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Allan, R, Sharples, AP, Close, GL, Drust, B, Shepherd, SO, Dutton, J, Morton, JP and Gregson, W

**Postexercise cold water immersion modulates skeletal muscle PGC-1 alpha mRNA expression in immersed and nonimmersed limbs: evidence of systemic regulation**

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### Article

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1 **Post-exercise cold-water immersion modulates skeletal muscle PGC-1 $\alpha$**   
2 **mRNA expression in immersed and non-immersed limbs: evidence of**  
3 **systemic regulation**

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29 **Running Head:** Cold-water immersion and PGC-1 $\alpha$

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42 **New & Noteworthy**

43 We report for the first time that post-exercise cold-water immersion of one limb also enhances  
44 PGC-1 $\alpha$  expression in a contra-lateral non-immersed limb. We suggest increased systemic  $\beta$ -  
45 adrenergic stimulation, and not localised cooling per se, exerts regulatory effects on local  
46 signalling cascades thereby modulating PGC-1 $\alpha$  expression. These data therefore have  
47 important implications for research designs that adopt contralateral non-immersed limbs as a  
48 control condition, whilst also increasing our understanding of potential mechanisms  
49 underpinning cold-mediated PGC-1 $\alpha$  responses.

50 **Author Contributions**

51 Conception and design of the experiments: RA, WG, JM, APS, BD; Collection, analysis and  
52 interpretation of data: RA, APS, JD, JM, WG, SS, GC; Drafting the article and Critical  
53 Revision of the article for important intellectual content: RA, APS, GC, SS, BD, JD, JM, WG.  
54 All authors approved the final version for publication and agree to be accountable for all aspects  
55 of the work.

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63 **Abstract**

64 Mechanisms mediating post-exercise cold-induced increases in PGC-1 $\alpha$  gene expression in  
65 human skeletal muscle are yet to be fully elucidated, but may involve local cooling effects on  
66 AMPK and p38 MAPK related signalling and/or increased systemic  $\beta$ -adrenergic stimulation.  
67 We aimed to therefore examine whether post-exercise cold-water immersion enhancement of  
68 PGC-1 $\alpha$  mRNA is mediated through local or systemic mechanisms. Ten subjects completed  
69 acute cycling (8x5 min at ~80% peak power output) followed by seated-rest (CON) or single-  
70 leg cold-water immersion (CWI; 10 min, 8°C). Muscle biopsies were obtained pre-, post- and  
71 3 h post-exercise from a single limb in the CON condition but from both limbs in CWI (thereby  
72 providing tissue from a CWI and non-immersed limb, NOT). Muscle temperature decreased  
73 up to 2 h post-exercise following CWI (-5°C) in the immersed limb, with lesser changes  
74 observed in CON and NOT (-3°C; P<0.05). No differences between limbs were observed in  
75 p38MAPK phosphorylation at any time point (P<0.05), whilst a significant interaction effect  
76 was present for AMPK phosphorylation (P=0.031). Exercise (CON) increased gene expression  
77 of PGC-1 $\alpha$  3 h post-exercise (~5-fold; P<0.001). CWI augmented PGC-1 $\alpha$  expression above  
78 CON in both the immersed (CWI; ~9-fold; P=0.003) and NOT limbs (~12-fold; P=0.001).  
79 Plasma Normetanephrine concentration was higher in CWI vs. CON immediately post-  
80 immersion (860 vs. 665 pmol/L; P=0.034). We report for the first time that local cooling of the  
81 immersed limb evokes transcriptional control of PGC1- $\alpha$  in the non-immersed limb, suggesting  
82 increased systemic  $\beta$ -adrenergic activation of AMPK may mediate, in part, post-exercise cold-  
83 induction of PGC-1 $\alpha$  mRNA.

84 **Key Words:** PGC-1 $\alpha$ , CWI, Normetanephrine, AMPK

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86

## 87 **Introduction**

88 It is well established that the transcriptional co-activator peroxisome proliferator-activated  
89 receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), the proposed “master regulator” of skeletal muscle  
90 mitochondrial biogenesis (37), is sensitively controlled by acute and chronic exercise (3, 4, 10,  
91 36). Upstream control of PGC-1 $\alpha$  includes phosphorylation by energy and stress sensing  
92 kinases AMP-activated protein kinase (AMPK) (23) and p38 mitogen-activated protein kinase  
93 (p38 MAPK) (2). Consistent with its initial discovery as cold-inducible (34, 38), recent studies  
94 have examined the potential of acute post-exercise cold exposure to also modulate PGC-1 $\alpha$   
95 expression. For example, in human tissue, both cold-ambient temperatures (42, 43) and post  
96 exercise cold-water immersion (18, 20) enhances the skeletal muscle PGC-1 $\alpha$  gene  
97 transcription and protein translational response versus exercise alone. The precise  
98 mechanism(s) mediating cold-induced regulation of the PGC-1 $\alpha$  transcriptional pathway have  
99 yet to be fully determined, though the cooling-induced alterations in muscle blood flow (14,  
100 30) are unlikely to mediate these effects (33, 45). It is noteworthy, however, that chronic cold-  
101 induced changes in PGC-1 $\alpha$  protein content arise in conjunction with increased activity of  
102 signalling kinases AMPK and p38 MAPK (18). These data suggest that cooling of the skeletal  
103 muscle tissue may play a role in mediating the post-exercise cold-induction of PGC-1 $\alpha$  mRNA  
104 through activation of local signalling kinases.

