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**Cool-water immersion reduces post-exercise quadriceps femoris muscle
perfusion more than cold-water immersion**

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Running Head: Muscle perfusion after cold-water immersion

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ABSTRACT

Purpose: The muscle perfusion response to post-exercise cold water immersion (CWI) is not well understood. We examined the effects of graded post-exercise CWI upon global and regional quadriceps femoris muscle perfusion using positron emission tomography (PET) and [^{15}O]H $_2$ O.

Methods: Using a matched-group design, 30 healthy men performed cycle ergometer exercise at 70% $\dot{V}\text{O}_{2\text{peak}}$ to a core body temperature of 38°C, followed by either 10 min of CWI at 8°C, 22°C or seated rest (control). Quadriceps muscle perfusion, thigh and calf cutaneous vascular conductance (CVC), intestinal, muscle, and local skin temperatures, thermal comfort, mean arterial pressure, and heart rate were assessed at pre-, post-exercise and following CWI.

Results: Global quadriceps perfusion was reduced beyond the pre-defined minimal clinically relevant threshold (0.75 mL·100 g·min $^{-1}$) in 22°C water versus control (difference [95% confidence interval (CI)]: -2.5 mL·100 g·min $^{-1}$ [-3.9 to -1.1]). Clinically relevant decreases in muscle perfusion were observed in the rectus femoris (-2.0 mL·100 g·min $^{-1}$ [-3.0 to -1.0]) and vastus lateralis (VL; -3.5 mL·100 g·min $^{-1}$ [-4.9 to -2.0]) in 8°C water, and in the vastus lateralis (-3.3 mL·100 g·min $^{-1}$ [-4.8 to -1.9]) in 22°C water versus control. The mean effects for vastus intermedius and vastus medialis perfusion were not clinically relevant. Clinically relevant decreases in thigh and calf CVC were observed in both cooling conditions.

Conclusion: The present findings revealed that less noxious CWI (22°C) promoted clinically relevant post-exercise decreases in global quadriceps muscle perfusion whereas noxious cooling (8°C) elicited no effect.

Key words: Cooling; Recovery; Blood flow; Exercise

1 INTRODUCTION

2 Cold-water immersion (cryotherapy) is widely applied after strenuous exercise to
3 facilitate recovery from exercise-induced muscle damage (1). It has been suggested that the
4 physiological effects associated with cryotherapy are partly underpinned by reductions in
5 microvascular blood flow to the exercised/injured muscle (2), which then subsequently reduce
6 edema and induction of inflammatory events (3). Given the potential importance of changes in
7 muscle perfusion in mediating the effects of post-exercise cold-water immersion on recovery,
8 further investigation is warranted to enhance the efficacy of such intervention strategies.

9 We and others have conducted a number of studies using continuous Doppler
10 ultrasound assessments of the femoral artery alongside simultaneous measures of cutaneous
11 blood flow, and demonstrated that limb blood flow at rest and following exercise can be
12 markedly reduced by cold-water immersion (4, 5, 6). These findings are consistent with other
13 studies, which employed venous occlusion plethysmography (7) and near infrared
14 spectroscopy (NIRS; 4, 8, 9). However, the above-mentioned techniques are limited by their
15 inability to provide a direct assessment of perfusion changes within the muscle, and therefore
16 permit only qualitative and indicative interpretations of the efficacy of cold-water immersion.

17 Recently, under resting conditions, we used positron emission tomography (PET) with
18 an oxygen-15-labelled water radiotracer ($[^{15}\text{O}]\text{H}_2\text{O}$) to provide a quantitative assessment of
19 quadriceps femoris muscle perfusion to different degrees of cold-water immersion applied over
20 10 minutes (10). We reported, for the first time, increased perfusion in deeper lying quadriceps
21 muscle following noxious (8°C) cold-water immersion, whereas superficial quadriceps muscle
22 perfusion was reduced in cooler (15°C) water. Furthermore, work from our laboratory
23 combining Doppler artery ultrasound alongside simultaneous cutaneous blood flow measures,
24 has indicated that the hemodynamic response to varying water immersion temperatures (8°C
25 and 22°C for 10 min) is different under resting (11) and post-exercise conditions (5, 12).

26 Moreover, blunting of the vascular response to sympathetic stimulation during exercise and
27 whole-body heat stress (13, 14, 15) may persist following exercise (13) and modify the muscle
28 perfusion response to cooling. Therefore, quantitatively determining the muscle perfusion
29 response to post-exercise cooling is necessary.

