1   **Title:** Reproducibility of cardiorespiratory and cellular responses to steady state exercise in

2   hypoxia and preloaded cycling time trial performance reliability in normoxia and acute

3   hypoxia.

4   **Running title:** Reproducibility of physiological performance and cellular measures during a

5   hypoxic exercise task.

6   **Key Words:** Cycling, normobaric hypoxia, reliability

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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 16.1km 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 $\dot{V}O_2$peak $= 3.50 \pm 0.60$ L.min^{-1}) were divided into three groups. Reliability of responses (HR, S_PO_2, $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}_E$ and RER) to the HST (F_IO_2 0.14; 15 minutes rest, 60 minutes cycling at 50% normoxic $\dot{V}O_2$peak) 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 $\dot{V}O_2$peak) was assessed across 3 repeat normoxic (N; F_IO_2 ≈ 0.21; n=6) and 3 repeat hypoxic ($F_IO_2 \approx 0.14$; 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, SpO$_2$, $\dot{V}_E$, $\dot{V}O_2$, $\dot{V}CO_2$ 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

62   preparation and management of exposed individuals and groups. Therefore it is important to

63   consider the utility of tests used to measure the magnitude of response to defined

64   environmental stressors. Accordingly a reasonable degree of confidence that the changes in

65   test measures are due to manipulation of environmental conditions in an acute setting or

66   chronic acclimation and/or training adaptation with repeat or continuous exposure rather than

67   measurement error (Atkinson and Nevill, 1998) or inherent high-test variability (Che Jusoh et

68   al., 2015) is important.

69   Fixed workload protocols are often used to provide 'steady-state' data in response to a

70   stimulus e.g. the hypoxic stress test used by Lee and colleagues (2015) to evaluate the impact

71   of short term heat acclimation on responses to exercise in hypoxia. It is generally assumed

72   that such protocols yield reproducible physiological data under defined conditions although

73   the test-retest variation in these measures is seldom reported. In addition to physiological

74   markers, aspects of the cellular response to stressors are also now often examined. For

75   example it has recently been demonstrated that steady state fixed workload exercise in both

76   acute heat and acute hypoxia increases the stress responsive monocyte heat shock protein 70

77   (mHSP70, Lee et al., 2014; Lee et al., 2015) concentration and that acclimation to both heat

78   and hypoxia elevates basal mHSP70 (McClung et al., 2008; Taylor et al., 2012; Lee et al.,

79   2015). Many studies have used flow cytometry to quantify HSP70 in different peripheral

80   blood mononuclear cell (PBMC) populations, such as monocytes (Taylor et al., 2010, 2011,

81   2012; Vince et al., 2011; Peart et al., 2013 Lee et al., 2014, 2014a). This technique was

82   shown to be a rapid, easy and sensitive technique for intracellular HSP70 quantification

83   (Bachelet et al., 1998). However, despite its wide spread use (Taylor et al., 2010; 2012; Lee

84   et al., 2014; 2015), no study has reported data on the test-retest reproducibility of HSP70

85   following environmental stress.

86   In order to provide an indicator of performance potential steady-state fixed workload

87   protocols may be extended by continuing the exercise bout until exhaustion. However this

88   approach often suffers from high test – retest variability and low ecological validity. For

89   example, trained cyclists had a test-retest coefficient of variation (CV) of ~27% when cycling

90   to exhaustion at 75% of maximal workload (W_{max}; Jeukendrup et al., 1996). Whereas, a

91   performance test (a self-paced 60-minute bout of cycling), conducted with the same

92   participants, returned a test-retest CV of 3.4% (Jeukendrup et al., 1996). This is typical of

93   self-paced tests with a defined end-point (i.e. time trials) that by definition provide more

94   ecologically valid data (Mee et al., 2015; Che Jusoh et al., 2015). Furthermore under heat

95   stress a comparable CV (3.6%) was reported by Che Jusoh and colleguess (2015) following a

96   45 minute cycling preload (cycling at 55% $\dot{V}O_2$peak). A similar performance test has been

97   used in hypoxic conditions (Beidleman et al., 2007) although no reliability data was

98   presented for the hypoxic performance test variables *per se*. As hypoxic tolerance before and

99   after an intervention is now often studied, it would be useful to determine the reproducibility

100   of a simple fixed-work protocol hypoxic stress test (HST) for use in physiological steady-

101   state conditions as well as the reliability of a preloaded self-paced cycling time trial cycling

102   test. Therefore, the purpose of this study was to assess the reproducibility of both

103   physiological and cellular mHSP70 responses to a HST (60 minute fixed load period of

104   cycling) and the reliability of a preloaded (40 minute bout of fixed-intensity cycling) 16.1km

105   TT conducted in moderate normobaric hypoxia.

106

107   **Methods**

108   *Participants*

109   The study received ethical approval from the Coventry University local Ethics Committee

110   (REF P2566/P6420) and was conducted in accordance with the declaration of Helsinki.

111   Eighteen healthy and regularly active males accustomed to cycling exercise provided their

112   informed consent to participate. Three groups of 6 participants were formed. Reproducibility

113   of physiological and cellular (HSP72) measurements to a fixed relative work-rate hypoxic

114   stress test (HST) where made on the HST group (age, 23 ± 3 years; height, 1.77 ± 0.05 m;

115   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

116   preloaded time trial (pTT) performance test was evaluated using separate groups under

117   normoxic ($F_IO_2 \approx 0.21$) conditions (NORM; age, 21 ± 2 years; height, 1.77 ± 0.04 m; body

118   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

119   (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 ±

120   0.60 L.min^{-1}).

121   *Experimental design*

122   Each participant visited the laboratory on 4 occasions (1 preliminary and 3 experimental)

123   each separated by at least 7 days (range 7 − 11 days). During the preliminary visit participants

124   gave informed consent and measures of height, body mass, skin folds (4 sites), normoxic

125   lactate threshold and $\dot{V}O_2$peak were made. Thereafter, on separate visits, participants in the

126   HST group completed 3 fixed work-rate (50% normoxic $\dot{V}O_2$peak, 60 minutes) exercise trials

127   whilst breathing hypoxia ($F_IO_2 \approx 0.14$) , and participants in NORM and HYP completed 3

128   preloaded time-trials whilst breathing $F_IO_2 \approx 0.21$ or 0.14, respectively.