105 Alternatively, systemic control via increased  $\beta$ -adrenergic activity is suggested to play a potent  
106 role in mediating the effects of cold exposure on PGC-1 $\alpha$  expression via AMPK (29) and  $\beta$ 2-  
107 adrenergic receptor (31) mechanisms. Indeed, plasma norepinephrine concentrations remain  
108 higher following high-intensity exercise after cold-water immersion versus control conditions  
109 (13). Studies to date have utilised the non-immersed limb as the control condition without the  
110 use of a true control (no cooling) condition (11, 18, 20). By assuming the response to cold-  
111 water immersion is mediated locally, such experimental designs do not permit examination of

112 the role of systemic versus localised mechanisms in mediating cold-induced changes in PGC-  
113  $1\alpha$ . Indeed, Ihsan et al. (20) observed that PGC- $1\alpha$  gene transcription was not induced in a non-  
114 immersed control limb, despite the limb having previously been exercised, and exercise being  
115 a potent stimulus to induce PGC- $1\alpha$  expression. This would therefore suggest that an induction  
116 of PGC- $1\alpha$  expression in an immersed limb occurred by way of cold-induced mechanisms. In  
117 this regard, we suggest that the increased systemic  $\beta$ -adrenergic activity associated with post-  
118 exercise cooling of the immersed limb could also modulate PGC- $1\alpha$  expression in the non-  
119 immersed contralateral limb.

120 Therefore, the aim of the present study was to examine whether the cold-water induced increase  
121 in PGC- $1\alpha$  mRNA observed post exercise is mediated through local or systemic mechanisms.  
122 To this end, we employed a novel experimental design (in a repeated measures crossover  
123 design) where ten recreationally active males completed an acute cycling protocol (8x5 min at  
124 ~80% peak power output) followed by a seated-rest condition (CON) or single-leg cold-water  
125 immersion (CWI; 10 min, 8°C). Muscle biopsies were obtained pre-, post- and 3 h post-exercise  
126 from a single limb in the CON condition but from both limbs post-exercise in the CWI trial  
127 (thereby providing tissue from a CWI and non-immersed limb, NOT). In this way, our design  
128 allowed us to obtain tissue from true control conditions but yet, also sample tissue from an  
129 immersed and non-immersed limb that was subject to the same hormonal milieu.

130

## 131 **Methods**

132 **Subjects.** Ten recreationally active healthy males (age  $26 \pm 4$  y; body mass  $79.29 \pm 6.73$  kg;  
133 height  $180 \pm 5$  cm;  $\dot{V}O_{2\text{peak}}$   $51.46 \pm 9.07$  mL.kg<sup>-1</sup>.min<sup>-1</sup>; peak power output (PPO)  $265.2 \pm 38.33$   
134 W; mean  $\pm$  SD) participated in this study. Subjects were instructed to refrain from exercise,  
135 alcohol and caffeine 48 hours prior to the test day. All subjects gave written informed consent

136 to participate after details and procedures of the study had been fully explained. Subjects had  
137 no history of neurological disease or musculoskeletal abnormality and none were under any  
138 pharmacological treatment during the course of the study. Each subject was medically screened  
139 by a practising GP prior to participation for their individual risk associated with high-intensity  
140 exercise, muscle biopsy procedures and immersion in cold water. All procedures performed in  
141 the study were approved by the Ethics Committee of Liverpool John Moores University and in  
142 accordance with the 1964 Helsinki declaration and its later amendments.

143 Experimental Design. In a repeated-measures crossover design, subjects completed two high-  
144 intensity intermittent cycling protocols followed either by 10 min seated rest (CON) or single-  
145 leg CWI (10 min at 8°C). Muscle biopsies were obtained from the vastus lateralis immediately  
146 before, post- and 3 h post-exercise. During the CWI trial muscle biopsies in the post-exercise  
147 period were taken from both the immersed and non-immersed leg. This design allowed us to  
148 assess the impact of the cold stimulus locally (immersed leg) and systemically (non-immersed  
149 leg) against a relevant resting control. Both experimental trials were conducted in a  
150 counterbalanced, randomized order with at least 10 days between conditions.

151 Experimental Protocol. Subjects attended the laboratory on 3 separate occasions. On the first  
152 occasion, subjects completed an incremental exercise test to fatigue for the determination of  
153  $\dot{V}O_{2max}$  and PPO (15). Results from this test were used to determine the power output necessary  
154 for cycling at a proportion of PPO on subsequent test days (detailed below). Prior to the first  
155 test day, subjects completed a 24-hour food diary to be replicated before the second trial. Upon  
156 arrival at the laboratory (0900h) subjects were fitted with a heart-rate monitor (Polar RS400,  
157 Kempele, Finland), skin and rectal temperature probes (MHF-18050-A and MRV-55044-A,  
158 Ellab, Rodovre, Denmark). Legs were marked for subsequent insertion of muscle temperature  
159 needles; area of insertion was calculated as half the length of the femur, over the 'belly' of the  
160 vastus lateralis. The needle thermistor was then placed at a depth of 3cm, plus one-half of the



