$)</td>
<td>0.62 (-3.5 – 0.95)</td>
<td>0.11</td>
</tr>
<tr>
<td>Peak $\dot{V}_O_2$ (L min$^{-1}$)</td>
<td>0.69 (-0.61 – 0.95)</td>
<td>0.17</td>
</tr>
<tr>
<td>Mean $\dot{V}_CO_2$ (L min$^{-1}$)</td>
<td>0.70 (-2.2 – 0.96)</td>
<td>0.12</td>
</tr>
<tr>
<td>Peak $\dot{V}_CO_2$ (L min$^{-1}$)</td>
<td>0.74 (-0.41 – 0.96)</td>
<td>0.09</td>
</tr>
<tr>
<td>Mean BLa (mmol L$^{-1}$)</td>
<td>0.90 (0.30 – 0.99)</td>
<td>0.62</td>
</tr>
<tr>
<td>Peak BLa (mmol L$^{-1}$)</td>
<td>0.75 (-0.29 – 0.96)</td>
<td>0.65</td>
</tr>
</tbody>
</table>
Table 2. Measures of reliability for mHSP70 (MFI A.U) for 5 repeated resting measurements (n = 5), prior to and after each HST (n = 6).

<table>
<thead>
<tr>
<th>Comparison</th>
<th>ICC</th>
<th>TEM</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1: Day 2</td>
<td>0.99 (0.95 – 0.99)</td>
<td>0.18</td>
<td>4.31</td>
</tr>
<tr>
<td>Day 1: Day 3</td>
<td>0.98 (0.87 – 0.99)</td>
<td>0.28</td>
<td>3.58</td>
</tr>
<tr>
<td>Day 1: Day 4</td>
<td>0.98 (0.82 – 0.99)</td>
<td>0.31</td>
<td>4.70</td>
</tr>
<tr>
<td>Day 2: Day 3</td>
<td>0.98 (0.83 – 0.99)</td>
<td>0.30</td>
<td>5.25</td>
</tr>
<tr>
<td>Day 3: Day 4</td>
<td>0.99 (0.97 – 0.99)</td>
<td>0.08</td>
<td>2.53</td>
</tr>
<tr>
<td>HST Rest</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HST1 : HST2</td>
<td>0.98 (0.88 – 0.99)</td>
<td>0.24</td>
<td>5.59</td>
</tr>
<tr>
<td>HST2 : HST3</td>
<td>0.98 (0.82 – 0.99)</td>
<td>0.21</td>
<td>5.25</td>
</tr>
<tr>
<td>HST After exercise</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HST1 : HST2</td>
<td>0.89 (0.09 – 0.99)</td>
<td>0.63</td>
<td>10.3</td>
</tr>
<tr>
<td>HST2 : HST3</td>
<td>0.87 (0.07 – 0.98)</td>
<td>0.62</td>
<td>10.0</td>
</tr>
</tbody>
</table>
Table 3. Measures of reliability in normoxic (n = 6) and hypoxic (n = 6) conditions during the time trial. Data are calculated from the mean TT power output (Watts) and heart rate.

<table>
<thead>
<tr>
<th></th>
<th>TT1 – TT2</th>
<th></th>
<th>TT2 – TT3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICC</td>
<td>TEM</td>
<td>CV</td>
<td>ICC</td>
</tr>
<tr>
<td><strong>Normoxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>0.79 (-0.52 – 0.97)</td>
<td>0.66</td>
<td>0.46</td>
<td>0.82 (0.05 – 0.99)</td>
</tr>
<tr>
<td>Watts</td>
<td>0.91 (0.35 – 0.99)</td>
<td>5.01</td>
<td>0.45</td>
<td>0.92 (0.41 – 0.99)</td>
</tr>
<tr>
<td>HR</td>
<td>0.94 (0.59 – 0.99)</td>
<td>3.0</td>
<td>1.56</td>
<td>0.97 (0.79 – 0.99)</td>
</tr>
<tr>
<td><strong>Hypoxia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>0.98 (0.86 – 0.99)</td>
<td>0.77</td>
<td>1.53</td>
<td>0.99 (0.97 – 0.99)</td>
</tr>
<tr>
<td>Watts</td>
<td>0.86 (-0.19 – 0.98)</td>
<td>2.30</td>
<td>2.30</td>
<td>0.79 (-0.65 – 0.97)</td>
</tr>
<tr>
<td>HR</td>
<td>0.92 (0.47 – 0.99)</td>
<td>3.0</td>
<td>0.43</td>
<td>0.98 (0.86 – 0.99)</td>
</tr>
</tbody>
</table>
Figure 1. A typical flow cytometry profile showing a) forward side scatter (FSC-H/SSC-H) with gated monocytes and b) florescence intensity (FL1-H nm; FITC stained samples on a linear scale) of monocytes incubated with isotype matched negative controls (black line) and anti-HSP70 (green line) antibodies.
Figure 2. Mean ± SD heart rate and SpO₂ (a), $\dot{V}_E$ (b), $\dot{V}_O_2$ (c), and $\dot{V}CO_2$ (d) during the 3 HST’s (n = 6). No differences were observed across trials (p > 0.05).
Figure 3. Repeat measurements of Monocyte HSP70 (n = 5) without (a) and with (b; n = 5) a HST in the previous 7 days. Panel c illustrates the reliable inverse response after exercise mHSP70 has with resting mHSP70. Lines show individual data and the bars represent the group mean.
Figure 4. Mean ± SD performance time, average power output at each km, and average HR for each km in both the NORM (n = 6, panel a, b, c) and HYP (n = 6, panel d, e, f) throughout the 16.1 km time trial.