30 We aimed to determine the effects of post-exercise lower body cooling with 8°C and
31 22°C water on global and regional quadriceps muscle perfusion, using [¹⁵O]H₂O and PET
32 imaging. We hypothesised that 8°C and 22°C water would elicit a similar reduction in muscle
33 perfusion in deep-lying and superficial quadriceps muscles following exercise.

34

35

36 **METHODS**

37 **Ethical Approval**

38 The Ethical Committee of the Hospital District of South-Western Finland approved this
39 study, with all study procedures performed in accordance with the standards set by the latest
40 revision of the declaration of Helsinki. All test procedures and potential risks were fully
41 explained prior to attaining each participant's written informed consent to participate.

42

43 **Participants**

44 Thirty recreationally active healthy males (means ± SD: age, 33 ± 8 yrs; body mass,
45 80.9 ± 9.5 kg; height, 183.9 ± 4.7 cm; percentage body fat, 12.9 ± 5.3%; $\dot{V}O_{2peak}$, 47.4 ± 8.1
46 mL·kg⁻¹·min⁻¹; peak power output on cycle ergometer (PPO), 343 ± 45 W) volunteered to
47 participate. The participants were requested to abstain from alcohol and caffeine containing
48 beverages for at least 24 h before the commencement of the experiments, and to avoid strenuous
49 exercise within 48 h of commencing the experimental protocol. Participants were screened for
50 history of cardiovascular disease, neurological disease, and skeletal muscle abnormality, and
51 were excluded if currently prescribed pharmacological medication.

52 **Study Design**

53 The present investigation formed part of a larger research project, which also examined
54 muscle perfusion under resting conditions using the same participant cohort (10). The design
55 adopted a principled approach to planning (16), with sample size decisions established on cost-
56 efficiency information and procedures relevant to subject condition allocation from existing
57 parallel-arm experiments in this area of research (17). After undertaking preliminary
58 assessments on their initial visit to the hospital, the participants were randomly allocated to one
59 of the three conditions: 8°C water immersion, 22°C water immersion, or a control (rest in a
60 semi reclined position), using covariate adaptive randomization (18). The nature of performing
61 repeated PET/CT measures has ethical considerations in regards to radioactive exposure limits
62 and invasive arterial cannulation. Therefore, a between subject design was employed to meet
63 the necessary ethical requirements, with the groups ($n = 10$) matched for confounding
64 covariates ($\dot{V}O_{2peak}$, height, body mass, body surface area, muscle mass and thigh skinfold
65 thickness), which could potentially influence changes in muscle perfusion (Table 1).

66

67 **Experimental Protocol**

68 The participants attended the hospital on two separate occasions: the first visit was a
69 preliminary test day to familiarize the participants with the experimental protocol, enable
70 anthropometric measurements to be taken, and to assess peak oxygen uptake ($\dot{V}O_{2peak}$). The
71 anthropometric assessments included taking measurements of the participants' height (KaWe
72 stadiometer, Asperg, Germany), body mass (Seca 703 electronic scales, Seca, Hamburg,
73 Germany), and limb circumferences at the right mid-thigh, forearm, and calf (Seca 201 tape
74 measure, Seca, Hamburg, Germany) (19). These measurements were subsequently used to
75 provide an estimation of each participant's muscle mass (20). In addition, skinfold measures
76 (HSK BI calipers; Baty International, West Sussex, U.K.) were taken across 7-sites (21) to

77 permit the calculation of each participant's body fat percentage (%Bfat) (22). Next, and as
78 previously described (10), a maximal incremental cycling protocol (Tunturi Ergometer E85,
79 Tunturi, Finland) was completed until volitional exhaustion was attained to enable the
80 assessment of each participant's Peak Power Output (PPO) and $\dot{V}O_{2peak}$ ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$).

81 On the second visit to the hospital, the participants were asked to undertake a number
82 of preparatory steps before conducting the main experimental test procedures. The participants
83 were asked to fast overnight, ingest a disposable temperature sensor pill (CorTemp, Human
84 Technologies Inc., Florida, USA) immediately prior to sleeping, and consume 5 mL·kg
85 bodyweight of water within two hours prior to arrival at the hospital (arrival: 0700-0800) to
86 help maintain hydration status (23). After changing into a pair of shorts, the participants were
87 asked to lay semi-reclined on a hospital bed to enable the attachment of equipment: heart rate
88 telemetry belt (Polar M400, Kempele, Finland), laser Doppler probes, and skin temperature
89 thermistors. An anaesthesiologist then cannulated the radial artery under local anaesthesia to
90 permit blood sampling during PET measurements. After providing ≥ 20 min to ensure
91 physiological status was stabilised, baseline thermometry measures were taken. The skin
92 thermistors were then unattached and the participant was taken by wheelchair to another room
93 (temperature $\sim 21.6^{\circ}\text{C}$) to undergo simultaneous PET/CT and laser Doppler measures.