161 skinfold measurement, for the determination of deep muscle temperature (3cm). Following 10-  
162 min resting in a supine position, baseline measures of heart rate (HR), temperature and oxygen  
163 uptake ( $\dot{V}O_2$ ; Oxycon Pro, Jaeger, Wuertzberg, Germany) were assessed. Resting venous blood  
164 samples were drawn from a superficial vein in the anti-cubital crease of the forearm using  
165 venepuncture cannulation (BD Nexiva Closed IV Catheter 22G Blue, Becton Dickinson,  
166 Oxford, UK). Resting muscle temperature was assessed using a needle thermistor (13050;  
167 Ellab, Rodovre, Denmark) inserted into the vastus lateralis at 3cm depth as previously  
168 described (30). Finally, resting muscle biopsy samples from the vastus lateralis (~30–50 mg  
169 wet wt) were obtained under local anaesthesia (0.5% marcaine) using a Pro-Mag 2.2 biopsy  
170 gun (MD-TECH, Manan Medical Products, Northbrook, IL). At rest only a single leg was  
171 biopsied in the cooling trial (CWI) in attempt to reduce the stress experienced by subjects. This  
172 leg was randomised between the immersed and non-immersed limbs to exclude potential  
173 variation caused by leg dominance; all subsequent biopsies were completed in both legs, at  
174 each time point, 2cm proximal to the previous incision.

175 Following the resting biopsy, subjects completed a high-intensity intermittent cycling protocol  
176 consisting of  $8 \times 5$  min bouts at 82.5% PPO separated by 1 min rest (adapted from 44) followed  
177 by either single-legged CWI (CWI: 10 min at 8°C; NOT: non-immersed leg i.e. 10 min at room  
178 temperature) or a control condition (CON; seated rest). CWI was conducted using a specialised  
179 seated mechanical hoist to lower the subject so that one leg was placed inside the cold-bath,  
180 allowing the other (NOT) to remain outside, and dry. Subjects then recovered in a semi-reclined  
181 position under normal laboratory temperatures (~21°C) until 3-hours post-exercise. Measures  
182 of heart rate, skin temperature (thigh and calf) and rectal temperature were recorded throughout  
183 the exercise and recovery periods. Oxygen uptake was measured during the final minute of  
184 each high-intensity bout of exercise, during immersion, immediately post-immersion and again  
185 at 1, 2 and 3 h post-exercise. Ratings of perceived exertion (RPE) were assessed during the

186 final minute of each exercise bout (5), whilst subjective measures of perceived shivering were  
187 assessed throughout water immersion and the 3h recovery period using a visual analogue scale  
188 from 1 (No shivering) to 5 (Intense Shivering) (24). Laboratory temperatures remained stable  
189 throughout ( $21.18 \pm 0.76^{\circ}\text{C}$ ) and at no point were subjects allowed to rub themselves dry or  
190 shower (changing into dry shorts after immersion was allowed). Subjects were advised to wear  
191 the same clothes between trials. Muscle temperature was assessed post-exercise, immediately  
192 post-immersion and at 1, 2 and 3h post-exercise. Venous blood samples were also drawn at  
193 these times. Bi-lateral muscle biopsies occurred immediately after exercise and 3h post-  
194 exercise.

195 Venous blood samples were drawn from a superficial vein in the anti-cubital crease of the  
196 forearm using standard venepuncture techniques (BD Nexiva Closed IV Catheter 22G Blue,  
197 Becton Dickinson, Oxford, UK). Blood samples (~10ml) were collected into vacutainer tubes  
198 (Becton Dickinson, Oxford, UK) containing EDTA and stored on ice until centrifugation at  
199  $1500 \text{ rev. min}^{-1}$  for 15-min at  $4^{\circ}\text{C}$ . Following centrifugation, aliquots of plasma were stored at  
200  $-80^{\circ}\text{C}$  for later analysis. Plasma metanephrine and Normetanephrine concentrations were  
201 measured using liquid chromatography tandem mass spectrometry as previously described  
202 (35). All samples were analysed in duplicate, with the mean value employed.

203 rt-qRT-PCR. Skeletal muscle samples (~30 mg) were transferred to 2 ml lysing tubes  
204 containing 1.4 mm ceramic beads (Lysing Matrix D, MP Biomedicals, UK) containing 1 ml  
205 ice-cooled TRI- reagent (Life Technologies Ltd, UK) and homogenised at 6 m/s for 3 x 40  
206 seconds, separated by 5 minutes cooling on ice (MP Fastprep-24, MP Biomedicals, UK). RNA  
207 was extracted according to the TRI-reagent manufacturer's instructions. RNA concentration  
208 and purity were assessed by UV spectroscopy at ODs of 260 and 280 nm using a Nanodrop  
209 3000 (Fisher, Roskilde, Denmark). A target of  $A_{260} / A_{280}$  ratio was set at 1.8 to 2.2. Seventy  
210 ng RNA was used for each PCR reaction. Primer sequences (Table 1) were identified using

211 Gene (NCBI, <http://www.ncbi.nlm.nih.gov/gene>) and designed using Primer-BLAST  
212 (NCBI, <http://www.ncbi.nlm.nih.gov/tools/primer-blast>). Sequence homology searches  
213 ensured specificity and that all primers had no potential unintended targets. The primers were  
214 ideally designed to yield products spanning exon-exon boundaries to prevent any amplification  
215 of gDNA. Three or more GC bases in the last five bases at the 3' end of the primer were  
216 avoided. Secondary structure interactions (hairpins, self-dimer and cross dimer) within the  
217 primer were avoided. All primers were between 16 and 25 bp, and amplified a product of  
218 between 67-212 bp. Primers were purchased from Sigma (Suffolk, UK).

