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Skeletal muscle ATP turnover by ³¹P magnetic resonance spectroscopy during moderate and heavy bilateral knee-extension

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35 **Key Points Summary**

- 36 • Heavy-intensity exercise causes a progressive increase in energy demand that
37 contributes to exercise limitation
- 38 • This inefficiency arises within the locomotor muscles and is thought to be due to an
39 increase in ATP cost of power production, – however the responsible mechanism is
40 unresolved
- 41 • We measured these by combined pulmonary gas exchange and muscle magnetic
42 resonance spectroscopy during moderate and heavy exercise in humans
- 43 • Muscle ATP synthesis rate increased throughout constant power heavy exercise, but
44 this increase was unrelated to the progression of whole body inefficiency
- 45 • Our data indicate that increased ATP requirement is not the sole cause of heavy
46 exercise inefficiency and other mechanisms, such as increased O₂ cost of ATP
47 resynthesis, may contribute

48 **Abstract**

49 During constant power high-intensity exercise, the expected increase in oxygen uptake ($\dot{V}O_2$) is
50 supplemented by a $\dot{V}O_2$ slow component ($\dot{V}O_{2sc}$), reflecting reduced work efficiency
51 predominantly within the locomotor muscles. The intracellular source of inefficiency is
52 postulated to be an increase in the ATP cost of power production (an increase in P/W) To test
53 this hypothesis, we measured intramuscular ATP turnover with ^{31}P magnetic resonance
54 spectroscopy (MRS) and whole-body $\dot{V}O_2$ during moderate (MOD) and heavy (HVY) bilateral
55 knee-extension in healthy participants (n=14). Unlocalised ^{31}P spectra were collected from the
56 quadriceps throughout using a dual-tuned (1H and ^{31}P) surface coil with a simple pulse and
57 acquire pulse sequence. Total ATP turnover rate (ATP_{tot}) was estimated at exercise cessation
58 from direct measurements of the dynamics of phosphocreatine (PCr) and proton handling.
59 Between 3 and 8 min during MOD there was no discernable $\dot{V}O_{2sc}$ (mean \pm SD: 0.06 ± 0.12
60 $L \cdot min^{-1}$) or change in [PCr] (30 ± 8 vs. 32 ± 7 mM) or ATP_{tot} (24 ± 14 vs. 17 ± 14 $mM \cdot min^{-1}$; each
61 $p = n.s.$). During HVY the $\dot{V}O_{2sc}$ was 0.37 ± 0.16 $L \cdot min^{-1}$ ($22 \pm 8\%$), [PCr] decreased (19 ± 7 vs.
62 18 ± 7 mM, or $12 \pm 15\%$; $p < 0.05$) and ATP_{tot} increased (38 ± 16 vs. 44 ± 14 $mM \cdot min^{-1}$, or $26 \pm$
63 30% ; $p < 0.05$) between 3 and 8 min. However, the increase in ATP_{tot} (ΔATP_{tot}) was not
64 correlated to the $\dot{V}O_{2sc}$ during HVY ($r^2 = 0.06$; $p = n.s.$). This lack of relationship between ΔATP_{tot}
65 and the $\dot{V}O_{2sc}$, together with a steepening of the [PCr]- $\dot{V}O_2$ relationship in HVY, suggests that
66 reduced work efficiency during heavy exercise arises from both contractile (P/W) and
67 mitochondrial (the O_2 cost of ATP resynthesis; P/O) sources.

68

69 **Abbreviations**

70 A, amplitude; ATP_{tot} , total ATP turnover rate; D , ATP production from PCr breakdown; k , rate
71 constant; L , ATP production from glycogenolysis; LT, lactate threshold; MRS, magnetic
72 resonance spectroscopy; PCr, phosphocreatine; PCr_{sc} , phosphocreatine slow component; pH_i ,
73 intramuscular pH; P_i , inorganic phosphate; P/O, ATP yield per $O \rightarrow H_2O$; P/W, ATP cost per unit
74 work; Q , ATP production from oxidative phosphorylation; RI, ramp incremental exercise; S_pO_2 ,
75 arterial oxygenation; τ , time constant; $V_{i[PCr]}$, initial rate of PCr resynthesis; $\dot{V}O_2$, rate of whole-
76 body O_2 uptake; $\dot{V}O_{2peak}$, peak rate of O_2 uptake; $\dot{V}O_{2sc}$, slow component of O_2 uptake

77 **Introduction**

78 During constant power exercise below the lactate threshold (LT; moderate intensity), the rate of
79 pulmonary oxygen uptake ($\dot{V}O_2$) increases exponentially, reaching a steady-state within 2-3 min.
80 A $\dot{V}O_2$ steady-state indicates that the exercise-related energy transfer is accounted for by
81 oxidative phosphorylation. However, above the LT (heavy intensity), the dynamics of $\dot{V}O_2$
82 become complicated by an additional, slow component ($\dot{V}O_{2sc}$) (Poole *et al.*, 1994). This
83 becomes especially important at power outputs above critical power, where the $\dot{V}O_{2sc}$ will draw
84 $\dot{V}O_2$ inexorably toward its physiologic maximum. In this intensity domain the limit of tolerance is
85 reached rapidly, and the exercise cannot continue unless the power output is reduced below
86 critical power (Coats *et al.*, 2003). Although the $\dot{V}O_{2sc}$ is intimately related to exercise
87 intolerance (Murgatroyd *et al.*, 2011), the aetiology of the $\dot{V}O_{2sc}$ remains poorly understood.

88
89 The $\dot{V}O_{2sc}$ represents an impairment of exercise economy, and is predominantly (~85%) due to
90 increased O_2 consumption in the muscles engaged in the locomotor work (Poole *et al.*, 1991;
91 Rossiter *et al.*, 2002; Krstrup *et al.*, 2009). However, the intracellular source of this inefficiency
92 is uncertain. It has been postulated that the $\dot{V}O_{2sc}$ is related to an increased phosphate cost of
93 force or power production. That is, an increase in the rate of ATP consumption per unit power
94 output (or P/W) is met instantaneously by phosphocreatine (PCr) (via the Lohmann reaction)
95 whose breakdown signals an increase in the rate of oxidative phosphorylation (Rossiter *et al.*,
96 2002). However, distinguishing between this and the alternative hypothesis, that supra-LT
97 exercise is associated with reductions in mitochondrial coupling (Krstrup *et al.*, 2003), i.e. the
98 ratio of the ATP resynthesized per oxygen converted to water (P/O), is technically challenging in
99 humans.

100

101 To test these two hypotheses requires knowledge of dynamic changes in total ATP turnover rate
102 (ATP_{tot}) in concert with power output and $\dot{V}O_2$. Specifically, were the intramuscular source of the
103 $\dot{V}O_2$ slow component to be caused by an increase in PW (in line with current views;(Rossiter,
104 2011; Poole & Jones, 2012)), then the slow component magnitude during heavy exercise would
105 be strongly related to the magnitude of the change in ATP_{tot} . Alternatively, if no proportionality
106 between the $\dot{V}O_2$ slow component and the change in ATP_{tot} were evident, then changes in PW
107 could not be the sole source of the $\dot{V}O_2$ slow component.

108

109 The technical challenge thus becomes, how best to establish ATP_{tot} during heavy intensity
110 exercise that elicits a $\dot{V}O_2$ slow component? One approach uses ^{31}P magnetic resonance
111 spectroscopy (MRS) (Kemp *et al.*, 2001; Layec *et al.*, 2009a) to partition ATP delivery from
112 oxidative phosphorylation, PCr breakdown, and glycogenolysis. ^{31}P MRS provides direct
113 measurement of [PCr], and allows glycogenolytic rate (a relatively minor component of ATP_{tot} in
114 exercise of this kind) to be estimated using reasonable assumptions about muscle H^+ buffering
115 (Kemp *et al.*, 2001; Kemp *et al.*, 2014). Several methods have been proposed to calculate
116 oxidative ATP yield using ^{31}P MRS, but these show poor agreement (Layec *et al.*, 2011).
117 Previous studies to estimate ATP_{tot} during supra-LT exercise have assumed linear $\dot{V}O_2/[PCr]$
118 and a fixed time constant (τ) of PCr breakdown and resynthesis (Meyer, 1988; Walter *et al.*,
119 1999; Lanza *et al.*, 2005; Faraut *et al.*, 2007) or first-order $[ADP]/\dot{V}O_2$ in order to transform [PCr]
120 into a rate of oxidative ATP turnover (Layec *et al.*, 2009a). However, it is clear that the $\dot{V}O_2$ -
121 [PCr] relationship is not linear through the intensity domains (Kemp, 2008; Wust *et al.*, 2011;
122 Kemp *et al.*, 2014), and accordingly τPCr is not invariant across exercise intensities (Yoshida &
123 Watari, 1993, 1994; Rossiter *et al.*, 2002; Jones *et al.*, 2008), making this an unreliable
124 assumption on which to base estimation of ATP_{tot} . Assuming τPCr to be invariant is equivalent
125 to assuming that any change in [PCr] is directly proportional to change in ATP_{tot} ; when τPCr

126 changes across exercise intensity and/or duration, this proportionality is lost (Kemp *et al.*, 2014).
127 These new findings mean that the close coherence between [PCr] and $\dot{V}O_2$ during the slow
128 component phase (Rossiter *et al.*, 2002; Layec *et al.*, 2009a) is no longer sufficient evidence to
129 imply that an increase in P/W alone is the responsible mechanism. Consequently, a direct
130 measurement of oxidative ATP yield during supra-LT exercise, that does not rely on these
131 assumptions, is required to distinguish whether change in P/W is the dominant mechanism for
132 the $\dot{V}O_{2sc}$.