94 In the same room (next to the PET/CT scanner), each participant was then asked to
95 undertake a submaximal exercise protocol on a cycle ergometer (Tunturi Ergometer E85,
96 Tunturi, Finland) at 70% $\dot{V}O_{2peak}$ until a core temperature of 38°C was obtained. This core
97 temperature was selected to examine whether a relatively small thermal load could override an
98 increase in deep muscle perfusion (speculated due to shivering) observed in cold-water (8°C)
99 under resting conditions (10). Upon completion, the participants were moved to the adjacent
100 PET/CT scanner to undertake post-exercise muscle perfusion measurements. Next, each
101 participant was then taken by wheelchair to undergo the assigned experimental treatment. The

102 skin thermistors were then re-attached and the participants were either immersed in a semi-
103 reclined position up to the navel in an inflatable water bath (iSprint, iCool, Queensland,
104 Australia) for 10 min, or rested in a semi-reclined position for the same duration (control).
105 Dependent on the participant's group allocation, the water temperature was pre-set to one of
106 the two temperatures ($8.8\pm 0.7^{\circ}\text{C}$, $21.8\pm 0.7^{\circ}\text{C}$), using a heating/chiller water system (Boyu CW
107 Series, Guangdong, China); and validated using a skin thermistor (MHF-18050-A, Ellab,
108 Rodovre, Denmark). Upon removal from the immersion bath, the participant's legs were
109 carefully dried as not to stimulate blood flow, and taken by wheelchair to undergo PET and
110 laser Doppler measures (commenced 10 min post-immersion). Post-immersion thermometry
111 measures were subsequently recorded. Heart rate was continuously measured, and ratings of
112 perceived exertion (RPE) (24) was recorded during the exercise protocol.

113

114 **Thermometry**

115 The temperature measures taken in this study (core, muscle, skin) are similar to that
116 described in our recent work (10). Briefly, after initially checking that the ingestible core
117 temperature sensor pill was located in the gastrointestinal tract, a data logger was positioned at
118 the waist (or near to) to permit continuous temperature measures during immersion, exercise
119 and PET/CT scans. Local skin temperature was measured at four sites (chest, forearm, thigh
120 and calf) using skin thermistors (MHF-18050-A, Ellab, Rodovre, Denmark), thus allowing for
121 weighted mean skin temperatures to also be calculated (25). Thigh muscle temperature was
122 assessed by initially measuring thigh skinfold thickness with calipers (HSK BI; Baty
123 International, West Sussex, U.K.) and dividing by 2 to take into account the subcutaneous fat
124 overlaying the vastus lateralis muscle. A needle thermistor (13050; Ellab, Rodovre, Denmark)
125 was then inserted into the vastus lateralis to a depth of 3 cm plus one-half of the skinfold
126 measurement to represent deep muscle temperature (26). Upon the values stabilizing, the

127 temperature was recorded using an electronic measuring system (CTF-9004, Ellab, Rodovre,
128 Denmark). The thermistor was then withdrawn at 1 cm increments and temperature was
129 recorded at 2 cm and 1 cm depths below the subcutaneous layer. Muscle temperature was
130 measured at baseline, pre-immersion, and post immersion.

131

132 **Blood Flow Measurements**

133 As recently described (10), positron emitting isotope [^{15}O] was produced using a
134 Cyclone 3 cyclotron (IBA Molecular, Belgium) to produce the radiowater tracer ($[^{15}\text{O}]\text{H}_2\text{O}$).
135 A PET/CT scanner (STE General Electric Medical systems, Milwaukee, USA) was used in
136 three-dimensional (3D) mode for image acquisition to measure muscle perfusion with
137 $[^{15}\text{O}]\text{H}_2\text{O}$. A dynamic PET scan (6 min) commenced 20 seconds after an intravenous injection
138 of ~ 455 MBq of $[^{15}\text{O}]\text{H}_2\text{O}$, with dynamic scanning performed in the following subsequent time
139 frames: 6x5 seconds, 12x10 seconds, 7x30 seconds and 12x10 seconds.

140 Input function was obtained from arterial blood, which was continuously withdrawn (5
141 $\text{ml}\cdot\text{min}^{-1}$) using an electronically operating pump during the PET scans. A two-channel online
142 detector system (Scanditronix, Uppsala, Sweden), cross-calibrated with an automatic gamma
143 counter (Wizard 1480 3", Wallac, Turku, Finland) and the PET scanner, measured radioactivity
144 concentration in blood. Arterial function was pre-processed with a delay correction. A 1-tissue
145 compartment model subsequently measured muscle perfusion. Image data analysis was
146 performed using an in-house developed program package (Carimas software,
147 <http://www.turkupetcentre.fi/carimas>), with muscle perfusion determined in a blinded fashion
148 by the same individual for the specific regions of the right quadriceps muscle group (rectus
149 femoris, vastus lateralis, vastus intermedius and vastus medialis). Blood pressure and MAP
150 were recorded using a blood pressure monitor (Apteq AE701f, APTEQ, Finland) during the
151 final 1 min of each PET scan.