219 rt-qRT-PCR amplifications were performed using QuantiFast™ SYBR® Green RT-PCR one  
220 step kit on a Rotor-gene 3000Q (Qiagen, Crawley, UK) supported by rotor-gene software  
221 (Hercules, CA, USA). rt-qTR-PCR was performed as follows: hold 50°C for 10 min (reverse  
222 transcription/cDNA synthesis), 95°C for 5 min (transcriptase inactivation and initial  
223 denaturation step) and PCR steps of 40 cycles; 95°C for 10s (denaturation), 60°C for 30s  
224 (annealing and extension). Upon completion, dissociation/melting curve analysis were  
225 performed to reveal and exclude non-specific amplification or primer-dimer issues (all melt  
226 analysis in this study presented single reproducible peaks for the reference gene and each target  
227 gene suggesting amplification of a single product). Following initial screening of suitable  
228 reference genes, GAPDH showed the most stable  $C_t$  values across all RT-PCR runs, subjects  
229 and regardless of experimental condition ( $23.54 \pm 1.69 C_t$ ; 7% Co-efficient of variation) and  
230 was selected as the reference gene in all RT-PCR assays. The average PCR efficiency was 90%  
231 and variation for all genes was less than 4.3%. The relative gene expression levels were  
232 calculated using the comparative  $C_t$  ( $\Delta\Delta C_t$ ) equation (41) where the relative expression was  
233 calculated as  $2^{-\Delta\Delta C_t}$  and where  $C_t$  represents the threshold cycle. mRNA expression for all target  
234 genes was calculated relative to the reference gene (GAPDH; subject's own samples reference)  
235 within same subject and condition and to a calibrator of Pre-exercise.

236 SDS-PAGE and Western Blotting. Approximately 30 mg of frozen muscle was homogenized  
237 using 2.4 mm ceramic beaded tubes (6 m/s for 3 x 40 seconds, separated by 5 minutes cooling  
238 on ice; MP Fastprep-24, MP Biomedicals, UK), in 500 µl of ice-cold lysis buffer [25 mM  
239 Tris·HCl (pH 7.4), 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10 mM Na-  
240 pyrophosphatase, 1% Triton X-100] and supplemented with a protease phosphatase inhibitor  
241 cocktail (Halt Protease and Phosphatase Inhibitor 186 Cocktail, Thermo Scientific, # 78442).  
242 The resulting homogenates were centrifuged at 14,000 g for 10 min at 4°C, and the supernatant  
243 was collected. The protein content of the supernatant was determined using a bicinchoninic  
244 acid assay (Sigma, UK). Each sample was diluted with an equal volume of 2X Laemmli buffer  
245 (National Diagnostics) and boiled for 10 min at 100°C. Forty µg of total protein from each  
246 sample was loaded (65 µg for phosphorylated analytes) and then separated in Tris-glycine  
247 running buffer (10x Tris/glycine, Geneflow, Staffordshire, UK) using self-cast 10% separating  
248 [33% Protogel; (30% w/v acrylamide: 0.8% (w/v) Bis-Acrylamide stock solution (37.5:1)),  
249 25% Protogel resolving buffer (1.5M Tris-HCL, 0.4% SDS, pH 8.8), 41% ddH<sub>2</sub>O, 100µl 10%  
250 APS, 20µl TEMED] and 4% stacking [13% Protogel, 25% Protogel Stacking buffer (0.5M Tris  
251 HCL, 0.4% SDS, pH 6.8), 61% ddH<sub>2</sub>O, 100µl 10% APS, 20µl TEMED] gels (National  
252 Diagnostics, Geneflow, UK). Gels were transferred semidry onto nitrocellulose membrane  
253 (Transblot Turbo, BioRad) for 30 min at 25V and 1.0 mA in transfer buffer [10% TRIS/glycine  
254 (Sigma), 20% methanol, 70% ddH<sub>2</sub>O). After transfer, membranes were briefly washed in TBST  
255 (0.19 M Tris pH 7.6, 1.3 M NaCl, 0.1% Tween-20] before being blocked for 1 h at room  
256 temperature in TBST with 1% BSA. The membranes were then washed for 3 x 5 min in TBST  
257 before being incubated overnight at 4°C with antibodies for anti-phospho-AMPK Thr172 (cat  
258 no: 2532), p38 MAPKThr180/Tyr182 (cat no: 9211) (Cell Signalling) as well as total protein  
259 content of AMPK (cat no: 2531), p38 MAPK (cat no: 9212) (Cell Signalling, UK), GAPDH  
260 (25778; Santa Cruz), and PGC-1α (Calbiochem, Merck Chemicals, UK) all at concentrations

261 of 1:1000 in 1 x TBS. The following morning, membranes were washed for a further 3 x 5 min  
262 in TBST and subsequently incubated with goat anti-rabbit horseradish peroxidase-conjugated  
263 secondary antibody (Bio-Rad, UK) for 1h at room temperature. After a further 3 x 5 min washes  
264 in TBST, membranes were exposed in a chemiluminescence liquid (SuperSignal, Thermo  
265 Fisher Scientific, Rockford, IL) for 5 min (30 sec for GAPDH). Membranes were visualized  
266 using a Bio-Rad Chemi-doc system, and band densities were determined using Image Lab  
267 image-analysis software (Bio Rad, UK). Samples from each subject for all exercise conditions  
268 were run on the same gel and statistical analysis conducted on raw densitometry data.  
269 Phosphorylated AMPK<sup>Thr172</sup> and p38 MAPK<sup>Thr180/Tyr182</sup> were normalised to their total protein,  
270 as these did not change significantly across blots or samples ( $P > 0.05$ ). PGC-1 $\alpha$  was  
271 normalised to GAPDH.