133

134 Oxidative ATP turnover (the dominant proportion of ATP_{tot}) at exercise cessation may be directly
135 measured from the initial rate of post-exercise PCr resynthesis ($V_{i[PCr]}$), easily measured by ^{31}P
136 MRS; the only assumptions required (the evidence for which is reviewed elsewhere (Kemp *et al.*
137 *et al.*, 2014)) are that PCr recovery is driven overwhelmingly by oxidative ATP synthesis, and that
138 any basal component of ATP turnover (i.e. ATP production not available for use by myosin
139 ATPase, SERCA, or Na^+/K^+ ATPase during exercise or PCr resynthesis during recovery) is
140 small and reasonably constant. Therefore, temporal characterisation of oxidative energy yield
141 during dynamic exercise can be made simply by halting the exercise and measuring $V_{i[PCr]}$.
142 Although this method has inherently poor temporal resolution (it is only valid at the instant of
143 exercise cessation), it provides the accuracy necessary to isolate the intracellular source of
144 inefficiency during high intensity exercise. The other, much smaller, components of ATP_{tot} can
145 be estimated at end-exercise by ^{31}P MRS in ways that are relatively robust against uncertainty
146 or changes in the underpinning assumptions.

147

148 Therefore, the purpose of this study was to characterise the rate of ATP turnover during sub-
149 and supra-LT exercise in human quadriceps during bilateral, prone, knee-extension exercise
150 using ^{31}P MRS. The rate of pulmonary oxygen uptake was measured under the same conditions

151 to quantify the $\dot{V}O_2$ slow component. We hypothesised that the close association between the
152 dynamics of the [PCr] and $\dot{V}O_2$ slow components during supra-LT exercise would be reflected in
153 the dynamics of ATP_{tot} (measured independently), thereby confirming the hypothesis that
154 increased P/W during heavy intensity exercise is the predominant mechanism of the $\dot{V}O_2$ slow
155 component.

156

157 **Materials and Methods**

158 *Ethical Approval*

159 The Biological Sciences Faculty Research Ethics Committee, University of Leeds, and the
160 University of Liverpool Committee on Research Ethics approved this study, and all procedures
161 complied with the latest revision of the *Declaration of Helsinki*. Written informed consent was
162 obtained from all volunteers prior to their participation in the study.

163

164 *Participants*

165 Fourteen healthy volunteers (1 female, 13 males) agreed to participate in this study (mean \pm
166 standard deviation (SD): age 27 ± 8 y; height 177 ± 8 cm; mass 75 ± 12 kg; bilateral knee-
167 extension $\dot{V}O_{2peak}$ 2.0 ± 0.5 L.min⁻¹). All participants were undertaking a regular exercise
168 regimen, ranging from recreational fitness to amateur competitive sport. Volunteers were
169 screened for cardiovascular disease risk with a resting ECG and a health history questionnaire.

170

171 *Exercise protocols*

172 All exercise tests were undertaken on an MR-compatible computer-controlled
173 electromagnetically-braked knee-extension ergometer (MRI Ergometer Up/Down, Lode BV,
174 Groningen, NL) customised for use at 3T by the addition of extended carbon fibre lever arms.
175 The participants lay prone with their feet strapped into moulded plastic stirrups, which were

176 attached to carbon-fibre/aluminium arms, linking to the ergometer crank arms. The participants'
177 hips were secured to the patient bed with nylon and Velcro straps in order to isolate power
178 production to the quadriceps and minimise movement from hip flexion/extension. Knee
179 movements were constrained by the scanner bore, allowing for approximately 35° of bilateral
180 knee-extension (Whipp *et al.*, 1999; Cannon *et al.*, 2013). No resistance was applied during
181 knee flexion, other than a constant work required to lift the mass of the lower leg.

182

183 The testing protocol began with a rigorous familiarisation phase that took place in a
184 temperature-controlled laboratory with pulmonary gas exchange measurements. Ramp
185 incremental and constant power protocols were completed until reproducible physiologic
186 measurements were obtained across two consecutive visits for each condition. The second
187 phase of the study took place within the bore of an MR scanner for measurement of muscle
188 phosphates. The same MRI ergometer was used for both phases of the protocol.

189

190 Initially, participants completed a ramp incremental (RI) exercise test to the limit of tolerance.
191 For this, participants lay at rest for ~3-4 min, followed by a low power exercise (5 W) for ~2-4
192 min. The power was then increased as a function of time at 2-5 W.min⁻¹ (the rate of increase
193 was dependent on the volunteer's size and strength) until the limit of tolerance was reached.
194 Ramp rates were adjusted using 'trial and error' to determine a ramp rate that resulted in a ramp
195 protocol of approximately 10-12 min. The frequency of knee-extension was constrained at 90
196 min⁻¹ with the use of a metronome. This cadence was chosen to allow synchronization with the
197 MR scanner acquisitions (1 pulse per 2 knee-extensions), and also acted to ensure that the
198 ergometer flywheel was maintained above its minimum operating speed. The RI was terminated
199 upon the participant being unable to maintain the required cadence, despite strong verbal
200 encouragement. The results of the RI were used to determine the $\dot{V}O_{2peak}$, and to calculate

201 power for subsequent tests. There is a substantial learning effect with the exercise model (large
202 gains in peak power were achieved with consecutive tests) and therefore typically more than 3
203 RI tests were completed by each participant until reproducible performances were achieved.

204

205 A series of constant power exercise tests were then undertaken. These consisted of an 8 min
206 moderate intensity bout, followed by a 6 min rest, and an 8 min heavy intensity exercise bout.
207 During moderate intensity exercise the target power was 80% of estimated LT (~60-70%
208 $\dot{V}O_{2peak}$), and during heavy intensity bouts the target power was half way between estimated LT
209 and $\dot{V}O_{2peak}$. These intensity domains were confirmed *post hoc* from the profile of $\dot{V}O_2$ during
210 constant power bouts (Whipp, 1996). If necessary, power was adjusted in subsequent
211 familiarisation tests to ensure the absence (moderate) or presence (heavy) of the $\dot{V}O_{2sc}$. Once
212 familiarised, participants repeated this protocol 3 times on separate days to combine respired
213 gas exchange data and improve signal:noise.

214

215 During the second phase of experiments, participants completed constant power bouts within
216 the bore of the superconducting magnet for ^{31}P MRS. Two trials of constant power tests were
217 completed in a random order consisting of: 1) 4 min of rest, followed by 3 min of moderate
218 exercise, 6 min rest, 3 min of heavy exercise; and 2) 4 min of rest, followed by 8 min of
219 moderate exercise, 6 min rest, 8 min heavy exercise. Each protocol was preceded by ~10 min
220 of magnet shimming to optimise the MRS signal, and separated by at least 30 min outside of the
221 MR scanner. Therefore approximately 60-90 min elapsed between the two exercise trials.

222

223 *Pulmonary gas exchange*

224 Participants breathed through a low resistance (< 0.1 KPa.L⁻¹.s⁻¹ at 15 L.s⁻¹), low dead space
225 (90 mL) mouthpiece for the measurement of respired gases. Flow rates and volumes were

226 measured with an infrared turbine flow sensor (Interface Associates, Laguna Niguel, CA, USA),
227 while a quadrupole mass spectrometer was used to measure respired gas concentrations after
228 sampling air at $0.5 \text{ mL}\cdot\text{s}^{-1}$ from the mouthpiece (MSX, nSpire Health Ltd, Hertford, UK). Gas
229 concentration signals were time aligned with the flow sensor signal using proprietary software
230 for the calculation of breath-by-breath gas exchange. These algorithms identified the end of
231 each breath with the flow sensor and time aligned the changes in respired gases.

232

233 Prior to each experiment the flow sensor and gas analysers were calibrated according to the
234 manufacturers' instructions. The turbine volume transducers were calibrated with a 3 L syringe
235 (Hans Rudolph Inc., Shawnee, KS, USA). The calibration was completed with flow rates ranging
236 from 0.2 to $6 \text{ L}\cdot\text{s}^{-1}$, mimicking flow rates expected for humans at rest and during exercise. After
237 the completion of the flow sensor calibration, the flow volumes were verified over ten syringe
238 strokes of varying flow rates, and accepted when the means were within $\pm 0.01 \text{ L}$, with a SD and
239 coefficient of variation of 0.02 L and 1% , respectively. Additionally, the mass spectrometer was
240 calibrated with atmospheric air and precision-verified gases with concentrations of O_2 , CO_2 and
241 N_2 spanning the physiologic range. Following each experiment, mass spectrometer calibration
242 factor drift was verified as negligible by sampling the calibration gases.