152 As previously described (10), integrated laser Doppler probes (Probe 455; Perimed,
153 Suffolk, U.K) were attached to thigh and calf sites to permit skin blood flow (red blood cell
154 flux) recordings via laser Doppler flowmetry (Periflux System 5001; Perimed Instruments,
155 Jarfalla, Sweden). The probes were unattached from the Doppler flowmetry unit during
156 exercise and immersion, however remained in situ on skin throughout the experimental testing.
157 Thigh and calf cutaneous vascular conductance (CVC) was calculated using laser Doppler
158 perfusion units (PU) and MAP (27) and expressed in percentage units as the difference between
159 the natural logarithms of PU and MAP to address the potential allometric relationship between
160 these variables.

161

162 **Statistical Analysis**

163 Summary statistics are presented as mean \pm SD for post-exercise data. Using a
164 constrained longitudinal model framework (28), within-subject linear mixed modelling with
165 restricted maximum likelihood and an unstructured covariance structure estimated post-
166 immersion *versus* post-exercise mean differences for primary and secondary outcome measures
167 between conditions. Primary outcome measures were global and individual muscle perfusion
168 and skin blood flow indices. Secondary outcome measures were MAP, heart rate, intestinal
169 temperature, mean skin temperature, thigh skin temperature, muscle temperature, and thermal
170 comfort. Condition, time, condition \times time interaction term and the post-exercise value of the
171 outcome were included as fixed effects, with individual specified as random effect plus a
172 random intercept. Standard residual diagnostics were undertaken to assess model specification
173 based on visual inspection of residual plots (29). The condition \times time interaction term
174 quantified post-immersion between-condition mean effects were interpreted against predefined
175 minimally clinically important differences (MCID) of 0.75 mL \cdot 100g \cdot min⁻¹ for muscle
176 perfusion (based upon a comparable reduction in resting muscle perfusion with nitric oxide

177 synthase inhibition) (30) and 19% CVC reduction in skin blood flow measures (5, 6, 12) with
178 no multiplicity adjustment (31). Effects were declared clinically relevant based on the location
179 of the 95% confidence interval (CI) for the between-condition mean difference to the
180 predefined MCID (32) and presented using density strips to illustrate the degree of uncertainty
181 surrounding the point estimates (33). Mean effects for the between-condition differences in
182 cardiovascular and thermoregulatory outcomes were interpreted as descriptive statistics based
183 on non-zero overlap of the 95%CI for the point estimate and presented with the respective *P*
184 values (34). Post-immersion versus post-exercise effects for CVC measures were summarised
185 as geometric mean differences. All analyses were performed using the MIXED procedure in
186 SAS OnDemand for Academics (SAS Institute[®]) and figures were produced using R (version
187 3.6.3, R Foundation for Statistical Computing).

188

189 **RESULTS**

190 *Exercise Protocol*

191 The exercise duration to attain a core temperature of 38°C was similar between
192 conditions (mean ± SD: 8°C, 17.2 ± 8.8 min; 22°C, 21.9 ± 6.23 min; control, 19.8 ± 6.1 min;
193 *P* = 0.420).

194

195 **Primary Outcome Measures**

196 *Muscle Perfusion*

197 Post-exercise and post-immersion muscle perfusion and temperature raw data are
198 illustrated in Table 2. The difference in global quadriceps muscle perfusion was clinically
199 relevant for 22°C versus control conditions (-2.5 mL·100g·min⁻¹; 95% CI: -3.9 to -1.1, *P* =
200 0.001; Figure 1) in relation to the 0.75 mL·100g·min⁻¹ MCID. There were no clinically relevant

201 differences in global quadriceps perfusion between the other cooling conditions ($P = 0.026$ to
202 0.214 ; Figure 1).