272 Statistical analysis. All data are presented as mean  $\pm$  SD, unless otherwise stated. Baseline  
273 data, distance cycled, exercise HR and RPE were compared between conditions using a Paired  
274 Samples T-test. A two-factor (two condition  $\times$  time) within-participants general linear model  
275 was used to evaluate the effect of time (baseline v post exercise) with shared baseline data used  
276 for NOT and CWI (Statistical Package for the Social Sciences version 21.0; SPSS Inc.,  
277 Chicago, IL). A two-factor (three condition  $\times$  time) within-participants general linear model  
278 was subsequently used to evaluate the influence of the cooling intervention following exercise  
279 and the 3h post exercise period. The main effects for condition and time was followed up using  
280 planned LSD multiple contrasts. Where a significant condition by time interaction was  
281 observed, the post exercise to 3h post exercise change scores were calculated and compared  
282 across the 3-conditions using LSD multiple contrasts. The ES magnitude was classified as  
283 trivial ( $<0.2$ ), small ( $>0.2-0.6$ ), moderate ( $>0.6-1.2$ ), large ( $>1.2-2.0$ ) and very large ( $>2.0-4.0$ )  
284 (17). The  $\alpha$  level for evaluation of statistical significance was set at  $P < .05$ .

285

286

## 287 **Results**

288 Exercise Response.

289 Distance cycled (CON  $32.52 \pm 4.21$  km, CWI  $32.33 \pm 4.33$  km;  $P = 0.629$ , ES 0.04 Trivial),  
290 heart rate ( $P = 0.309$ , ES 0.13 Trivial),  $\dot{V}O_2$  (ml/kg.min<sup>-1</sup>;  $P = 0.855$ , ES 0.02 Trivial) and RPE  
291 ( $P = 0.637$ , ES 0.08 Trivial) were similar between CON and CWI trials (data not shown). Mean  
292 HR during the final minute of exercise was  $182 \pm 8$  beats.min<sup>-1</sup> in CON and  $183 \pm 8$  beats.min<sup>-1</sup>  
293 <sup>1</sup> in CWI, equating to ~94% HR max. The RPE in the final exercise bout was  $19 \pm 1$  AU and  
294  $19 \pm 1$  AU in the CON and CWI trials respectively.

295

296 Recovery Response.

297 Metabolic Responses. Heart rate (ES 0.90 Moderate) and  $\dot{V}O_2$ , (ES 1.20 Moderate) was higher  
298 in CWI vs CON during the post-exercise recovery period (Table 2;  $P < 0.001$ ). The change in  
299 HR and  $\dot{V}O_2$ , over time was also different between conditions ( $P < 0.001$ ) with increases in HR  
300 and  $\dot{V}O_2$ , occurring during the initial 2 minutes of immersion. Following immersion, HR and  
301  $\dot{V}O_2$  dropped below pre-immersion values and remained lower throughout the 3h recovery  
302 period (HR, ES  $>0.92$  Moderate;  $\dot{V}O_2$ , ES  $>1.25$  Large) ( $P < 0.05$ ).

303 Thermoregulatory Responses. Rectal temperature was similar between conditions throughout  
304 the post-exercise period (CON  $37.52 \pm 0.24^\circ\text{C}$ , CWI  $37.48 \pm 0.05^\circ\text{C}$ , ES 0.49 Small,  $P = 0.217$ ).

305 The change in rectal temperature over time was different between conditions, with a small  
306 decline in rectal temperature occurring after 3 minutes of immersion until 3h post-exercise  
307 ( $P=0.034$ , ES  $>0.22$  Small). Thigh skin temperature was generally lower throughout the post-  
308 exercise recovery period in CWI versus CON (ES 6.26 Very Large) and NOT (ES 6.46 Very

309 Large) (Figure 1a,  $P < 0.001$ ). The change over time was also different between conditions,  
310 with thigh skin temperature continually decreasing in CWI and remaining lower than pre-  
311 immersion values until 1h post exercise (ES 3.0 Very Large,  $P < 0.001$ ). Values in CON and  
312 NOT limbs remained similar to pre-immersion throughout the 3h recovery period ( $P = 0.10$ ,  
313 ES 0.57 Small).

314 Post-exercise muscle temperature (3 cm depth) was similar between CON ( $38.75^{\circ}\text{C}$ ), CWI  
315 ( $38.86^{\circ}\text{C}$ ) and NOT ( $38.54^{\circ}\text{C}$ ) (Figure 1b;  $P > 0.05$ ). During the 3 h recovery period muscle  
316 temperature (3cm) was lower in CWI versus CON (ES 1.60 Large) and NOT (ES 1.77 Large)  
317 ( $P < 0.001$ ). The change in muscle temperature over time was also different between conditions  
318 ( $P < 0.001$ ). Muscle temperature declined to a large extent immediately after immersion in the  
319 CWI limb, followed by a further gradual reduction during the remaining 3h post-exercise  
320 period ( $P < 0.001$ ). In CON and NOT conditions, muscle temperature was reduced to a lesser  
321 extent immediately following immersion followed by a further gradual reduction during the 3h  
322 post-exercise period ( $P = 0.246$ , ES 0.34 Small) (See Figure 1b).