243

244 *Data analyses for pulmonary measures*

245 Breath-by-breath $\dot{V}\text{O}_2$ was filtered for errant breaths (i.e. values resulting after sighs, swallows,
246 coughs etc., defined as residing outside of 99% prediction limits) (Lamarra *et al.*, 1987).
247 Responses from like transitions were combined to improve signal:noise using an averaging
248 technique that preserves the breath-by-breath density measured during the exercise transition.
249 This method requires time aligning and sorting of all $\dot{V}\text{O}_2$ data from exercise transitions in the
250 time domain. Time and $\dot{V}\text{O}_2$ are then averaged into bins of n breaths, where n is the number of

251 exercise transitions completed (Murgatroyd *et al.*, 2011). The magnitude of the $\dot{V}O_{2sc}$ was
252 expressed as the difference in $\dot{V}O_2$ between 3 min and 8 min of exercise.

253

254 Power output and flywheel speed from the ergometer were sampled continuously and digitised
255 by a data recording system and stored on a PC (PowerLab 8/30 with LabChart Pro,
256 ADInstruments Pty Ltd, Bella Vista, NSW, AU).

257

258 *³¹P magnetic resonance spectroscopy*

259 Muscle phosphorus-containing metabolites were measured with a 3T superconducting magnet
260 (Magnetom Trio, Siemens AG, Erlangen, DE). A one-pulse MRS acquisition was employed
261 using a dual tuned (¹H and ³¹P) 15 cm and 18 cm diameter surface RF coil (RAPID Biomedical
262 GmbH, Rimpar, DE), which was placed under the knee extensors, half way between the hip and
263 knee. The concave RF coil was stabilised with sandbags and was secured to the table once the
264 participants' hips were strapped to the scanner table. A series of axial, sagittal and coronal
265 gradient-recalled echo images of the thigh were acquired to confirm the placement of the RF
266 coil relative to the knee extensor muscles and to prescribe the volume over which shimming
267 was achieved. Subsequently, a standard ¹H shimming protocol was used to optimise the
268 homogeneity of the magnetic field (β_0). A fully relaxed spectrum (repetition time (TR) of 10 s;
269 number of scans = 4) was initially obtained to provide a high-resolution unsaturated resting
270 spectrum along with a 32 scan spectrum with a TR of 2 s. Following this, free induction decays
271 for ³¹P spectra were collected every 2 s with a spectral width of 3200 Hz and 1024 data points
272 throughout the rest-to-exercise-to-rest transitions. ³¹P data were averaged over four acquisitions
273 yielding a datum every 8 s.

274

275 *Kinetic analysis of ³¹P MRS data*

276 Signal intensities, frequencies and line widths of inorganic phosphate (Pi), PCr, γ -ATP, α -ATP,
277 and β -ATP, were determined using Java-based Magnetic Resonance User Interface (jMRUI)
278 (Naressi *et al.*, 2001) in order to transform the raw data into a time series for each of the
279 phosphates of interest. Intramuscular pH (pH_i) was estimated from the chemical shift of Pi
280 (Moon & Richards, 1973):

281

$$282 \text{pH}_i = 6.75 + \log(\bar{\delta} - 3.27/5.69 - \bar{\delta}) \quad \text{Equation 1}$$

283

284 where $\bar{\delta}$ is the chemical shift of the Pi peak, relative to PCr.

285

286 PCr kinetics were modelled using non-linear least squares regression (OriginPro 7.5, OriginLab
287 Corp., Northampton, MA, USA). ³¹P MRS data were filtered for errant values resulting from
288 artefact (Rossiter *et al.*, 2000) prior to characterisation with the following function:

289

$$290 [\text{PCr}]_{(t)} = [\text{PCr}]_0 + A \cdot (1 - e^{-t/\tau}) \quad \text{Equation 2}$$

291

292 where τ is a time constant, $[\text{PCr}]_{(t)}$, $[\text{PCr}]_0$, and A are the time variant form, baseline, and
293 fundamental amplitude, respectively. The fitting window was determined from an iterative
294 process (Rossiter *et al.*, 2001) to ensure the exclusion of phase III (steady state or slow
295 component, depending on the intensity domain). The magnitude of the PCr slow component
296 ($[\text{PCr}]_{\text{sc}}$) was expressed as the difference in $[\text{PCr}]$ between the 3rd and 8th min of exercise.

297

298 ATP_{tot} was estimated from the contributions from oxidative phosphorylation (Q), PCr breakdown
299 (D), and glycogenolysis (L), which were determined from the ³¹P MRS data acquired during

300 exercise and recovery, using methods described elsewhere (Kemp *et al.*, 2001; Kemp *et al.*,
301 2007; Layec *et al.*, 2011; Kemp *et al.*, 2014), and outlined below.

302

303 *ATP production from PCr breakdown (D)*

304 The rate of PCr breakdown by creatine kinase (*D*) yields one component of ATP production (in
305 mM.min⁻¹), and was determined over 32 s (4 spectra) immediately prior to exercise cessation

306

$$307 \quad D = d[\text{PCr}]/dt \quad \text{Equation 3}$$

308

309 In the present experiments, where [PCr] is either close to steady state or changing only slowly
310 by the end of exercise, *D* is a very small component of end-exercise ATP_{tot}.

311

312 *ATP production from oxidative phosphorylation (Q)*

313 The rate of oxidative ATP yield (*Q*) is reflected in the rate of [PCr] recovery at the instant of
314 exercise cessation (*V*_{i[PCr]}), and was calculated (mM.min⁻¹) as:

315

$$316 \quad V_{i[\text{PCr}]} = k \cdot A \quad \text{Equation 4}$$

317

318 where *A* is the amplitude of [PCr] change (in mM). The rate constant (*k*) was estimated by fitting
319 the PCr recovery kinetics with the following function:

320

$$321 \quad [\text{PCr}]_t = [\text{PCr}]_{\text{end}} - A \cdot (1 - e^{-kt}) \quad \text{Equation 5}$$

322

323 where [PCr]_t is the time-dependent variant of [PCr], and [PCr]_{end} is the concentration of PCr
324 measured at the end of exercise. We make the well-evidenced assumption (Kemp *et al.*, 2014)
325 that the rate of suprabasal oxidative synthesis at the start of recovery (*V*_{i[PCr]} from Equation 4) is
326 a good estimate of the suprabasal rate of oxidative synthesis at the end of exercise (*Q*_{end}).

327

328 *ATP production from anaerobic glycolysis (L)*

329 During exercise, glycogenolysis and the resulting lactate and H⁺ production cause disturbances
330 in pH_i. These changes in pH_i are readily measured by ³¹P MRS data and can therefore be used
331 to estimate ATP production from glycogenolysis; 1 mol of H⁺ resulting in 1.5 mol of ATP. This
332 requires estimation of the flux rates: H⁺ production accompanying changes in PCr concentration
333 via the creatine kinase reaction (H⁺_{CK}, which is positive, i.e. H⁺ 'consumption', when [PCr] is
334 falling in exercise, and negative, i.e. H⁺ generation, when [PCr] is rising in recovery), by the
335 buffers of the muscle cytosol (H⁺_β which is positive, i.e. H⁺ 'buffering', when pH_i is falling in
336 exercise, and negative, i.e. H⁺ 'unbuffering', when pH_i is rising in recovery) and proton efflux
337 from the cells (H⁺_{efflux}). Together these sum to the total proton yield (P) during exercise:

338

$$339 \quad P = H_{CK}^{+} + H_{\beta}^{+} + H_{efflux}^{+} \quad \text{Equation 6}$$

340

341 From which

342

$$343 \quad L = 1.5 \times P \quad \text{Equation 7}$$

344

345 The number of protons consumed at the creatine kinase reaction was calculated from the time
346 dependent changes in [PCr] using the proton stoichiometric coefficient γ (Kushmerick, 1997):

347

$$348 \quad H_{CK}^{+} = -\gamma D \quad \text{Equation 8}$$

349

350 Protons buffered (H⁺_β mM.min⁻¹) was calculated from the apparent buffering capacity, β_{total}
351 (mmol acid added per unit change in pH_i) and from the (smoothed) rate of pH change during
352 exercise:

353

354 $H^+_{\beta} = -\beta_{total}(dpH_i/dt)$ Equation 9
355

356 where

357

358 $\beta_{total} = \beta_{non-bicarbonate-non-Pi} + \beta_{Pi}$ Equation 10
359

360 The intrinsic cytosolic buffering capacity $\beta_{non-bicarbonate-non-Pi}$ is calculated from initial-exercise data:

361

362 $\beta_{non-bicarbonate-non-Pi} = \beta_a - \beta_{Pi}$ Equation 11
363

364 where the apparent β (β_a) is obtained from the initial rate of change in [PCr] (ΔPCr_i) and
365 alkalisation of pH (ΔpH_i):

366

367 $\beta_a = \gamma \Delta PCr_i / \Delta pH_i$ Equation 12
368

369 β_{Pi} was calculated as:

370

371 $\beta_x = (2.303 \times H^+ \times K \times [P_i]) / (K + H^+)^2$ Equation 13
372

373 where $K = 1.77 \times 10^{-7}$ (Conley *et al.*, 1998). $\beta_{bicarbonate}$ was neglected, which assumes that
374 muscle is a closed system during short duration exercise *in vivo* (Kemp *et al.*, 1993). Proton
375 efflux (H^+_{efflux} mM.min⁻¹) was estimated for each time point of exercise assuming a linear pH-
376 dependence constant λ :

377

378 $H^+_{efflux} = -\lambda \Delta pH_i$ Equation 14
379

380 This proportionality constant λ (mM.min⁻¹.pH unit⁻¹) was estimated from initial recovery after
381 exercise cessation:

382

383 $\lambda = -V_{\text{eff}}/\Delta\text{pH}_i$ Equation 15
384

385 At the cessation of exercise, the PCr resynthesised at the creatine kinase reaction is essentially
386 solely a consequence of oxidative ATP production (Kemp *et al.*, 2014). Therefore, $\text{H}^+_{\text{efflux}}$ can be
387 calculated from the rate of proton production from creatine kinase (H^+_{CK}) and the rate of pH
388 change on the other side.