203 A clinically relevant decrease in rectus femoris ($-2.0 \text{ mL}\cdot 100\text{g}\cdot \text{min}^{-1}$; 95% CI: -3.0 to -
204 $1.0 \text{ mL}\cdot 100\text{g}\cdot \text{min}^{-1}$; $P < 0.001$) and vastus lateralis ($-3.5 \text{ mL}\cdot 100\text{g}\cdot \text{min}^{-1}$; 95% CI: -4.9 to -2.0
205 $\text{mL}\cdot 100\text{g}\cdot \text{min}^{-1}$; $P < 0.001$) muscle perfusion was observed in the 8°C versus control conditions
206 (Figure 2B). A clinically relevant decrease in vastus lateralis muscle perfusion was also
207 observed in the 22°C versus control conditions ($-3.3 \text{ mL}\cdot 100\text{g}\cdot \text{min}^{-1}$; 95% CI: -4.8 to -1.9
208 $\text{mL}\cdot 100\text{g}\cdot \text{min}^{-1}$; $P < 0.001$; Figure 2C). There were no clinically relevant differences in vastus
209 intermedius ($P = 0.014$ to 0.784 ; Figure 2) or vastus medialis ($P = 0.028$ to 0.414 ; Figure 2)
210 muscle perfusion irrespective of the experimental group.

211

212 *Skin Blood Flow*

213 There was a clinically relevant reduction in thigh CVC observed between the 8°C
214 versus control (-69.3% ; 95% CI: -76.1 to -60.7% ; $P = 0.001$; Figure 3A) and 22°C versus
215 control conditions (-52.1% ; 95% CI: -62.9 to -38.1% ; $P < 0.001$ Figure 3A) when compared to
216 the predefined -19% MCID. A clinically relevant reduction in calf CVC was also found
217 between the 8°C versus control (-57.1% ; 95% CI: -66.0 to -45.8% ; $P < 0.001$; Figure 3B) and
218 8°C versus 22°C conditions (-36.4% ; 95% CI: -50.0 to -19.0% ; $P < 0.001$; Figure 3B),
219 respectively.

220

221 **Secondary Outcome Measures**

222 *Muscle Temperature*

223 At 1 cm depth, the change in muscle temperature was -4.3°C (95% CI: -5.3 to -3.4°C
224 $P < 0.001$) for the 8°C versus control condition, and -2.1°C (95% CI: -2.9 to -1.2°C ; $P < 0.001$)
225 for the 22°C versus control condition (Figure 4A). At 2 cm depth, the change in muscle

226 temperature was -3.3°C (95% CI: -3.7 to -2.8°C ; $P<0.001$) for the 8°C versus control condition,
227 and -1.2°C (95% CI: -1.6 to -0.7°C ; $P<0.001$) for the 22°C versus control condition (Figure
228 4B). At 3 cm depth, a larger change in muscle temperature was observed for the 8°C versus
229 control (-1.9°C ; 95% CI: -2.3 to -1.5°C ; $P<0.001$) compared with 22°C versus control (-0.7 ;
230 95% CI: -1.1 to -0.3°C ; $P<0.001$) conditions (Figure 4C).

231

232 *Intestinal and Skin Temperature*

233 The mean change in thigh skin temperature (Figure 5) was 3.9°C ; (95% CI: -4.4 to -
234 3.4°C ; $P<0.001$) for the 8°C versus control condition, and was larger than effects for the 22°C
235 versus control condition (-2.6°C ; 95% CI: -3.1 to -2.1°C ; $P<0.001$). The change in mean body
236 temperature was -0.9°C (95% CI: -1.1 to -0.6°C ; $P<0.001$) for the 8°C versus control, and -
237 0.5°C (95% CI: -0.8 to -0.2°C ; $P<0.001$) for 22°C versus control conditions (Figure 5). There
238 were no clear differences in intestinal temperature or mean skin temperature between any group
239 comparisons (Figure 5).

240

241 *Mean Arterial Pressure and Heart rate*

242 The change in MAP for the 8°C versus control condition was 6 mmHg (95% CI: 2 to
243 10 mmHg; $P=0.003$), whereas effects were trivial for the 22°C versus control (-1 mmHg; 95%
244 CI: -5 to 3 mmHg; $P=0.727$). The change in MAP for the 8°C versus 22°C condition was 7
245 mmHg (95% CI: 3 to 11 mmHg; $P=0.011$). There were no clinically relevant differences in
246 heart rate responses between any group comparisons.

247

248 **DISCUSSION**

249 We demonstrated, for the first time, that non-noxious (22°C) cold-water immersion was
250 more effective than noxious cooling (8°C) for reducing global quadriceps muscle perfusion

251 beyond a clinically relevant threshold after exercise. The difference in the magnitude of
252 reduction in global perfusion between the cooling conditions was reflected in the profound
253 effect that colder water (8°C) had on maintaining deeper vastus intermedius and vastus medialis
254 muscle perfusion, while similar reductions in perfusion were observed in both cooling
255 conditions across superficial muscles (rectus femoris & vastus lateralis). These findings have
256 practical implications for practitioners who apply cold-water immersion after exercise to
257 facilitate recovery.