129   *Preliminary testing*

130   Anthropometric data were collected in accordance with the International Society for the

131   Advancement of Kinathroprometry (ISAK) guidelines (Marfell-Jones et al., 2006). Lactate

132   threshold and peak oxygen consumption ($\dot{V}O_2$peak) were determined via an incremental

133   exercise test to volitional exhaustion on calibrated SRM cycle ergometer (Schoberer Rad

134   Meßtechnik, Welldorf, Germany). Resting blood lactate (Biosen C-Line analyser, EKF

135   Diagnostics, Germany) was determined from a finger capillary whole blood sample following

136   a 10 minute seated rest period. The test began at a workload of 70W for 4-minutes and was

137   then increased by 35W every 4 minutes until a blood lactate value of > 4mmol.L^{-1} was

138   reached. Thereafter, workload increased 35W every 2 minutes until volitional exhaustion. A

139   cadence of 70 revolutions per minute (RPM) was maintained throughout. Expired gas was

140   collected during the last minute of each exercise stage via a two-way nonrebreathable valve

141   (Harvard Ltd, Eldenbridge, UK) and 1.5 m of 30mm diameter polyvinyl tubing supplying a

142   200L Douglas bag (Cranlea & Co, Birmingham, UK). Heart rate (Polar FT1, Polar Electro

143   OY, Kempele, Finland) was recorded and perceived exertion (Borg, 1976) sought within the

144   last 10 seconds of each gas collection. Respiratory gas analysis was completed as previously

145   described (Lee et al., 2014, Lee et al., 2015). $\dot{V}O_2$peak was considered to be achieved if two

146   of the following criteria were met: i) a respiratory exchange ratio of >1.1, ii) a heart rate

147   greater than 95% of age predicted maximum (220−age) and iii) a final blood lactate value in

148   excess of 8 mmol.mL^{-1}.

149   *Experimental trials*

150   Laboratory attendance time was consistent for each participant in order to minimize the

151   effects of circadian variation on performance and the known diurnal variation in performance

152   (Drust et al., 2005) and HSP70 (Taylor et al., 2009; Vince et al., 2010). Participants were

153   requested to abstain from caffeine (Luo et al., 2008) and alcohol consumption for 72 h prior

154   to each laboratory visit and required to maintain a food and activity diary as accurately as

155   possible for 3 days prior to each experimental visit (Morton et al., 2006). Participants

156   refrained from all supplementation (i.e. vitamins, ergogenic aids) throughout the study period

157   and were requested to abstain from prolonged thermal exposures (baths, saunas, steam rooms,

158   and tanning devices) and vigorous physical activity for seven days prior to each laboratory

159   visit. Persons whom had visited or resided at altitudes in excess of 1000 m (Taylor et al.,

160   2010) or climates with ambient temperatures in excess of 30°C (Sandstrom et al., 2009;

161   Selkirk et al., 2009; Lee et al., 2014) or had experienced high pressure environments, for

162   example, hyperbaria (Taylor et al., 2013), within the three months prior to study

163   commencement were excluded during recruitment due to the possible influence of such

164   environments on basal HSP70 expression.

165   Participants adhered to an overnight fast (Febbraio et al., 2002) and consumed 500 ml of

166   plain water one hour before visiting the laboratory in accordance with the American College

167   of Sports Medicine position stance on hydration (Sawka *et al*., 2007). Upon arrival (0630 –

168   0730 hrs), participants voided their bladder to provide a sample for hydration assessment via

169   urine specific gravity (USG; Atago Refractomer, Jencons Pls, Leighton Buzzard, UK) and

170   urine osmolarity (U_{osmo}; Advanced 3300 Micro-Osmometer, Advanced Inc, Massachusetts,

171   USA). Euhydration was assumed for urine specific gravity values of ≤ 1.020 g·ml^{-1} and

172   osmolarity values of ≤ 700 mOsm·kg^{-1} (Armstrong et al., 1994). This control was not violated

173   by any participant during any trial. Following this, participants measured their own nude

174   body mass (Seca 880, Seca, Hamburg, Germany), inserted a calibrated rectal thermistor probe

175   (Grant Squirrel 2020, Grant Instruments, Shepreth, UK) 10 cm past the anal sphincter, and

176   fitted a telemetric heart rate monitor around their chest (Polar FT1, Polar Electro OY,

177   Kempele, Finland).

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

179   Participants in the HST group completed 15 minutes of seated rest in normoxic conditions

180   prior to a resting venous blood sample being drawn into an EDTA vacutainer (n = 5;

181   Vacuette®, Greiner Bio-one, UK) for quantification of mHSP70. A 15 minute resting 'wash-

182   in' period of breathing hypoxic gas ($F_IO_2 = 0.14$) was then completed. The gas was delivered

183   to the participant via a mouthpiece and 30 mm diameter connector (Harvard Ltd,

184   Eldenbridge, UK) connected to a two-way non-rebreathable valve (Harvard Ltd, Eldenbridge,

185   UK). Ethylene clear vinyl tubing was used to connect the inspiratory side of the valve to a

186   series of 1000L Douglas bags which were filled via a hypoxicator (Hypoxico HYP123

187   Hypoxicator, New York, USA). At the end of the wash-in period participants began 60

188   minutes of cycling exercise at an intensity corresponding to 50% normoxic $\dot{V}O_2peak$ (145 ±

189   16 Watts) on an SRM ergometer (Schoberer Rad Meßtechnik, Welldorf, Germany) at a

190   cadence of 70 RPM. Measures of heart rate (HR), arterial heamoglobin oxygen saturation

191   (SpO_2), respiratory variables ($\dot{V}_E$, $\dot{V}O_2$, $\dot{V}CO_2$) via Douglas bag collections, and fingertip

192   capillary blood samples for determination of glucose and lactate concentrations were taken at

193   the start and end of each 15 minute resting period and every 10 minutes throughout exercise.

194   Upon termination of the exercise bout a further 7mL venous blood sample was collected to

195   assay for post exercise mHSP70 concentrations.

Preloaded time trial (pTT)

197   Participants undertook 30 min of seated rest (NORM $F_IO_2 = 0.21$ throughout; HYP 15 min

198    $F_IO_2 = 0.21$ followed by 15 min $F_IO_2 = 0.14$ 'wash in' period) prior to undertaking a 40

199   minute period of cycling exercise at an intensity corresponding to 50% normoxic $\dot{V}O_2$peak

200   (144 ± 18 Watts) whilst breathing either $F_IO_2 = 0.21$ (NORM) or $F_IO_2 = 0.14$ (HYP).