389 $V_{\text{eff}} = \beta_{\text{total}} (d\text{pH}_i/dt) = \gamma V_{i,\text{PCr}}$ Equation 16
390

391 Where ΔpH_i is very low, Equation 15 becomes unreliable, and the end-exercise rate of $\text{H}^+_{\text{efflux}}$ is
392 simply assumed to be equal to $\text{H}^+_{\text{efflux}}$ calculated from the initial recovery data by Equation 14.

393 In the present experiments, where pH_i is close to steady state or changing only slowly by the
394 end of exercise, L is a very small component of ATP_{tot} .

395

396 *Statistical analyses*

397 Relationships between variables were assessed with a Pearson correlation coefficient, where
398 appropriate. The difference between ^{31}P measures at discrete time points and across exercise
399 intensities were compared with a two-factor (time \times intensity domain) repeated measures
400 ANOVA. Bonferroni-corrected paired t-tests were used *post hoc* to identify simple effects in the
401 case of a significant interaction. For all tests, $\alpha = 0.05$. Analyses were completed using the
402 Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA).

403

404 **Results**

405 During RI exercise, participants attained a peak power output of 47 ± 11 W at a $\dot{V}\text{O}_{2\text{peak}}$ of 2.00
406 ± 0.48 L.min⁻¹. Based on peak power output and estimated LT ($\sim 60\text{-}70\%$ $\dot{V}\text{O}_{2\text{peak}}$), moderate
407 (sub-LT; 19 ± 4 W) and heavy (supra-LT; 46 ± 11 W) constant power exercise bouts were

408 assigned. The dynamics of $\dot{V}O_2$ were examined *post hoc* to confirm the appropriate intensity
409 assignment (Whipp, 1996; Rossiter, 2011).

410

411 During moderate exercise, there was no discernable pulmonary $\dot{V}O_{2sc}$ (0.06 ± 0.12 L.min⁻¹).
412 However, during heavy exercise the $\dot{V}O_{2sc}$ was 0.37 ± 0.16 L.min⁻¹ (**Figure 1A**), or a 22 ± 8 %
413 increase. [PCr] did not change between 3 and 8 min of moderate intensity exercise (30 ± 8 vs.
414 32 ± 7 mM; $p = n.s.$). Conversely, during heavy exercise [PCr] fell from 3 to 8 min (19 ± 7 vs. 18
415 ± 7 mM or 12 ± 15 % fall; $p < 0.05$; **Figure 1B**).

416

417 ATP yield during moderate and heavy exercise from oxidative phosphorylation (Q), PCr
418 hydrolysis (D), lactate production (L) and, consequently, ATP_{tot} are presented in Table 1. $V_{i[PCr]}$,
419 calculated as described in Equation 4 is shown, along with the rate constant of PCr resynthesis
420 (k) and the amplitude of PCr recovery (A), in **Figure 2** (moderate) and **Figure 3** (heavy).

421

422 Comparisons of ATP_{tot} revealed a significant interaction (time \times intensity domain; $F[1,13] = 17.2$;
423 $p < 0.01$; $\eta^2 = 0.57$). ATP_{tot} was not different between 3 and 8 min of moderate exercise ($p =$
424 $n.s.$; Figure 2, Table 1), but ATP_{tot} increased (ΔATP_{tot}) during heavy exercise from 3 to 8 min
425 ($CI_{Difference}$ 1.9, 12.6 mM.min⁻¹; $p < 0.05$; **Figure 3, Table 1**), equating to a 26 ± 30 % increase in
426 ATP_{tot} from 3 to 8 min (**Figure 4A**). This percentage change in ATP_{tot} was not different to that
427 measured in both the $[PCr]_{sc}$ and the $\dot{V}O_{2sc}$ ($F[2,26] = 2.4$; $p = n.s.$; $\eta^2 = 0.16$) (**Figure 4A**).
428 However, among participants the individual values of ΔATP_{tot} during heavy exercise were not
429 significantly correlated with the magnitude of the $\dot{V}O_{2sc}$ (**Figure 4B**).

430

431 To examine the relationship of $\dot{V}O_2$ and [PCr], a correction for the transit delay from muscle to
432 lung was applied. $\dot{V}O_2$ data were time-corrected using 12 s difference with respect to ³¹P

433 measures (Rossiter *et al.*, 1999; Krstrup *et al.*, 2009). The relationship between $\dot{V}O_2$ and [PCr]
434 was linear during moderate exercise and the first 3 min of heavy exercise ($r^2 = 0.94$; **Figure 5**).
435 However, the slope of the [PCr]- $\dot{V}O_2$ relationship was significantly steeper when data from 8 min
436 of heavy exercise was included (-67 ± 25 vs. -61 ± 25 mL.min.mM⁻¹; $p < 0.05$).

437

438 **Discussion**

439 The [PCr] slow component ([PCr]_{sc}), like the $\dot{V}O_2$ slow component ($\dot{V}O_{2sc}$), is only present during
440 exercise above LT. That the [PCr]_{sc} and $\dot{V}O_{2sc}$ are of similar magnitude (Rossiter *et al.*, 2002)
441 led to the argument that the $\dot{V}O_{2sc}$ is caused by an increased phosphate cost of power
442 production (P/W) during heavy intensity exercise. However, this is at odds with the observed
443 dissociation between the [PCr]_{sc} and $\dot{V}O_{2sc}$ in endurance-trained individuals (Layec *et al.*, 2009b;
444 Layec *et al.*, 2012), and both observations relied upon equivocal assumptions about the
445 dynamic relationships between [ADP]- $\dot{V}O_2$ or $\dot{V}O_2$ -[PCr] (Yoshida & Watari, 1993, 1994;
446 Rossiter *et al.*, 2002; Jones *et al.*, 2008; Kemp, 2008; Wust *et al.*, 2011). Our present data
447 agree with previous reports that mean [PCr]_{sc} and $\dot{V}O_{2sc}$ magnitudes were not statistically
448 different. Crucially, however, the data add that, among individuals, the increase in the $\dot{V}O_{2sc}$
449 during heavy intensity exercise (averaging ~22%) is not correlated with the increase in the
450 phosphate cost of power production, ATP_{tot} (average ~26%). Thus, while the exercising limb
451 remains likely the major source of the $\dot{V}O_{2sc}$ (Poole *et al.*, 1991; Rossiter *et al.*, 2002; Bailey *et al.*
452 *et al.*, 2010; Dimenna *et al.*, 2010), the observed dissociation between $\dot{V}O_{2sc}$ and ΔATP_{tot} (Figure
453 4B) strongly suggests that the progressive increase in $\dot{V}O_2$ during heavy exercise is not solely
454 due to contractile inefficiency (P/W). Thus, other explanations, such as a reduction in
455 mitochondrial efficiency (P/O), should also be considered.

456

457 *ATP turnover during moderate and heavy constant power exercise*

458 The primary aim of this investigation was to estimate the ATP turnover rate for exercise below
459 and above LT and over time without assumptions about the $[ADP]-\dot{V}O_2$ or $\dot{V}O_2-[PCr]$
460 relationship. By using the most robust estimations of ATP_{tot} (Kemp *et al.*, 1995; Walter *et al.*,
461 1999; Lanza *et al.*, 2005; Faraut *et al.*, 2007), we provided ^{31}P MRS-derived estimates of ATP
462 yield from oxidative phosphorylation, lactate production, and PCr hydrolysis at 3 and 8 min of
463 exercise that were unencumbered by the recently-challenged assumptions about the $[ADP]-\dot{V}O_2$
464 relationship (Kemp, 2008; Wust *et al.*, 2011; Glancy & Balaban, 2012; Kemp *et al.*, 2014).