258 The present study is the first to directly and quantitatively determine the perfusion
259 response to post-exercise cooling. In contrast to 8°C immersion, the application of cool water
260 (22°C) reduced global quadriceps muscle perfusion beyond a clinically relevant threshold (>
261 0.75 mL·100g·min⁻¹; Figure 1). The observed difference in global quadriceps perfusion
262 between cooling conditions post-exercise contrasts with previous work from our laboratory (5,
263 6, 12) and with others (4) that employed simultaneous Doppler ultrasound alongside cutaneous
264 blood flow measurements [4, 5, 6, 12) and NIRS (4) to provide indirect estimates of muscle
265 perfusion. While we previously reported similar reductions in limb blood flow/volume between
266 the different cooling conditions (range: 8-22°C), Doppler ultrasound assessment of the femoral
267 artery only provides an indirect estimate of muscle flow in the lower limbs. This includes
268 supply to tissue capillaries (nutritive capillary blood flow) and flow into veins via shunts that
269 bypass the capillary bed (non-nutritive blood flow); for example, to muscle connective tissue,
270 fat tissue and skin circulation (35, 36). In contrast, the PET [¹⁵O]H₂O radiotracer technique
271 excludes the non-nutritive fraction of blood flow; suggesting that downstream changes in limb
272 blood flow, or muscle blood volume, are not representative of the changes in the muscle
273 microcirculation (37). Our data suggest that the measured blood flow response to cooling
274 depends on measurement site, e.g., actually within the skeletal muscle itself (capillary level) or
275 in conduit vessel proximal to the muscle bed (arterial level). These observations therefore

276 support the application of the PET [¹⁵O]H₂O radiotracer method to obtain a true reflection of
277 perfusion changes within muscle vasculature itself.

278 In the present study, the decrease in thigh skin blood flow exceeded the threshold of
279 clinical relevance ($\Delta < 19\%$) in both cooling conditions (Figure 3A). However, the skin blood
280 flow response was not consistent across the leg, with calf skin blood flow only decreased
281 beyond a clinical threshold in 8°C water (Figure 3B). This finding contrasts with previous
282 work, which has reported similar reductions in lower limb skin blood flow after different
283 degrees (range: 8-22°C) of lower-body cold-water immersion (4, 5, 12). Adopting a similar
284 exercise model and cooling stimuli (12), we previously speculated that the similar skin blood
285 flow response to different degrees of cooling was related to reduced vasoconstrictor
286 responsiveness in the skin. While the magnitude of sympathetic nervous activity may be greater
287 at colder water temperatures, any potential increase in vasoconstriction in the cutaneous vessels
288 is blunted in the presence of whole body heat stress (15). Therefore, the inconsistency between
289 our current findings and past observations make our data difficult to interpret. Furthermore, the
290 difference between our findings are also likely related to employing a MCID for our primary
291 outcomes in this study. In support of this, the magnitude and precision (95% CI) of the
292 percentage change in skin blood flow between the different conditions encompassed values
293 observed in our previous work.

294 We observed different perfusion mechanisms between individual quadriceps muscles
295 with 8°C and 22°C cooling. Clinically relevant perfusion reductions in the superficial rectus
296 femoris and vastus lateralis muscles were generally observed under both water temperatures
297 versus control (Figure 2B & C), with only the decline in rectus femoris perfusion in 22°C water
298 close to, but not exceeding, the clinical threshold (Figure 2C). A similar directional response
299 to cooler water temperatures (8°C and 15°C) has been documented under resting conditions,
300 though declines in superficial muscle perfusion were only clinically relevant in the rectus

301 femoris muscle (10). This greater scope to decrease perfusion towards maximal
302 vasoconstriction after exercise may simply reflect the greater absolute capacity to reduce
303 muscle perfusion (i.e., higher perfusion values after exercise compared with baseline at rest)
304 (38).