323 Subjective shivering ratings were greater in CWI vs CON during the post-exercise recovery  
324 period (ES 1.20 Large,  $P = 0.067$ ). The change in shivering over time also tended to be different  
325 between conditions ( $P = 0.062$ ), with 'slight' shivering observed in the CWI condition during  
326 the first 2 minutes following immersion (ES  $>0.60$  Moderate). Slight shivering was also  
327 observed 2h post exercise in the CWI condition (ES 0.95 Moderate).

328 AMPK and P38 MAPK activity and total abundance

329 Phosphorylation of AMPK<sup>Thr172</sup> was not increased post-exercise ( $P = 0.242$ , ES 0.20 Small).  
330 At post-exercise and 3h post-exercise phosphorylation of AMPK<sup>Thr172</sup> was similar between  
331 conditions ( $P = 0.846$ , ES 0.03 Trivial). However, the change in AMPK<sup>Thr172</sup> between these  
332 time points was different between conditions ( $P = 0.031$ ; Figure 2). AMPK<sup>Thr172</sup>

333 phosphorylation increased in CWI vs. CON (P = 0.027, ES 1.22 Large) with a moderate  
334 increase in AMPK<sup>Thr172</sup> phosphorylation also observed in NOT vs. CON (P = 0.145, ES 0.70  
335 Moderate). Representative Western blots are shown in Figure 5.

336 Exercise induced a small increase in phosphorylation of p38MAPK<sup>Thr180/Tyr182</sup> (P = 0.056, ES  
337 0.44 Small, Figure 3). At post exercise and 3h post-exercise phosphorylation of  
338 p38MAPK<sup>Thr180/Tyr182</sup> was similar between conditions (P = 0.672; ES 0.03, Trivial). No  
339 differences in the change in phosphorylation between these time points was observed between  
340 conditions (P = 0.268, Figure 3). Representative Western blots are shown in Figure 5.

341 PGC-1alpha mRNA and protein abundance.

342 PGC-1 $\alpha$  mRNA expression was moderately increased with exercise (P = 0.066, ES 0.92  
343 Moderate, Figure 4a). At 3h post-exercise, expression was greater in CWI (ES 1.2 Moderate,  
344 P = 0.003) and NOT (ES 1.6 Large, P = 0.001) versus CON, but was similar between CWI and  
345 NOT (ES 0.6 Small, P = 0.141) (Figure 4a). This reflected the greater change in expression in  
346 CWI and NOT conditions between post exercise and 3h post exercise time points (P = 0.001,  
347 Figure 4a). PGC-1 $\alpha$  protein content was not influenced by exercise (P = 0.092) or CWI (P =  
348 0.471, Figure 4b). Representative Western blots are shown in Figure 5.

349 Additional gene expression

350 Exercise induced increases in SIRT1 (P = 0.057, ES 0.8 Moderate) and NRF2 (P = 0.028, ES  
351 0.6 Moderate) mRNA (data not shown). No changes were seen between conditions, or between  
352 conditions over time (P > 0.05). Gene expression analysis for p53, COXIV, CS, TFAM, SIRT1,  
353 NRF2 and ERR $\alpha$  mRNA was not influenced by exercise or CWI (P > 0.05; data not shown).

354

355 Plasma Metanephrine and NorMetanephrine.



356 Metanephrine concentrations were similar between conditions ( $P = 0.159$ , ES 0.15 Trivial).  
357 The change in metanephrine over time was also similar between conditions ( $P = 0.299$ ).  
358 Metanephrine concentration was increased post-exercise (ES 2.46 Very Large) and post-  
359 immersion (ES 0.77 Moderate) vs. baseline ( $P \leq 0.02$ ). Normetanephrine values were greater  
360 in CWI vs. CON ( $P = 0.034$ , ES 0.43 Small) with the largest difference seen post-immersion  
361 (860 vs. 665 pmol/L, CWI vs. CON, respectively). The change in Normetanephrine over time  
362 was similar between conditions ( $P = 0.821$ ). Normetanephrine concentrations increased with  
363 exercise (ES  $>4.70$ ;  $P < 0.001$ ) and remained above baseline post-immersion (ES 1.52 Large)  
364 and 1hr post-exercise (ES 1.06 Moderate) ( $P < 0.001$ ). Concentrations returned to baseline at  
365 2hr post-exercise (See Table 3).

366

## 367 **Discussion**

368 The aim of the present study was to examine whether the post-exercise cold-water induced  
369 increase of PGC-1 $\alpha$  mRNA is mediated through local or systemic mechanisms. Using a novel  
370 experimental design, we report for the first time the appearance of systemic “cross-talk”  
371 between immersed and non-immersed limbs, as evidenced by the similar increase in PGC-1 $\alpha$   
372 mRNA in these limbs after single-legged CWI. Additionally, we suggest that this effect could  
373 be mediated by  $\beta$ -adrenergic induced stimulation of AMPK. In addition to providing novel  
374 data on the potential mechanisms mediating post-exercise cold-induced enhancement of PGC-  
375 1 $\alpha$  expression, our data also have potential implications for research designs that utilise as non-  
376 immersed limbs as control conditions.