465

466 Unsurprisingly, there were no changes in ATP_{tot} during exercise below the lactate threshold,
467 where negligible muscle fatigue is expected (Sargeant & Dolan, 1987; Yano *et al.*, 2001),
468 reflecting a steady-state condition. Conversely, during heavy exercise in which the $\dot{V}O_{2sc}$ and
469 $[PCr]_{sc}$ were present, ATP_{tot} was increased between 3 and 8 min of exercise. This is consistent
470 with the suggestions that the $\dot{V}O_{2sc}$ is consequent to increased P/W in the large locomotor
471 muscles during supra-LT exercise (Rossiter *et al.*, 2002), perhaps associated with muscle
472 fatigue and a reduction in contractile efficiency. However, the lack of relationship between
473 ΔATP_{tot} and $\dot{V}O_{2sc}$ is in contrast to this postulate, and challenges the current understanding of
474 the $\dot{V}O_{2sc}$ aetiology (Rossiter, 2011; Poole & Jones, 2012).

475

476 Dissociation of the $\dot{V}O_{2sc}$ and changes in the phosphate cost of exercise may have a few
477 different explanations. It may indicate $\dot{V}O_2$ originating from regions within the knee extensors
478 that are not interrogated by the surface coil. While we can only speculate on this, a similar
479 finding has been reported where the $\dot{V}O_2$ and $[PCr]$ slow components were dissociated in
480 endurance trained participants but not in sedentary controls, despite increasing EMG activity in
481 both participant groups during the $\dot{V}O_{2sc}$ (Layec *et al.*, 2009b; Layec *et al.*, 2012). It was

482 hypothesised that the exercise-trained volunteers may be better able to optimise motor unit
483 recruitment patterns to maintain high-intensity exercise (e.g. compared to active but untrained
484 subjects (Rossiter *et al.*, 2002)), thereby recruiting motor unit pools that reside outside of the
485 muscle volume being interrogated by MRS. It should be noted, however, that our surface coil
486 interrogated a large muscle volume (~300 g) compared to alternative techniques, e.g. biopsy
487 (~200 mg). Additionally, controversy exists whether progressive recruitment itself is even
488 responsible for the slow component (Zoladz *et al.*, 2008; Cannon *et al.*, 2011; Vanhatalo *et al.*,
489 2011), in which case recruitment of muscle outside the surface coil view would seem to be an
490 unlikely explanation if the motor program and recruitment pattern is stable.

491
492 The source of the $\dot{V}O_{2sc}$ may even reside outside of the locomotor muscles. Progressive
493 increases in respiratory (Wasserman *et al.*, 1995; Zoladz & Korzeniewski, 2001) or cardiac
494 work, or even work from non-power-producing musculature, such as stabilising effort during
495 cycling (Billat *et al.*, 1998) may contribute to a reduction in exercise efficiency during the slow
496 component. It is unlikely that the latter source would contribute to prone knee-extension where
497 the work of stabilising the torso is minimised by the body position, the ergometer, and the heavy
498 strapping used to isolate quadriceps activity. Nonetheless, the work of ventilation during prone
499 knee-extension may still contribute a meaningful proportion; particularly as the locomotor
500 muscle mass in our study is relatively small in comparison to cycling or running.

501
502 Finally, dissociation of the $\dot{V}O_{2sc}$ and ΔATP_{tot} could result from mitochondrial uncoupling
503 (reduced P/O;
504 Figure 5). In this scenario, an increased O_2 cost of ATP resynthesis may contribute to driving
505 the increase in $\dot{V}O_2$ during heavy exercise, rather than it coming exclusively from an increased
506 ATP cost of muscle power generation.

507

508 *$\dot{V}O_2$ -[PCr] relationship and mitochondrial coupling during heavy intensity exercise*

509 Without an invasive measure of $\dot{V}O_2$ across the volume of tissue interrogated by MRS, the
510 relationship between whole body $\dot{V}O_2$ and localised [PCr] is the next best estimate for coupling
511 of O_2 uptake and ATP turnover. Our data show that the mean $\dot{V}O_2$ -[PCr] relationship was linear
512 over the moderate intensity, and during the first minutes of heavy exercise ($r^2 = 0.94$;
513 **Figure 5**). Importantly, this relationship became steeper ($p < 0.05$) with the inclusion of data
514 from the final minute of heavy exercise. With some important assumptions, these data suggest
515 a reduced P/O between 3 and 8 minutes of heavy exercise, implicating mitochondrial
516 uncoupling as an additional mechanism of the $\dot{V}O_2$ slow component.

517

518 It is important to recognise that the $\dot{V}O_2$ /[PCr] slope reflects the combined influence of
519 mitochondrial density, the rate constant (k) of [PCr] breakdown relative to k of $\dot{V}O_2$, the total
520 [creatine], and the P/O (Meyer, 1988; Kemp *et al.*, 2014). Mitochondrial density and total
521 [creatine] are constant during acute exercise, and therefore any divergence in $\dot{V}O_2$ -[PCr] slope
522 would result from changes in k [PCr] and/or P/O over the exercise intensities. While the k [PCr]
523 was not different between 3 and 8 min of heavy intensity exercise ($p = n.s.$), there was variance
524 among individuals (**Figure 3A**). Therefore, while we base our interpretation on the group mean,
525 we cannot rule out the influence of variance in the individual changes in k [PCr] in interpreting
526 the $\dot{V}O_2$ -[PCr] slope. In addition, we used a fixed transit delay to phase align the $\dot{V}O_2$ and [PCr]
527 measurements in the time domain. This correction provided the best fit to the kinetics that we
528 could make, but it is a limitation for interpreting the $\dot{V}O_2$ -[PCr] relationship. Specifically, small
529 errors in transit delay adjustment result in non-linear distortion when plotting single participant
530 data, although this influence is greater during the early kinetics (first 2 min) than between 3 and
531 8 minutes of exercise where the kinetics are slower. Finally, the progressive intramuscular

532 acidification during exercise would be expected to dissociate the dynamics of $\dot{V}O_2$ and [PCr],
533 speeding the former, and slowing the latter (Iotti *et al.*, 1993; Gerbino *et al.*, 1996; Layec *et al.*,
534 2013). Therefore, while substantial assumptions necessarily underlie the interpretation of the
535 $\dot{V}O_2$ -[PCr] relationship, it is currently the only way to examine change in P/O as a potential
536 mechanism explaining the lack of relationship between the magnitude of the $\dot{V}O_{2sc}$ and ΔATP_{tot} .
537 These data suggest that P/O is stable during moderate intensity exercise and the first 3 min of
538 heavy intensity exercise, in agreement with the other ^{31}P MRS studies [e.g. where the $\dot{V}O_2$ -[PCr]
539 relationship is strikingly linear throughout the metabolic rate range (Bailey *et al.*, 2010)], but that
540 sustained heavy intensity exercise beyond 3 min may be accompanied by a reduction in P/O.
541 Consequently, contrary to the prevailing hypothesis (Rossiter *et al.*, 2002), the $\dot{V}O_{2sc}$ may at
542 least, in part, be a result of mitochondrial uncoupling in the active muscle during acidifying
543 exercise.

544

545 *Potential mechanisms of mitochondrial uncoupling*

546 There are various mechanisms that might cause the mitochondrial trans-membrane proton
547 gradient to dissipate during exercise. This proton 'leak' is regulated by uncoupling proteins and
548 contributes to setting the resting P/O. If this process is augmented during exercise, the ATP
549 yield per atomic oxygen consumed would fall. Others have shown upregulation of uncoupling
550 proteins 2 and 3 with an acute bout of exercise (UCP2, UCP3; both expressed in skeletal
551 muscle) and these can induce mitochondrial uncoupling, likely to minimise production of, and
552 damage from, reactive oxygen species (ROS) (Brand *et al.*, 2004; Bo *et al.*, 2008; Jiang *et al.*,
553 2009). This effect may be akin to the chronic uncoupling reported with ageing, posited as a
554 protective mechanism against ROS damage (Brand *et al.*, 2004; Amara *et al.*, 2007), particularly
555 as leak respiration comprises a large proportion of resting $\dot{V}O_2$. However, the kinetics of UCP
556 upregulation are relatively slow in comparison with the exercise duration in our study;

557 upregulation of uncoupling proteins is typically present ~45-90 min post-acute exercise.
558 Additionally, investigations into mitochondrial uncoupling have relied on relatively long bouts of
559 exercise (> 30 min), and evidence from human muscle suggests that acute exercise may not be
560 sufficient to elicit the same effect size for upregulation seen in the rat (Fernstrom *et al.*, 2004).
561 Therefore, upregulation of uncoupling proteins seems less likely to fully explain the lack of
562 relationship between $\dot{V}O_{2sc}$ and ΔATP_{tot} during heavy exercise.