305 In contrast to the uniform reduction in superficial muscle perfusion in both cooling
306 conditions, a different perfusion response was observed in the deeper lying quadriceps muscles.
307 While the degree of decline in perfusion in the deeper-lying vastus intermedius and vastus
308 medialis muscles in 22°C water (Figure 2C) would not exclude the presence of a potential
309 effect yet not exceeding our pre-defined MCID value (39), perfusion remained unchanged after
310 8°C cooling (Figure 2B). The perfusion response in deeper muscle supports our previous work
311 under resting conditions (10), where increases in vastus intermedius muscle perfusion were
312 speculated to reflect the occurrence of low-intensity shivering in the deep-lying type 1 muscle
313 fibers (40, 41). This putative mechanism stimulates metabolism and oxygen consumption and
314 increases blood supply to meet the higher metabolic demand (42, 43). Taken together, the
315 decline in superficial muscle perfusion in both cooling conditions, and the inconclusive effects
316 observed for perfusion in deeper located muscles in 8°C water, collectively underpin the greater
317 magnitude of global perfusion reduction with less noxious water (22°C). This suggests that
318 non-noxious cooling (15-22°C) may have greater efficacy following exercise compared with
319 more noxious water temperatures (<8°C). This is due to causing reductions in superficial muscle
320 perfusion while simultaneously minimising any increases in deeper muscle perfusion that are
321 observed at colder water temperatures. Indeed, when considered in line with previous
322 observations at rest (10), the present data suggest that non-noxious cooling is likely to be more
323 effective from a muscle perfusion perspective when applied either at rest or following exercise.
324 It should be noted that the changes in perfusion must be interpreted in the context of when
325 PET/CT measures were taken, i.e., 10 min post immersion. Since deep (3 cm) muscle

326 temperature continues to decrease for at least 30 min post-immersion under similar conditions
327 (12), these perfusion responses are, however, likely to extend beyond the current 10 min period
328 studied. Interestingly, our current findings correspond with recent work using phase change
329 material (44), which demonstrates prolonged (>3 hours) mild cooling (15°C) ameliorates the
330 loss in functional strength and improves subjective recovery after muscle damaging exercise.
331 Thus, our findings may be extrapolated to other forms of cryotherapy, such as ice application,
332 whole body cryotherapy or phase change material, which also attempt to manipulate muscle
333 temperature and perfusion to enhance recovery.

334 The potential benefits of reducing muscle perfusion using cold-water immersion, are
335 often cited to be related to minimizing the underlying infiltration and accumulation of pro-
336 inflammatory cells (45, 46); likely mediated via reductions in tissue temperature (47). In
337 comparison with the control, the marked reductions in superficial muscle (1 cm, Figure 4A)
338 and skin temperature (Figure 5B & C) across both cooling conditions, appeared to have been
339 of sufficient magnitude to reduce superficial muscle perfusion to a clinically relevant degree.
340 In the absence of any objective measurement of shivering, the difference in deeper muscle
341 temperature (2 and 3 cm; Figure 4B & C) between the 8°C and 22°C conditions supports the
342 occurrence of shivering, and the explanation for preservation of vastus intermedius and vastus
343 medialis perfusion in the colder water. Indeed, work from our laboratory (6) has demonstrated
344 that a difference in deep muscle temperature, similar to that observed in this study (~1°C), can
345 modify femoral artery blood flow (i.e., total flow to the leg musculature). Nevertheless, without
346 taking muscle temperature measurements across deeper lying individual quadriceps muscles,
347 potential temperature-dependent perfusion changes cannot be directly confirmed.

348 It currently remains unclear whether higher exercise-induced elevations in core and
349 deep muscle temperatures would be of sufficient magnitude to completely override any potential
350 shivering (and therefore perfusion) response after 8°C water exposure. For example, in

351 comparison with our recent observations under resting conditions (10), where we reported a
352 clinically relevant increase in vastus intermedius muscle perfusion after 8°C cooling, there was
353 some evidence that the relatively small heat load placed upon the body (i.e., core temperature
354 ~38°C) negated this mechanism (i.e., vastus intermedius perfusion increase did not attain
355 clinical relevance). Therefore, future work is required to investigate the influence of graded
356 thermal loads upon the body (i.e., higher core and muscle temperatures) prior to being exposed
357 to different degrees of cold-water immersion, and examine muscle perfusion responses. This
358 will be beneficial in helping to provide individualized cold-water immersion prescriptions after
359 different types of exercise of varying durations and intensity (i.e., different thermal loads);
360 likely more representative of athletic training and competition.

361 We have previously discussed the limitations of our applied experimental approach and
362 the potential confounding effects of muscle activation on perfusion measures (10). In particular,
363 a key limitation was the inability to assess the shivering response in deep muscle to provide an
364 objective interpretation of our perfusion findings. Therefore, future studies may attempt to
365 relate changes in deep muscle perfusion in the quadriceps femoris muscle after post-exercise
366 cold-water immersion (or cryotherapy) using suitable radiotracers (i.e., ¹⁸FDG) to examine the
367 shivering response (48). Likewise, the nature of the measurements we undertook in our
368 investigation prevented use of a within-subject crossover design from an ethical standpoint,
369 thereby contributing to render the width of the uncertainty around the estimated mean
370 differences in perfusion prone to sampling error. Nevertheless, the degree of uncertainty in the
371 effects we presented can inform sample size planning based on criteria of precision (49) for
372 future investigations with similar, or alternative (50), experimental designs to our study. We
373 also selected an all-male participant cohort to make it somewhat easier to conduct covariate
374 adaptive randomization. Thus, extrapolating our perfusion data to females who typically
375 possess different anthropometrical characteristics represents another study limitation. Finally,

376 it is recommended that future studies utilize more strenuous exercise protocols in order to better
377 understand perfusion changes promoted by different degrees of cold-water immersion under
378 conditions which more closely simulate those experienced by athletes.