201   Physiological variables were collected every 10 minutes as previously described. At the end

202   of the 40 minute fixed load exercise bout and after5 min of passive recovery the self-paced

203   16.1km cycling pTT was commenced. The pTT was controlled within the SRM ergometers

204   open-ended mode, which creates a braking force that has a cubic relationship with speed,

205   mimicking the effect of air resistance on a moving bicycle. Participants were instructed to

206   complete distance as quickly as possible. The only feedback available to participants was the

207   distance completed at any given time. During the time trial only measures of HR and power

208   output were collected in order to avoid providing any external time cues.

Determination of monocyte HSP70

210   Flow cytomeery was used to quantify intracellular HSP70 in peripheral blood mononuclear

211   cells (PBMC's). Whole blood (100μL) from EDTA tubes was transferred into 2mL of red

212   cell lysing buffer (Erythrolyse, AbD Serotec, UK) and incubated at room temperature for 10

213   minutes. The sample was centrifuged at 500g for 5 minutes and the supernatant discarded.

214   Cells were then washed by 2mL of phosphate buffer saline (PBS) and centrifuged for 5

215   minutes at 500g. Cells obtained following red cell lysis were fixed for 15 minutes by adding

216   100 μL of fixative (Reagent A, Leukoperm, AbD Serotec, UK) at room temperature and then

217   washed with PBS as previously described. Cells were then permeablised (Reaged B,

218   Leukoperm, AbD Serotc, UK) and a IgG1 negative control (4 μL; FITC, AbD Serotec, UK)

219   or an anti-HSP70 antibody (4 μL; SPA-810, Assay Designs, USA) was added to a final

220   concentration of 100 μg/mL to label 1×10^6 cells according to manufactures instructions. The

221   stained cells were then incubated in the dark for the 30 minutes and washed with PBS.

222 Samples were analysed on a BDFACScaliber (BD Bioscience, UK) with monocytes gated by

223 forward/side scatter properties and further discriminated by CD14 expression. Mean

224 flouresence intensity (MFI) was calculated using CELLQuest software (BD Biosciences, UK)

225 with a total of 15,000 cells counted. Figure 1 illustrates a typical forward side scatter plot

226 showing different cell types, and a frequency histogram for cells stained with the negative or

227 HSP70 positive antibody.

228 *Statistical analysis*

229 Data are reported as mean ± SD unless otherwise stated. Physiological data were analysed

230 using trial x time ANOVA, with repeated measures over time. Between-day resting, and

231 between-trial resting mHSP70 was analysed via a one-way repeated measures ANOVA. In

232 order to assess test-retest reproducibility in the mean physiological and cellular measures

233 obtained at rest and during exercise throughout HST, NORM and HYP, the mean difference

234 between trials (change in mean), intra-class correlation coefficient (ICC) and coefficient of

235 variation (CV) was calculated between the 3 repeat conditions in each group (Hopkins,

236 2000). The technical error of measurement between trials was calculated for all participants

237 by calculating the difference scores and standard deviation of difference scores between trials

238 and dividing the square root of the standard deviation of the difference by 2 (Hopkins, 2000).

239 **Results**

240 All participants adhered here to the activity and nutrition controls specified in the participant

241 information sheet prior to each testing session.

242 *Physiological responses to the HST*

243 The cardiorespiratory responses to each HST can be seen in Figure 2. Based on participants

244 mean oxygen consumption and mean exercising heart rate, participants were exercising at an

245 intensity eliciting 62 ± 11, 58 ± 9 and $61 \pm 16\%$ $\dot{V}O_2$peak at the end of the HST. No main

246 effect for trial (p > 0.05) was observed for heart rate, S_PO_2, $\dot{V}_E$, $\dot{V}O_2$, $\dot{V}CO_2$, and RER over

247 the course of the 3 trials. The technical error of measurement, ICC and coefficient of

248 variation for mean exercise measurements are shown in Table 1. Heart rate increased from 62

249 ± 7, 63 ± 8, and 66 ± 9 beats.min^{-1} at rest to 153 ± 12, 154 ± 14, and 152 ± 17 beats.min^{-1} at

250 the end of the 40 minute exercise period for TT1, TT2 and TT3 respectively. Arterial oxygen

251 saturation decreased from 98 ± 1, 98 ± 0.5, and $98 \pm 0.5\%$ at rest to 89 ± 2, 90 ± 2, and $90 \pm$

252 4% at the end of rest and 79 ± 4, 79 ± 3, and $80 \pm 2\%$ at the end of exercise. Oxygen

253 consumption increased from 0.32 ± 0.1, 0.36 ± 0.1 and $0.33 \pm 0.1 L.min^{-1}$ at the end of rest to

254 2.11 ± 0.26, 1.98 ± 0.22, and 2.06 ± 0.24 $L.min^{-1}$ at the end of the exercise period.

255 *Monocyte HSP70* Five participants from the HST group attended the laboratory on 4 separate

256 occasions at 10:00am to provide a resting blood sample in the 2 weeks prior to the exercise

257 testing. There was no main effect for time ($f = 0.18$, $p = 0.945$) for mHSP70. Table 2 shows

258 the technical error of measurement, ICC and CV between each time point.

259 Resting mHSP70 was also consistent between HST trials 1 - 3 (T1, 3.85 ± 1.47 AU; T2, 3.75

260 ± 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

261 HST1 and HSTT2, HSTl and HST3 and HST2 and HST3 respectively. The post HST

262 response of mHSP70 was also consistent between TT1, TT2, and TT3, increasing post

263 exercise by $31 \pm 12\%$, $29 \pm 13\%$ and $25 \pm 13\%$ after T1, T2 and T3 ($p < 0.05$, Figure 3),

264 although post exercise mHSP70 in HST3 was lower than after HST1 ($p < 0.05$). The post

265 HST mHSP72 response was shown to be inversely related to basal mHSP72 in each HST

266 (Figure; HST1, $R^2 = 0.85$; HST2, $R^2 = 0.86$; HST3, $R^2 = 0.71$).