377 Since its initial discovery (38) the importance of the post-exercise PGC-1 $\alpha$  response to the  
378 oxidative adaptive process has been examined extensively, with mRNA increases ranging 5 to  
379 10-fold commonly observed at 3-4 hours following exercise (3, 4, 21, 36). More recently, cold-

380 ambient temperatures (42, 43) and post exercise cold-water immersion (18-20, 24), have also  
381 been shown to enhance (~2- to 4- fold greater) the skeletal muscle PGC-1 $\alpha$  gene transcription  
382 and protein translational response versus exercise alone. In line with such observations, the  
383 high-intensity intermittent cycling protocol used in the present study elicited a ~5-fold increase  
384 in PGC-1 $\alpha$  mRNA at 3 hours post-exercise. Furthermore, CWI enhanced this response to  
385 a greater extent than exercise alone (~9-fold increase vs. Pre in the CWI immersed limb).

386 There have been suggestions that reduced tissue temperature may be responsible for the  
387 differences observed between cold-treated and control limbs. This stems from the initial  
388 discovery that PGC-1 $\alpha$  was cold inducible in animals (6, 9, 38). In humans, recent data  
389 from Ihsan and colleagues (18, 20) implicates a reduction in tissue temperature in the cold-  
390 induced increases of PGC-1 $\alpha$ , as increases in mRNA (3h post-exercise) and protein content  
391 (after 4 weeks of training) were seen only in a cooled limb, and not in the contralateral non-  
392 immersed limb. Potential mechanisms underpinning such responses include activation of non-  
393 noxious thermoreceptors via reduced skin temperature (Hensel & Boman, 1960, 22). Within  
394 the present study, both the NOT and CWI limbs displayed similar acute PGC-1 $\alpha$  mRNA  
395 expression in the 3 h recovery period, whilst skin and muscle temperature were significantly  
396 reduced in the CWI limb only. Indeed, the non-immersed limb (NOT) showed a similar  
397 temperature profile (skin and muscle temperature) to that of CON, where the magnitude of  
398 PGC-1 $\alpha$  mRNA response was almost half when compared with CWI and NOT. When taken  
399 together, these data suggest that alterations to local muscle temperature do not play a significant  
400 role in cold-induced regulation of PGC-1 $\alpha$  expression.

401 As a result of increased local PGC-1 $\alpha$  gene expression in the non-immersed exercised limb to  
402 a similar magnitude as the immersed limb, we sought to consolidate the role of the upstream  
403 kinases AMPK and p38-MAPK (7, 23, 47) in their ability to regulate PGC-1 $\alpha$  transcription.  
404 p38MAPK is a stress activated kinase that has been shown extensively to be phosphorylated

405 after acute exercise, independent of intensity (10). Moreover, p38MAPK can exert its effect  
406 upon PGC-1 $\alpha$  transcription via upregulated ATF2 activity at the PGC-1 $\alpha$  promoter (2). In the  
407 current study, exercise induced a small (ES 0.44, 1.5-fold) increase in phosphorylation with no  
408 further response to cooling. Moreover, our data supports previous data showing acute post-  
409 exercise phosphorylation of p38MAPK locally in skeletal muscle tissue occurs systemically  
410 (46). Exercise-induced intensity dependant AMPK phosphorylation is a well reported  
411 phenomenon (8, 12, 28) in rodent and human studies (34, 10). Moreover, AMPK is implicated  
412 in PGC-1 $\alpha$  activity via direct phosphorylation, initiating many of the important gene regulatory  
413 functions of PGC-1 $\alpha$  in skeletal muscle (23). The post-exercise increase in phosphorylation of  
414 AMPK in the present study was similar in magnitude to previous work (3) from our laboratory,  
415 albeit failing to achieve statistical significance. Notwithstanding this, large and moderate effect  
416 sizes were observed at 3 h post-exercise in the CWI and NOT limbs vs. CON, respectively.  
417 These greater increases from post-exercise to 3 h post-exercise in the immersion trial (CWI  
418 and NOT limbs), compared to a slight decline in CON suggest the increases in phosphorylation  
419 of AMPK during the post-cooling period are controlled by a systemic mechanism, possibly  
420 adrenergic control via cold-augmented plasma Normetanephrine.

421 Epinephrine and norepinephrine are both dual  $\alpha$ - and  $\beta$ - adrenergic agonists. Previous in vivo  
422 and in vitro incubation techniques utilising  $\alpha$ - and  $\beta$ - adrenergic agonists have reported  
423 increased AMPK activation in rodent skeletal muscle (32), adipose tissue (26) and cell cultures  
424 (48) implicating catecholamines as a potential AMPK activator. Despite this, support for the  
425 above hypothesis is currently conflicting, and is limited by distinct differences in  
426 methodological design and species studied. In rodent muscle, acute infusion of adrenergic  
427 agonists/antagonists have previously shown to be ineffective at altering PGC-1 $\alpha$  transcriptional  
428 activity (39, 40) and its upstream effector p38-MAPK (25). In contrast, treatment with  $\beta$ -  
429 adrenergic agonists/antagonists has been shown to induce and inhibit PGC-1 $\alpha$  respectively

430 (31), whilst incubation of rodent skeletal muscle with the adrenergic agonist phenylephrine  
431 increased the activity of the upstream regulator of PGC-1 $\alpha$ , AMPK (32). When results from  
432 these studies are taken together, it could be suggested that adrenergic activation of AMPK is a  
433 potential mechanism to explain the systemic increases in PGC-1 $\alpha$  gene expression described  
434 herein.