563

564 Alternatively, dissociation of the $\dot{V}O_{2sc}$ and ΔATP_{tot} may result from high $[H^+]$ or $[Pi]$ during
565 exercise (Walsh *et al.*, 2002). Low pH can reduce $[ADP]$ from a shift in the creatine kinase
566 equilibrium (Conley *et al.*, 2001), and also serve to dissociate creatine kinase from the
567 mitochondrial membrane, leading to a disruption in oxidative phosphorylation (Walsh *et al.*,
568 2002). While evidence for a direct effect of acidosis is certainly not conclusive (Suleymanlar *et*
569 *al.*, 1992; Kemp *et al.*, 2014), numerous studies show disturbances to oxidative phosphorylation
570 through the inhibition of respiratory enzymes or reductions in the proton motive force (Hillered *et*
571 *al.*, 1984; Harkema & Meyer, 1997; Jubrias *et al.*, 2003), but fail to result in change to P/O alone
572 (Tonkonogi & Sahlin, 1999). Nevertheless, the variable relationships between the magnitude of
573 the $\dot{V}O_{2sc}$ and ΔATP_{tot} , together with a steepened $\dot{V}O_2$ -[PCr] relationship, suggest P/O change
574 as a possible scenario during heavy exercise.

575

576 *Technical considerations and study limitations*

577 While limitations accompany the estimations, our study design provides advantage over
578 previous reports of ATP turnover rate in the literature. Prior estimations have relied on
579 extrapolation of $V_{i[PCr]}$, which is assumed to be only affected by the [PCr] recovery amplitude.
580 This model constrains P/O with a linear $\dot{V}O_2$ -[PCr] relationship, by definition (Layec *et al.*,
581 2009a), which is in contrast with recent findings (Kemp, 2008; Wust *et al.*, 2011; Glancy &

582 Balaban, 2012)) and the observations in this study (**Figure 5**). Conversely, [PCr] recovery
583 dynamics may be plastic during supra-LT exercise where intracellular acidification (Yoshida &
584 Watari, 1993, 1994), fatigue related metabolite accumulation (Jones *et al.*, 2008), and muscle
585 fatigue (Yano *et al.*, 2001; Cannon *et al.*, 2011) have been reported. While the group mean for
586 $k[\text{PCr}]$ resynthesis (or time constant, $\tau = 1/k$) is not different following sub- and supra-LT
587 exercise in this study and others (Rossiter *et al.*, 2002), our data suggests that $k[\text{PCr}]$ is not
588 constant within an individual. Therefore, in our study, $V_{i[\text{PCr}]}$ (and, thus, Q and ATP_{tot}) were not
589 constrained to increase in response only to changes in [PCr]. In other words, the augmented
590 amplitude of [PCr] during the slow component did not result in an obligatorily faster initial rate of
591 change following the cessation of exercise; our measurement was dependent on the recovery
592 dynamics characterised and specific to that moment in time. Consequently, the estimations
593 provided for oxidative ATP yield in our study are devoid of the assumptions about the $\dot{V}\text{O}_2$ -
594 [ADP] and $\dot{V}\text{O}_2$ -[PCr] relationships.

595

596 ATP_{tot} is most heavily weighted on changes in $V_{i[\text{PCr}]}$, a measure that is sensitive to noise in the
597 MRS signal [e.g. Figure 7 in (Rossiter *et al.*, 2000)]; this initial rate is derived from
598 characterisation of the kinetics of [PCr] recovery. The influence of noise in [PCr] recovery
599 kinetics, particularly in the early transient, is likely the largest source of variability to resolve
600 ATP_{tot} . Conversely, the confidence in characterising [PCr] off-kinetics is substantially greater
601 than for pulmonary $\dot{V}\text{O}_2$ or even [PCr] during the on-transient. Any improvement in the
602 characterisation of ^{31}P dynamics will take a considerable leap in signal:noise and more rapid
603 spectra acquisition.

604

605 The heterogeneous nature of skeletal muscle metabolism (Koga *et al.*, 2007; Damon *et al.*,
606 2008; Saitoh *et al.*, 2009; Cannon *et al.*, 2013) may have obscured the characterisation of [PCr]

607 dynamics, and therefore ATP_{tot} . ^{31}P MRS measures a volume of tissue (~300 g) which may not
608 be representative of the entire knee extensor group responsible for the power output, or the
609 diversity of metabolic strain within this group. Finally, the unmeasured work of knee flexion is
610 not accounted for with our ergometer. Therefore, the work of knee flexion (to lift the leg) is
611 assumed to be constant in our experiments, but does contribute to the pulmonary $\dot{V}O_2$ signal.

612

613 **Conclusions**

614 Similar to previous studies, the mean magnitude of the $\dot{V}O_2$ and [PCr] slow components were
615 not different during heavy exercise, consistent with the prevailing hypothesis for the
616 intramuscular source of the $\dot{V}O_{2sc}$: an increase in the phosphate cost of force production.
617 Although the magnitude of the $\dot{V}O_2$ slow component (~22%) was similar to the increase in ATP_{tot}
618 (~26%) from 3 to 8 min during heavy exercise, there was no relationship detected between
619 these measures among individuals. Therefore, our data suggest that the pulmonary $\dot{V}O_2$ slow
620 component does not solely originate from increases in the phosphate cost of power production
621 (increased P/W). Other mechanisms, such as an increased O_2 cost of ATP resynthesis (reduced
622 P/O) during acidifying exercise, may also contribute to generating the $\dot{V}O_2$ slow component.

623

624 **Acknowledgements**

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626 all volunteers for their time and dedication.

627 **Figure Legends**

628

629 **Figure 1.** $\dot{V}O_2$ (panel A), [PCr] (panel B), and pH (panel C) plotted as a function of time for
630 moderate (●) and heavy (○) intensity prone bilateral knee-extension exercise. Black rectangle
631 denotes exercise bout from time 0 to 8 min. Data points are 8 s means with error bars
632 representing SD.

633

634 **Figure 2.** Moderate exercise recovery rate constant (k ; Panel A), amplitude of PCr resynthesis
635 (A; Panel B), initial rate of PCr resynthesis ($V_{i[PCr]}$; Panel C), and ATP_{tot} (Panel D) at 8 min of
636 exercise, plotted as a function of 3 min of exercise. Dashed line is $y = x$.

637

638 **Figure 3.** Heavy intensity rate constant (k ; Panel A), amplitude of PCr resynthesis (A; Panel B),
639 initial rate of PCr resynthesis ($V_{i[PCr]}$; Panel C), and ATP_{tot} (Panel D) at 8 min of exercise, plotted
640 as a function of 3 min of exercise. Dashed line is $y = x$.

641

642 **Figure 4.** Magnitudes of the $\dot{V}O_{2sc}$ and $[PCr]_{sc}$ and ΔATP_{tot} from min 3 to 8 of heavy exercise
643 expressed as a % change (Panel A). Panel B is ΔATP_{tot} during heavy exercise plotted as a
644 function of the $\dot{V}O_{2sc}$.

645

646 **Figure 5.** Relationship between pulmonary $\dot{V}O_2$ and [PCr] during moderate (●) and heavy (○)
647 exercise. The regression shown (solid line) was fit to moderate and the first 3 min of data from
648 heavy exercise and extrapolated (dashed line) to 8 min of heavy exercise. Error bars represent
649 SD. $\dot{V}O_2$ data were phase aligned with respect to [PCr] measurements.

650

651 **Tables**

652 Table 1. Rates of ATP turnover from oxidative phosphorylation (*Q*), PCr hydrolysis (*D*), lactate
 653 production (*L*) and the sum (ATP_{tot}) during moderate and heavy constant power exercise at two
 654 time points. Values are presented in mM.min⁻¹ as means (SD).

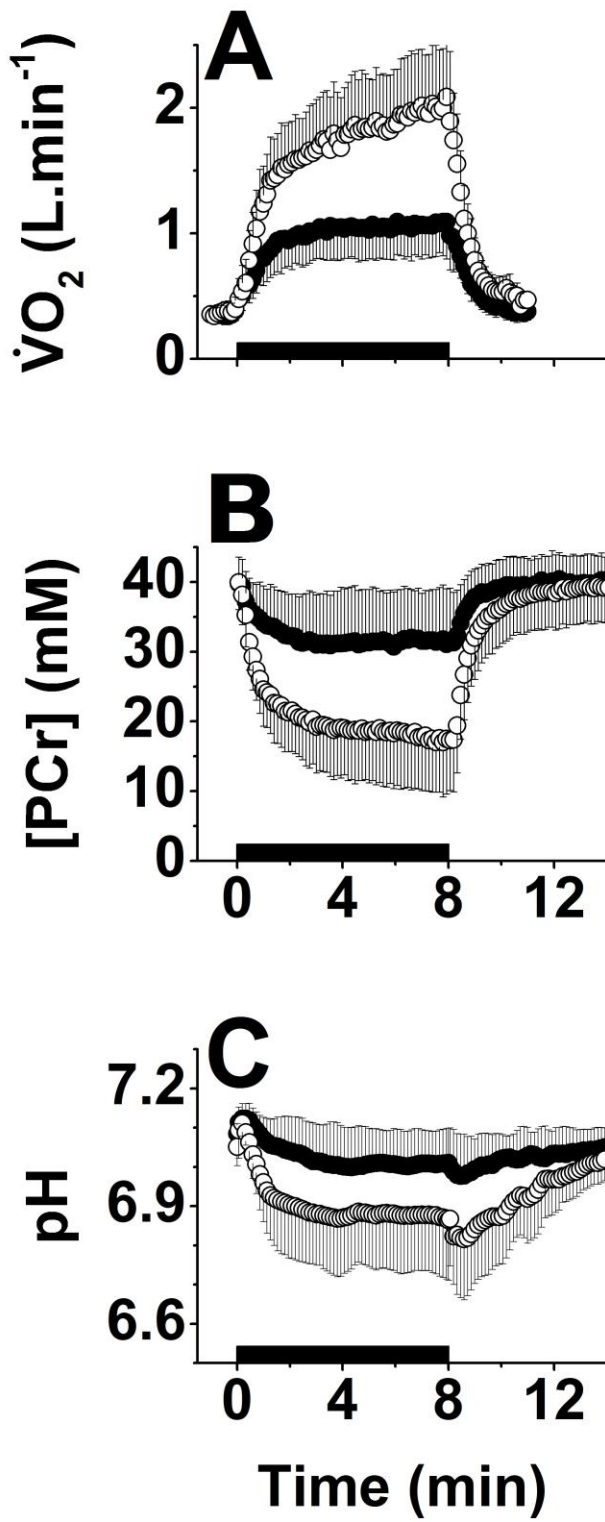
	Moderate		Heavy	
	3 min	8 min	3 min	8 min
<i>Q</i> (mM.min ⁻¹) §	23 (14)	17 (13)	35 (17)	42 (13)*
<i>D</i> (mM.min ⁻¹)	0.6 (1.2)	0.2 (1.0)	1.1 (2.6)	0.7 (0.9)
<i>L</i> (mM.min ⁻¹)	1.0 (1.3)	0.3 (0.6)	1.5 (1.3)	1.3 (1.7)
ATP_{tot} (mM.min ⁻¹) #	24 (14)	17 (14)	38 (16)	44 (14)*