267 *Preloaded exercise – physiological responses*

268 Based on participants mean $\dot{V}O_2$ and HR participants in the NORM group were exercising at

269 an intensity eliciting $51.1 \pm 8\%$, 51.3 ± 11 and $52 \pm 10\%$ $\dot{V}O_2peak$, and participants in the

270 HYP group at $67 \pm$, 65 ± 9 and $65 \pm \%$ $\pm$ for T1, T2, and T3 respectively. Cardiorespiratory

271 responses are shown in Figure 4. Main effects for time ($p < 0.0001$) but not trial ($p > 0.05$) or

272 interaction ($p > 0.05$) were observed for HR, SpO_2, $\dot{V}O_2$, $\dot{V}_E$ and RER during the 40 minute

273 preload. HR was unchanged during the rest period in NORM (TT1, 72 ± 8 to 75 ± 14; TT2,

274 73 ± 10 to 76 ± 4; TT3, 73 ± 9 to 78 ± 15 beats.min^{-1}) and increased to 140 ± 12, 142 ± 7

275 and 140 ± 10 beats.min^{-1} at the end of the 40 minute preload. HR increased from 66 ± 5, $67 \pm$

276 5, and 76 ± 9 beats.min^{-1} at the beginning of rest to 78 ± 8, 78 ± 9 and 86 ± 10 beats.min^{-1} at

277 the end of the rest period ($p < 0.05$), which corresponded to the decrease in SpO_2 at the end of

278 the rest period (98 ± 1 to $90 \pm 3\%$ across all trials, $p < 0.001$). Heart rate was 156 ± 6, $156 \pm$

279 9, and 158 ± 4 beats.min^{-1} at the end of the 40 minute exercise period in HYP, for TT1, TT2

280 and TT3 respectively, which was greater than that in NORM ($p < 0.05$).

281 *Time trial performance*

282 Time to complete the 16.1km course was longer in HYP compared to NORM during TT1

283 (42.3 ± 5.5 versus 39.4 ± 1.1 minutes), TT2 (41.4 ± 5.5 versus 39.1 ± 1.5 minutes) and TT3

284   (41.8 ± 5.3 versus 38.8 ± 0.6 minutes), although no TT x condition effect was observed ($F_{(2,}$

285    $_{20)}$ 1.36, p = 0.250). The typical error for completion time, mean power output and mean HR

286   during the 3 time trials are presented in Table 2. Based on mean power output during each

287   TT, the NORM group completed the distance at 63 ± 5%, 63 ± 6% and 66 ± 9% and the HYP

288   group at 60 ± 6%, 62 ± 6% and 61 ± 6%% of normoxic W_{max} for TT1, TT2 and TT3

289   respectively.

290   The TEM for completion time between TT1 and TT2 was between 59 and 79 seconds in

291   NORM, and 68 – 92 seconds in HYP. The TEM between TT2 and TT3 was 34 – 46 seconds

292   and 32 – 43 seconds for NORM and HYP respectively, with a CV of 0.6%. The smallest

293   worthwhile change in TT performance is > 46 seconds.

294

295   **Discussion**

296   This study reports that physiological and cellular (mHSP70) responses to a 60 minute steady-

297   state HST are reproducible and that preloaded (40 minutes of steady state cycling) 16.1km

298   TT performance, conducted under both normoxic and acute normobaric hypoxic conditions

299   ($FiO_2 \approx 0.14$), is reliable. Importantly these data demonstrate that the reproducibility of the

300   measurements in HST trials and reliability of pTT performance (under both N and H

301   conditions) improved between the second and third trials. Accordingly when using such

302   protocols in future investigations we would advise conducting at least one familiarisation trial

303   were participants carryout the full protocol under the proposed experimental conditions.

304   Furthermore an awareness of the typical variance of each of these tests under the conditions

305   studied can help provide context to the effect of an intervention on defined outcome

306   measures.

307   During each HST trial classic markers of hypoxic physiology (HR, SpO_2, $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}_E$,

308   RER and blood lactate) were measured. Our data indicate that this simple to conduct fixed-

309   workload test had good agreement for all the physiological variables studied (Table 1) when

310   tests are conducted one week apart, supporting its use in future studies investigating hypoxic

311   tolerance. Typically, a correlation coefficient of over 0.90 is considered high, 0.70-0.80

312   moderate, and below 0.70 considered too low for a reliable and sensitive physiological test

313   (Vincent, 1995). In the present investigation the ICC for each physiological variable was over

314   0.80 when HST2 and HST3 were considered. Based on this observation, the whole body

315   physiological measurements of HR, SpO_2, $\dot{V}O_2$, $\dot{V}CO_2$, RER and $\dot{V}_E$ all have an acceptable

316   level of reproducibility between repeated trials in the conditions studied if one familiarisation

317   visit is conducted. With reference to the TEM data we suggest that changes in HR, SpO$_2$, $\dot{V}_E$

318   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

319   statistically meaningful change in these variables between HST tests conducted before and

320   after an intervention (e.g. an acclimation period).

321   The reproducibility of a commonly used marker of cellular stress mHSP70 was assessed at

322   rest and following exercise in 5 of the HST participants (Figure 1). Our data show that there

323   is a considerable inter-subject variation in resting mHSP70. However within-subject

324   reproducibility is shown to be high both at rest, and following the HST. Based on these

325   observations we suggest that researchers presenting mHSP70 data (or indeed any continuous

326   data variable) should present data in a way that allows for a visual analysis of data

327   distribution (Weissgerber et al., 2015; Figure 3). In small sample size studies such as this one,

328   and others (Taylor et al., 2010, 2011, 2012), a more appropriate data presentation can allow

329   readers to assess the distribution of the data set visually (Weissgerber et al., 2015). The low

330   CV ($< 6\%$) and high ICC ($> 90\%$) for resting mHSP70 suggests that this measure is sensitive

331   to changes in resting data. It also indicates that a washout period of 7 days allows for the

332   'resetting' of baseline mHSP70 following a hypoxic exercise induced increase. In support of

333   this our data show that the variation in repeated resting measurements, made without any

334   form of exercise stressor in between them, was similar to the repeated measurements obtained

335   prior to all 3 HSTs (Table 2). If any residual effect of the previous weeks HST were still

336   present a higher CV would be expected. In addition, we showed a consistent yet more

337   variable (CV ~8 – 10.3%) post exercise increase in mHSP70 of ~25-30% following each

338   trial. However it is noted that our post hypoxic exercise mHSP70 response is lower than the

339   increase observed following a 75 minute period of hypoxic rest in which mHSP70 was

340   assessed using the same method described herein (Taylor et al., 2011; 2012). Furthermore the

341   well reported inverse relationship between basal HSP72 and its induction post stressor (Vince

342   et al., 2010; Taylor et al., 2010, 2011) was evident with the post HST mHSP72 response

343   being inversely related to basal mHSP72 in each HST. This also occurred in a repeatable

344   manner following the 7 day wash-out period.

345   The mHSP70 data highlight implications that should be considered when designing and

346   implementing experiments that measure the heat shock response. In order to avoid the known

347   influence of basal values on stressor mediated increases in mHSP72, the blood sample

348   collection and scheduling of interventions require careful planning in order to mitigate the

349 known effects of circadian variation on basal values (Taylor et al., 2009, Vince et al., 2010).