435 In humans, one study has investigated the impact of higher catecholamine levels on AMPK  
436 phosphorylation in human skeletal muscle (27). These authors assessed muscle biopsies from  
437 an exercised and non-exercised limb in conditions of heightened catecholamine release. Results  
438 showed AMPK activity was restricted to contracting muscle only, with no systemic effects  
439 notable in the non-exercised limb despite the increased catecholamine levels. Importantly, in  
440 our study all limbs were exercised before undergoing cold exposure. It therefore may be that  
441 the cold induction of  $\beta$ -adrenergic pathways (via increased catecholamines) presented in this  
442 manuscript allows an additive response to exercise stimulated AMPK phosphorylation. Further  
443 studies are now required to verify this signalling response in related experimental conditions  
444 in human skeletal muscle.

445 Another pathway by which increased catecholamine's may enhance PGC-1 $\alpha$  transcription is  
446 via increased activation of  $\beta$ -adrenergic receptors. Activation of these receptors increases  
447 intracellular cAMP, which could ultimately activate CREB function on the PGC-1 $\alpha$  promoter  
448 (1). However, evidence exists to show  $\beta$ -adrenergic stimulation does not activate a p38MAPK  
449 - ATF2 - CREB - PGC-1 $\alpha$  signalling axis in skeletal muscle (25). These findings resonate with  
450 results from the present study as no changes in phosphorylation of p38MAPK were observed  
451 alongside increased plasma Normetanephrine concentrations. Further work is required to  
452 investigate the influence of the dual stress of exercise and cold temperature upon this signalling  
453 axis.

454 Downstream of PGC-1 $\alpha$ , Slivka and colleagues (42) noted that recovery in cold ambient  
455 temperatures (4h in 7°C ambient air) reduced the expression of the transcription factors  
456 Nuclear-respiratory factor 2 (NRF2) and estrogen-related receptor  $\alpha$  (ERR $\alpha$ ), whilst having no  
457 effect on mitochondrial transcription factor a (TFAM). Their importance to the adaptive  
458 response is highlighted by their roles in oxidative metabolism. Our data suggest the immersion  
459 protocol used herein was not sufficient to induce such changes. Indeed, 10 minutes of single-  
460 legged immersion offers a much smaller cooling stimulus than the 4 hours in cool ambient  
461 temperatures, as used by Slivka et al. (42). In addition, an acute increase in PGC-1 $\alpha$  gene  
462 expression was not followed by changes in PGC-1 $\alpha$  total protein content, perhaps due to the  
463 acute time-frame of sampling applied in the present study. With this lack of change in total  
464 protein content it is however, unlikely that changes in gene expression of downstream genes  
465 such as NRF2, TFAM, COXIV, CS, ERR $\alpha$  presented here, would be affected by its upstream  
466 protein function as a transcription factor (PGC-1 $\alpha$ ). More research is required to understand  
467 the effect and dose response of a cold stimulus on downstream targets of PGC-1 $\alpha$ .

468 It is difficult to explain the differences in PGC-1 $\alpha$  mRNA results between the present study  
469 and Ihsan et al. (20), particularly the difference in non-immersed limbs. Moreover, reasons as  
470 to why Ihsan and colleagues failed to see the expected exercise-induced response in their non-  
471 immersed control limb, with minor changes from baseline at 3h post-exercise (1.5-fold increase  
472 vs. 3-5-fold increase usually seen), remain unclear. One possible explanation might include the  
473 differing muscle recruitment patterns occurring between the exercise protocols utilised (cycling  
474 vs. running). Ultimately, results from the present study have future implications on scientific  
475 study designs. Those wishing to investigate cold induced post-exercise responses in skeletal  
476 muscle must be aware of the evidence that implicates a systemic response of Normetanephrine,  
477 and a local response of p-AMPK in both immersed and non-immersed limbs. Further evidence  
478 is required to support the impact of systemic transcriptional responses in unilateral research

479 designs, as such designs may be liable to error; potentially underestimating the actual response  
480 occurring in the immersed limb if relativized to a non-immersed limb instead of a resting  
481 control. Ultimately, the choice of scientific design lies with the question posed, as contralateral  
482 designs remain useful for understanding both local and systemic responses.

483 In summary, the present study characterises for the first time the mechanistic control of cold  
484 induced PGC-1 $\alpha$  mRNA expression. Data herein indicate a reduction in tissue temperature (2-  
485 3°C) plays a limited role as similar levels of PGC-1 $\alpha$  mRNA expression are observed in an  
486 immersed and non-immersed limb despite a reduction in tissue temperature in the CWI limb  
487 only. Moreover, a cold-induced systemic increase in plasma Normetanephrine may impact  
488 localised phosphorylation events of the signalling kinase AMPK<sup>Thr172</sup>, with potential  
489 downstream effects upon rates of PGC-1 $\alpha$  mRNA expression. Future studies should investigate  
490 the role of  $\beta$ -adrenergic receptors in Normetanephrine induced AMPK phosphorylation and the  
491 signalling role of MEF2 and CRE/ATF2 sites to confirm a link between catecholamines and  
492 PGC-1 $\alpha$ . Moreover, due to the acute nature of the present study more work is required to  
493 investigate whether the response seen herein is maintained over a more chronic term.

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#### 499 **Disclosures**

500 The authors report no conflicts of interest.

501

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