655 §Time × Intensity interaction; $p < 0.01$; $F[1,13]=17.2$; $\eta^2=0.57$

656 #Time × Intensity interaction; $p < 0.01$; $F[1,13]=17.2$; $\eta^2=0.57$

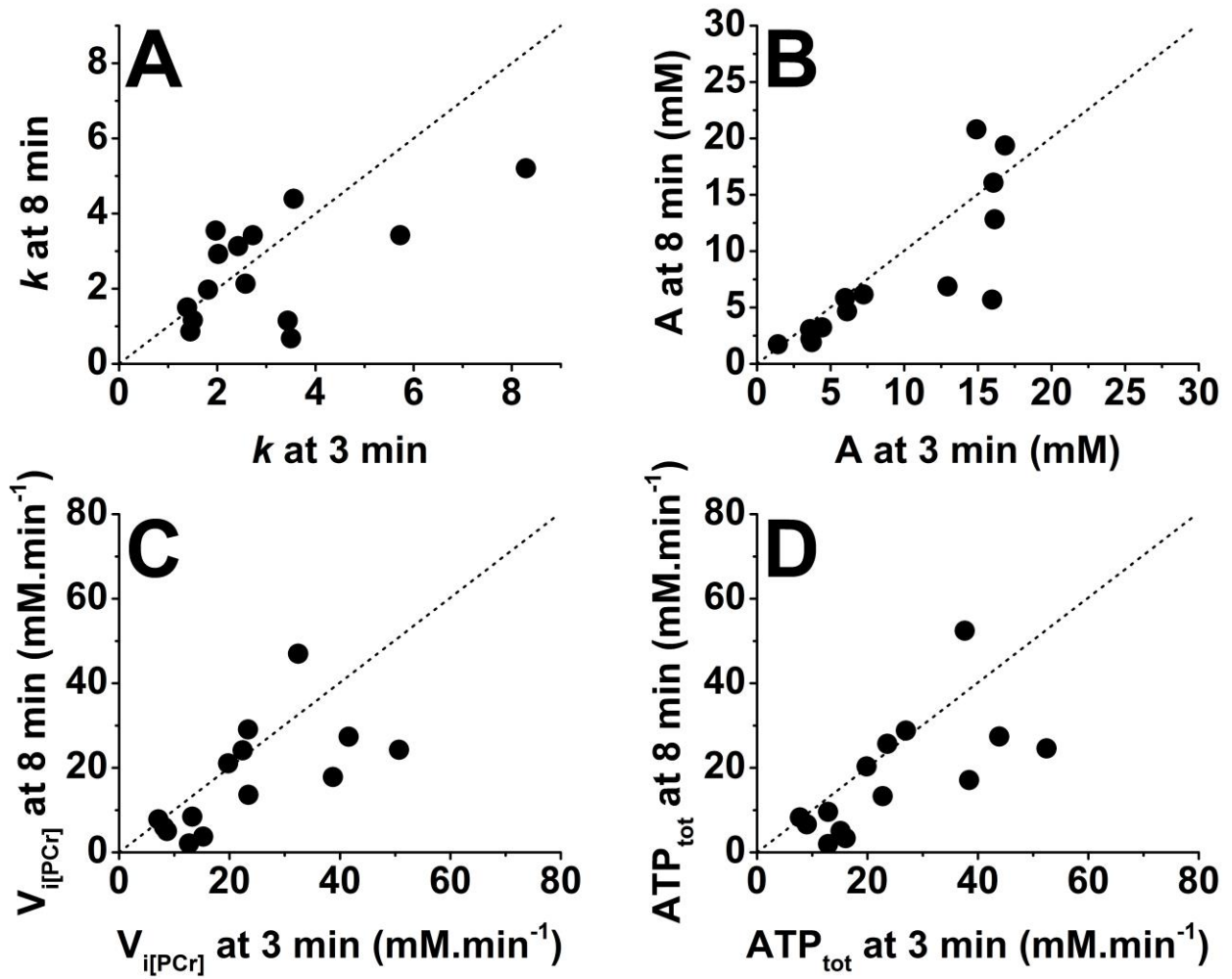
657 *Different from 3 min; $p < 0.05$

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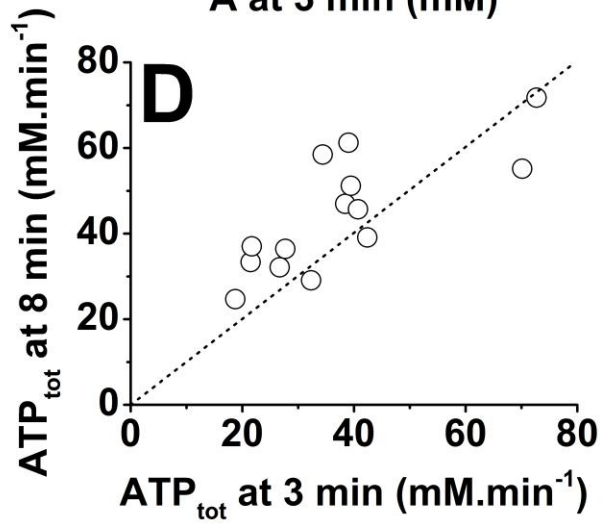
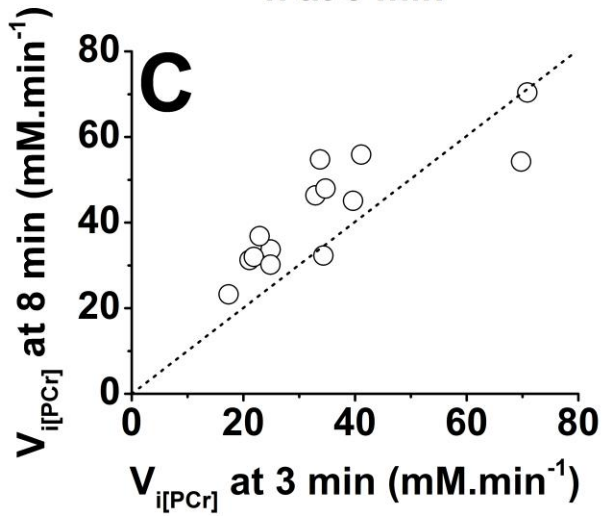
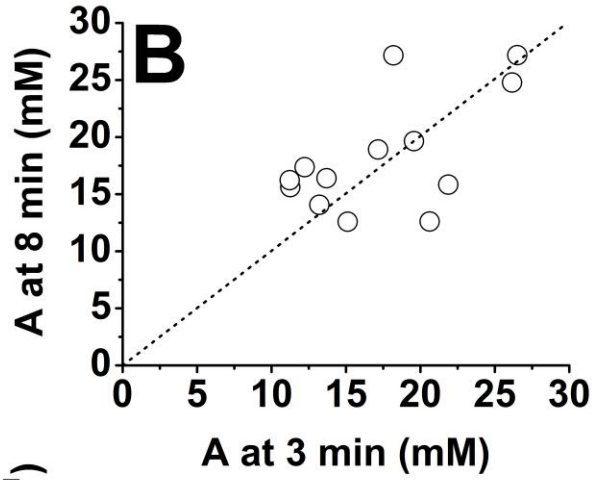
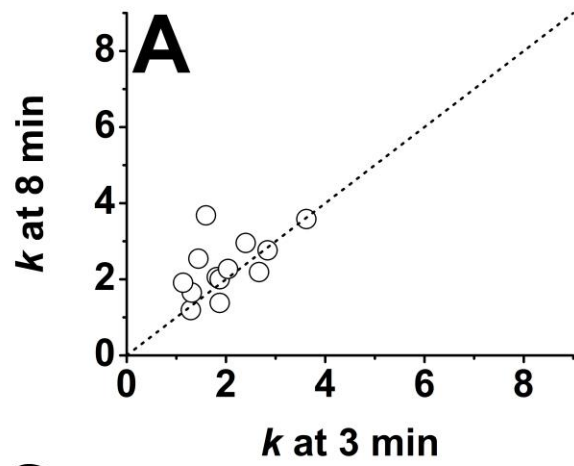
661 Figure 1