350 Thus when attempting to manipulate basal mHSP72 *in vivo* via an environmental stressor,

351 different interventions must be administered at identical times within the experimental

352 design. This ensures that changes in mHSP72 are due to the intervention and not as a result of

353 circadian variation. Therefore care should be taken to schedule all testing sessions at the same

354 time of day for a given participant, and to allow at least 7 days between the end of one

355 experimental trial and the commencement of the next.

356

357 In addition to the fixed workload trial we also examined the reliability of a 16.1km TT

358 conducted immediately following a 40-minute, submaximal preload. This type of

359 experimental design allows researchers to assess both steady state responses, and incorporate

360 a measure of physical performance. As the TEM and CV for NORM and HYP are similar this

361 indicates that any familiarisation sessions can be completed in normoxic conditions, thereby

362 reducing the potential for conferred acclamatory effect prior to subsequent hypoxic exposure.

363 It should be noted that, while not trained cyclists, all participants used in the present

364 investigation were not naive to laboratory testing protocols. To avoid further confounds care

365 was taken to remove the influence of diurnal variation, previous activity, and diet, as these

366 may all effect endurance performance (Atkinson et al., 2005; Rauch et al., 2005). Like other

367 time trial procedures, feedback in the form of distance completed was provided (Jeukendrup

368 et al., 1996; Tyler and Sutherland et al., 2008; Biedleman et al., 2014). This increases the

369 external validity of the test and allows participants to use a pacing strategy to a known end-

370 point (Che Jusoh et al., 2015). One criticism levelled at the use of fixed intensity steady-state

371 preload periods is that by the time participants begin the self-paced performance period they

372 are already near to their physiological limit (Che Jusoh et al., 2015). To minimise this

373 potential confound a workrate of 50% normoxic $\dot{V}O_2$ max was selected as pilot data indicated

374 this allowed the participants to perform exercise below both the normoxic and hypoxic lactate

375 threshold. At the end of the preload period, HR was 156, 156, and 158 for the HYP group,

376 and 140, 142, and 140 for the NORM group (Figure 4), which would suggest that while this

377 40 minute period presents a moderate physiological challenge, participants began the time

378 trial with considerable capacity to increase physiological strain, i.e., from 156 beats.min^{-1} to

379 180 beats.min^{-1} at the end of the TT. The TEM for performance time following one

380 experimental visit (TT1 and TT2) was between 59 – 79 seconds and 68 – 92 seconds for the

381 NORM and HYP time trials respectively. However if one trial is conducted prior to an

382   experimental trial the TEM is reduced to 34 – 46 seconds (NORM) and 32 – 43 seconds

383   (HYP) indicating that a statistically significant change in performance time induced about by

384   an intervention would need to be > 46 seconds.

385

386   **Conclusion**

387   Knowledge of the variance in physiological, cellular and performance measures between

388   repeated trials is essential to inform the number of familiarisation trials required prior to

389   exposing participants to an intervention. Without such information confidence that any

390   changes in test measures are due to the manipulation of environmental conditions rather than

391   measurement error and / or inherent high test variability is limited. Such an approach may

392   potentially highlight hitherto identified potential confounders that could potentially influence

393   one or more outcome measures. The current data demonstrate that the reproducibility of the

394   physiological and cellular measurements used in HST trials and reliability of pTT improved

395   between the second and third repeats. Therefore one familiarization visit is recommended

396   prior to employing these protocols in future studies.

397

402   **Conflict of interest**

403   The authors declare no conflicts of interest

404

405   **References**

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

|	HST1 – HST 2			HST2 – HST 3		
	ICC	TEM	CV	ICC	TEM	CV
Mean HR (bts·min^{-1})	0.99 (0.93 – 0.99)	1.6	0.91	0.99 (0.96 – 0.99)	1.7	0.90
Peak HR (bts·min^{-1})	0.98 (0.89 – 0.99)	2.44	1.15	0.97 (0.81 – 0.99)	3.92	1.84
Mean SpO$_2$	0.95 (0.65 – 0.99)	0.91	0.81	0.97 (0.73 – 0.99)	0.50	0.61
Peak SpO$_2$	0.96 (0.69 – 0.99)	0.83	1.15	0.83 (0.50 – 0.97)	1.44	1.37
Mean $\dot{V}_E$BTPS (L·min^{-1})	0.75 (0.74 – 0.97)	3.92	4.84	0.82 (0.59 – 0.97)	2.37	2.90
Peak $\dot{V}_E$BTPS (L·min^{-1})	0.64 (-2.7 – 0.95)	6.20	2.23	0.97 (0.83 – 0.99)	1.25	1.53
Mean $\dot{V}O_2$ (L·min^{-1})	0.62 (-3.5 – 0.95)	0.11	4.10	0.85 (-19 – 0.98)	0.16	5.64
Peak $\dot{V}O_2$ (L·min^{-1})	0.69 (-0.61 – 0.95)	0.17	5.75	0.74 (0.72 – 0.95)	0.22	8.06
Mean $\dot{V}CO_2$ (L·min^{-1})	0.70 (-2.2 – 0.96)	0.12	5.65	0.87 (0.15 – 0.98)	0.10	2.41
Peak $\dot{V}CO_2$ (L·min^{-1})	0.74 (-0.41 – 0.96)	0.09	8.61	0.85 (0.33 – 0.97)	0.19	12.04
Mean BLa (mmol^{-1})	0.90 (0.30 – 0.99)	0.62	15.0	0.96 (0.72 – 0.99)	0.39	8.7
Peak BLa (mmol^{-1})	0.75 (-0.29 – 0.96)	0.65	19.2	0.98 (0.85 – 0.99)	0.26	3.04

549   **Table 2.** Measures of reliability for mHSP70 (MFI A.U) for 5 repeated resting measurements

550   (n = 5), prior to and after each HST (n = 6).

Comparison	ICC	TEM	CV
Rest			
Day 1: Day 2	0.99 (0.95 – 0.99)	0.18	4.31
Day 1: Day 3	0.98 (0.87 – 0.99)	0.28	3.58
Day 1: Day 4	0.98 (0.82 – 0.99)	0.31	4.70
Day 2: Day 3	0.98 (0.83 – 0.99)	0.30	5.25
Day 3: Day 4	0.99 (0.97 – 0.99)	0.08	2.53
HST Rest			
HST1 : HST2	0.98 (0.88 – 0.99)	0.24	5.59
HST2 : HST3	0.98 (0.82 – 0.99)	0.21	5.25
HST After exercise			
HST1 : HST2	0.89 (0.09 – 0.99)	0.63	10.3
HST2 : HST3	0.87 (0.07 – 0.98)	0.62	10.0

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564 **Table 3.** Measures of reliability in normoxic (n = 6) and hypoxic (n = 6) conditions during

565 the time trial. Data are calculated from the mean TT power output (Watts) and heart rate.