379

380 **CONCLUSION**

381 We used [¹⁵O]H₂O PET/CT to quantitatively measure quadriceps muscle perfusion
382 after different degrees of post-exercise cold-water immersion. In contrast to noxious water
383 (8°C), we observed non-noxious water (22°C) to decrease global quadriceps perfusion beyond
384 a clinically relevant threshold. Despite both cooling temperatures reducing superficial muscle
385 perfusion, the degree of decline in perfusion for the deeper located vastus intermedius and
386 vastus medialis muscle with colder water would not exclude the presence of a potential effect
387 yet not exceeding our pre-defined MCID value. Our findings therefore suggests that the
388 selection of non-noxious water temperatures (22°C) may be more suitable for post-exercise
389 recovery after performing exercise, which places a relatively small thermal load (< 38°C core
390 temperature) upon the body.

391

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395

396 **Author Contributions**

397 I.H., D.A.L., H.J., A.K., J.K., V.D.S., T.C., and W.G., conceived and designed the study; C.M.,
398 C.H., and L.L., analyzed the data; C.M., I.H., D.A.L., H.J., K.K.K., and W.G., interpreted the
399 results of the experiments; L.L., prepared figures; C.M, I.H., and W.G., drafted the manuscript;
400 C.M., I.H., D.A.L., H.J., A.K., K.K.K., J.K., L.L., and W.G. edited and revised the manuscript;

401 C.M., I.H., D.A.L., C.H., H.J., K.K.K., A.K., J.K., V.D.S., L.L., N.T.C., and W.G., approved
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406

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408

409 The results of the present study do not constitute endorsement by the ACSM.

410

411 We declare that the results of the study are presented clearly, honestly, and without fabrication,
412 falsification, or inappropriate data manipulation.

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545 **Figure captions**

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Figure 1. The mean difference (Δ) in global quadriceps muscle perfusion between the 8°C, 22°C and control conditions ($n = 10$ per condition; mean \pm 95% confidence interval (CI)). Clinical relevance was assessed against a minimal clinically important difference in muscle perfusion of $\pm 0.75 \text{ mL}\cdot 100\text{g}\cdot \text{min}^{-1}$ (dashed lines). The colour intensity of the density strip represents the degree of uncertainty around the point estimate for the mean effect.

Figure 2. The mean difference (Δ) in individual muscle perfusion between a) 8°C versus 22°C; b) 8°C versus control; and c) 22°C versus control conditions ($n = 10$ per condition; mean \pm 95% confidence interval (CI)). Clinical relevance was assessed against a minimal clinically important difference in muscle perfusion of $\pm 0.75 \text{ mL}\cdot 100\text{g}\cdot \text{min}^{-1}$ (dashed lines). The colour intensity of the density strip represents the relative frequency of the data. The colour intensity of the density strip represents the uncertainty around the point estimate for the mean effect.

Figure 3. The mean percentage difference in a) thigh and b) calf cutaneous vascular conductance, between the 8°C, 22°C and control conditions ($n = 10$ per condition; mean \pm 95% confidence interval (CI)). Clinical relevance was assessed against a minimal clinically important difference in muscle perfusion of $\pm 19\%$ (dashed lines). The colour intensity of the density strip represents the uncertainty around the point estimate for the mean effect.

Figure 4. The mean difference (Δ) in muscle temperature at a depth of a) 1 cm, b) 2 cm, and c) 3 cm, between the 8°C, 22°C and control conditions ($n = 10$ per condition; mean \pm 95% confidence interval (CI)). Non-zero overlap of the 95%CI for the mean represents clear difference between conditions. The colour intensity of the density strip represents the uncertainty around the point estimate for the mean effect.

Figure 5. The mean difference (Δ) in the secondary outcome variables of core temperature, thigh temperature, mean skin temperature and mean body temperature between the between the 8°C, 22°C and control conditions ($n = 10$ per condition; mean \pm 95% confidence interval (CI)). Non-zero overlap of the 95%CI for the mean represents clear difference between conditions. The colour intensity of the density strip represents the uncertainty around the point estimate for the mean effect.