663

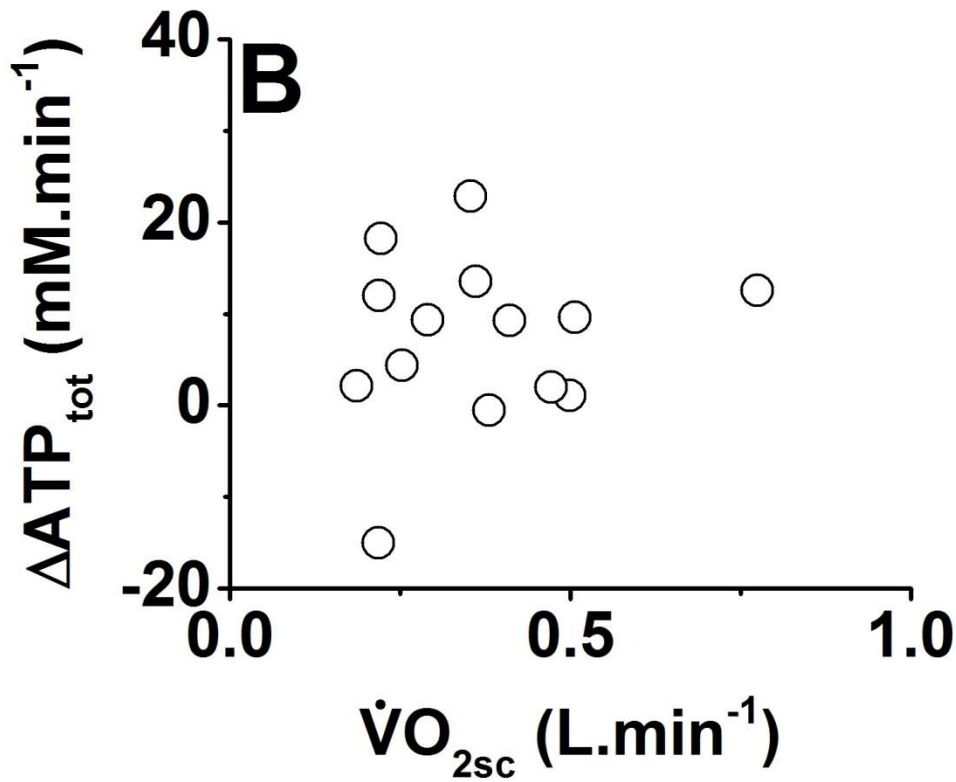
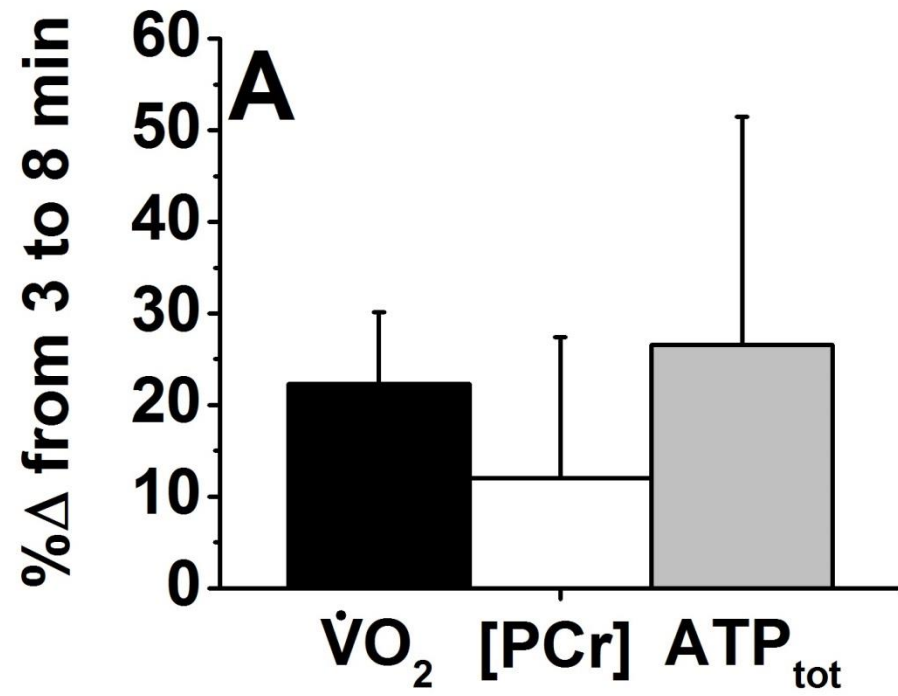
664 Figure 2

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666

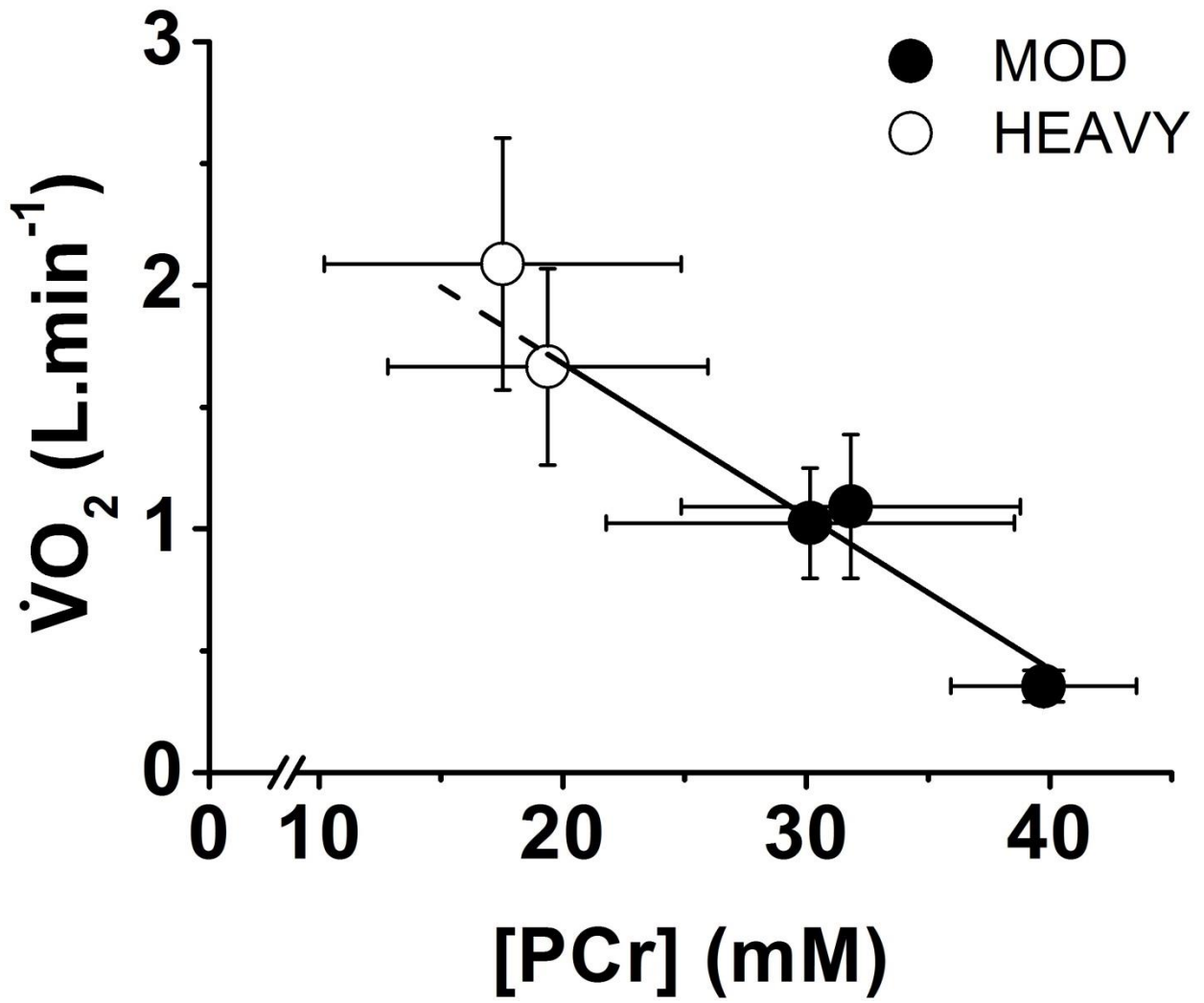
667 **Figure 3**



668

669 Figure 4

670



671

672 Figure 5

673 **References**

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