	TT1 – TT2			TT2 – TT3		
	ICC	TEM	CV	ICC	TEM	CV
Normoxia						
Time	0.79 (-0.52 – 0.97)	0.66	0.46	0.82 (0.05 – 0.99)	0.39	0.62
Watts	0.91 (0.35 – 0.99)	5.01	0.45	0.92 (0.41 – 0.99)	5.7	3.01
HR	0.94 (0.59 – 0.99)	3.0	1.56	0.97 (0.79 – 0.99)	2.0	1.06
Hypoxia						
Time	0.98 (0.86 – 0.99)	0.77	1.53	0.99 (0.97 – 0.99)	0.36	0.63
Watts	0.86 (-0.19 – 0.98)	2.30	2.30	0.79 (-0.65 – 0.97)	6.30	0.71
HR	0.92 (0.47 – 0.99)	3.00	0.43	0.98 (0.86 – 0.99)	3.00	0.71

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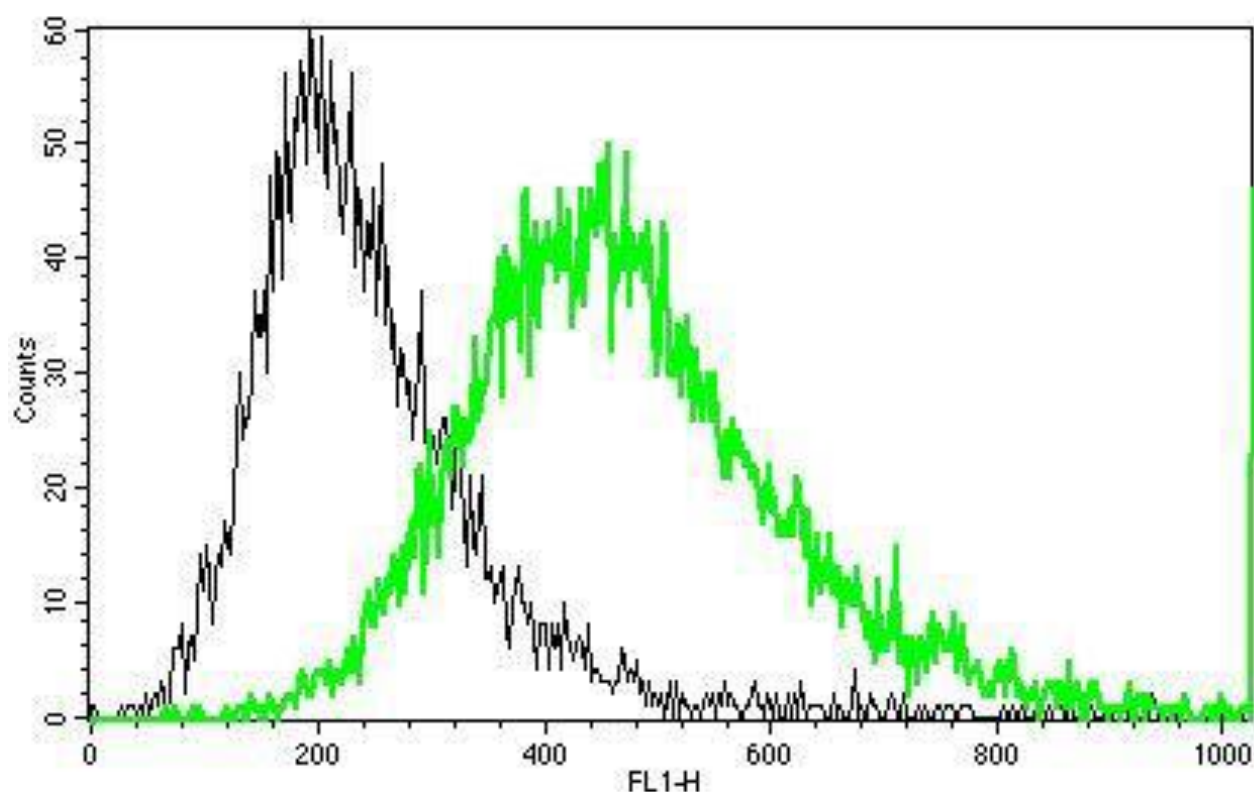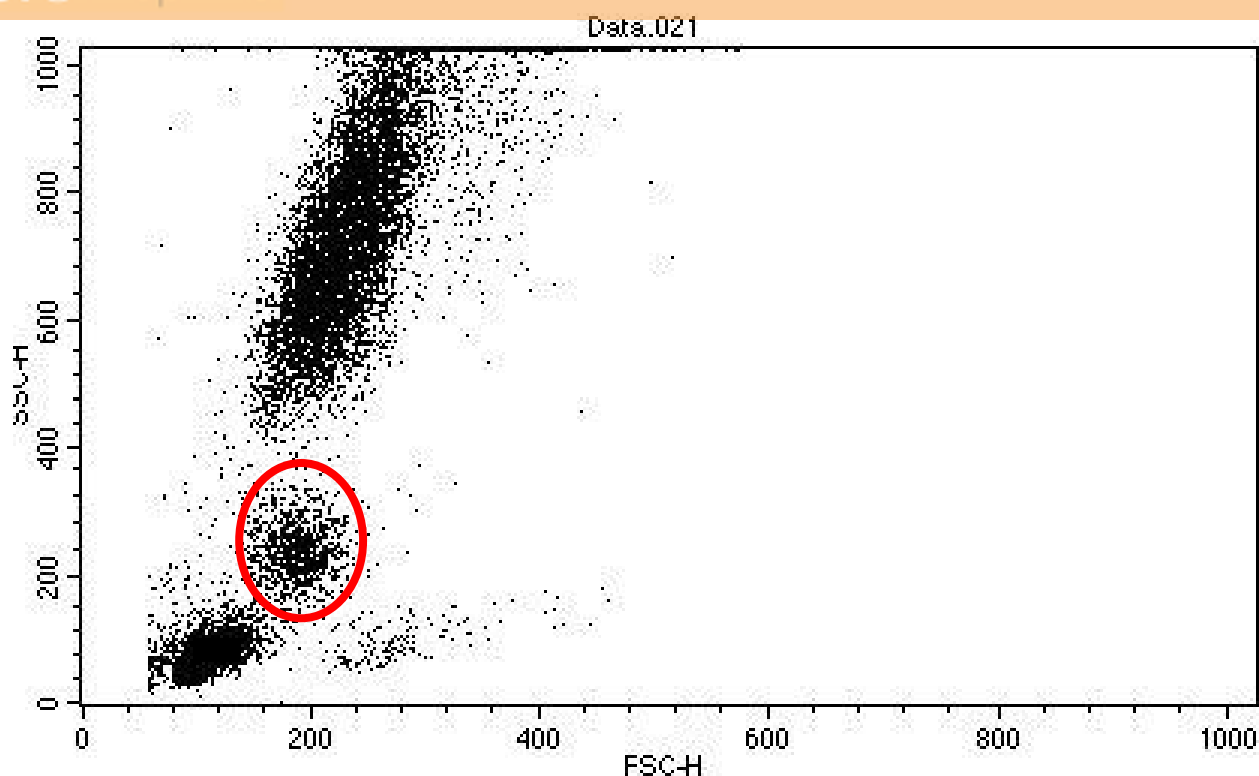
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573 **Figure 1.** A typical flow cytometry profile showing a) forward side scatter (FSC-H/SSC-H)

574 with gated monocytes and b) florescence intensity (FL1-H nm; FITC stained samples on a

575 linear scale) of monocytes incubated with isotype matched negative controls (black line) and

576 anti-HSP70 (green line) antibodies.
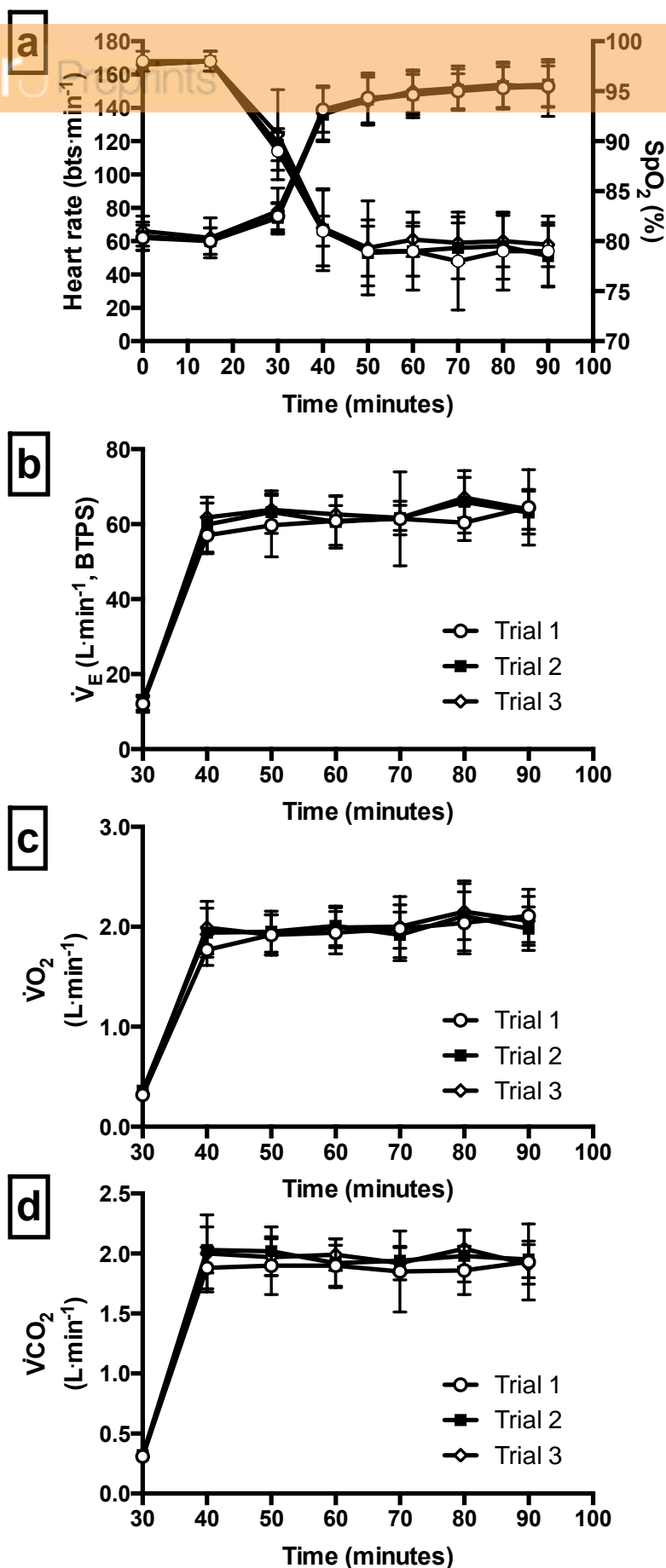
Figure 2. Mean ± SD heart rate and SpO_2 (a), $\dot{V}_E$ (b), $\dot{V}O_2$ (c), and $\dot{V}CO_2$ (d) during the 3 HST's (n = 6). No differences were observed across trials (p > 0.05).
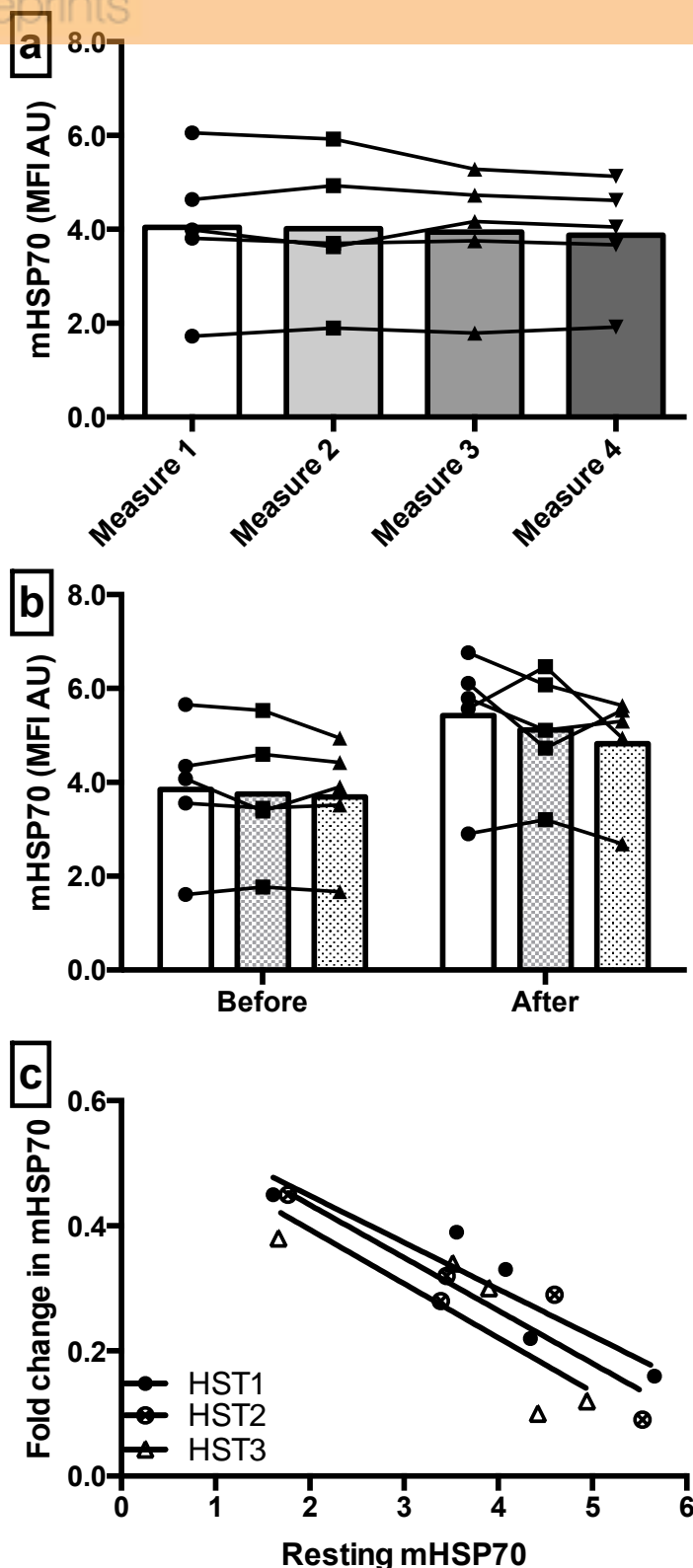
Figure 3. Repeat measurements of Monocyte HSP70 (n = 5) without (a) and with (b; n = 5) a

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.
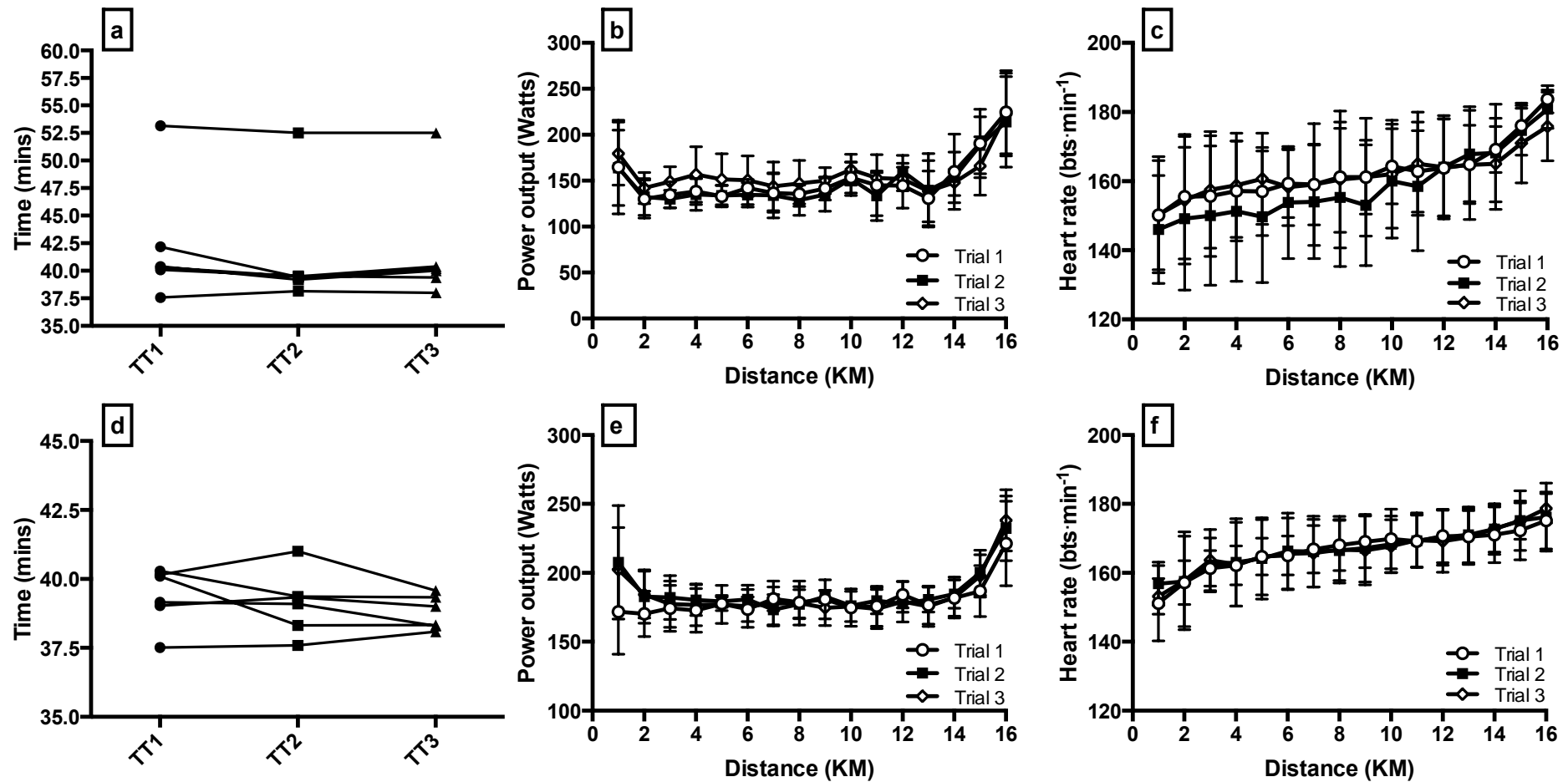
Figure 4. Mean ± SD performance time, average power output at each km, and average HR for each km in both the NORM (n = 6, panel a, b, c) and HYP (n = 6, panel d, e, f) throughout the 16.1 km time trial.