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Running Head:

Molecular response to post-exercise cold-water immersion

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ABSTRACT

We investigated the underlying molecular mechanisms by which post-exercise cold-water immersion (CWI) may alter key markers of mitochondrial biogenesis following both a single session and six weeks of sprint interval training (SIT). Nineteen males performed a single SIT session, followed by one of two 15-min recovery conditions: cold-water immersion (COLD; 10°C) or a passive room-temperature control (CON; 23°C). Sixteen of these participants also completed six weeks SIT, each session followed immediately by their designated recovery condition. Four muscle biopsies were obtained in total, three during the single SIT session (pre-exercise, post-recovery, and 3 h post-recovery), and one 48h after the last SIT session. Following a single SIT session, phosphorylated (p-) AMPK, p-p38 MAPK, p-p53, and *PGC1α* mRNA were all increased ($P < 0.05$). Post-exercise CWI had no effect on these responses. Consistent with the lack of a response following a single session, regular post-exercise CWI had no effect on PGC-1α or p53 protein content. Six weeks of SIT increased peak aerobic power, $\dot{V}O_{2peak}$, maximal uncoupled respiration (complexes I and II), and 2-km time-trial performance ($P < 0.05$). However, regular CWI had no effect on changes in these markers, consistent with the lack of response in the markers of mitochondrial biogenesis. While these observations suggest CWI is not detrimental to endurance adaptations following six weeks of SIT, they question whether post-exercise CWI is an effective strategy to promote mitochondrial biogenesis and improvements in endurance performance.

INTRODUCTION

Despite the popularity of post-exercise cold-water immersion (CWI) in athletic training regimes, few studies have investigated its effect on the adaptive response to endurance-related training. Notably, there have been no studies investigating the use of CWI in conjunction with sprint-interval training (SIT). Some research involving lower intensity exercise indicates CWI may stimulate a number of cellular responses related to skeletal muscle mitochondrial biogenesis following a single exercise session in humans (1, 33, 34). Furthermore, improvements in skeletal muscle recovery following CWI (67) may allow athletes to train with a higher quality and/or load in subsequent sessions, potentially providing a greater stimulus for adaptation (29). However, these benefits may be counteracted by the attenuation of molecular responses related to vascular remodeling and heat-shock protein induction (74), resulting in long-term detrimental effects on skeletal muscle adaptations and endurance performance. Given the contrasting findings reported to date, clarification of the effects of CWI on both molecular responses to a single exercise session and adaptations to exercise training are warranted.

In humans, post-exercise exposure to cold has been reported to augment increases in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*) mRNA (34, 61, 62) - an important regulator of skeletal muscle mitochondrial biogenesis (41). Increases in *PGC-1α* mRNA following post-exercise muscle cooling have been attributed to both shivering and non-shivering thermogenesis (34, 61, 62). For example, 1 h of exercise followed by 3 h of recovery in a cold (7°C) environment induced significantly greater increases in *PGC-1α* mRNA in human skeletal muscle, compared with a room-temperature (20°C) control condition (62). The increase in *PGC-1α* mRNA reported by Slivka et al. (62) was attributed in part to contraction-induced increases in calcium (Ca^{2+}) and adenosine monophosphate (AMP) as a result of shivering. Apart from producing heat via repetitive

muscle contraction and subsequent energy expenditure, shivering may also serve to activate PGC-1α via the upstream kinases calcium/calmodulin-dependent protein kinase and AMP-activated protein kinase (AMPK) (62). Consistent with work in adipose tissue and muscle cells (53, 72), *PGC-1α* mRNA has also been reported to increase in human skeletal muscle following post-exercise cold-water immersion (CWI) via non-shivering thermogenesis (34) - a process by which heat is produced from shivering-independent mechanisms such as mitochondrial uncoupling and substrate cycling (70). This cold-induced increase in *PGC-1α* mRNA may be related to *β*-adrenergic stimulation and nitric oxide synthesis (34), and/or altered calcium handling and subsequent p38 MAPK/AMPK phosphorylation (33). These studies suggest that cold application may be a novel method to promote exercise-induced mitochondrial biogenesis in humans, but further research is required to identify additional underlying mechanisms.

p53 is another key transcription factor involved in the regulation of exercise-induced mitochondrial biogenesis (58). As shown in a number of different cell and tissue types, p53 plays an important role in regulating mitochondrial content, oxidative capacity, and exercise performance (44, 50, 56, 58, 59). Apart from its well-defined role in response to genotoxic stress and associated DNA damage, research has also implicated p53 activation in response to everyday stressors (e.g., metabolic stress from exercise) (9). p53 activation has also been reported following cold stress (28 °C) in cultured cells (43), implicating the cold stress imposed by post-exercise CWI as a potential stimulus to augment exercise-induced increases in p53 activation. Once activated, p53 transcriptionally regulates genes associated with energy metabolism and mitochondrial biogenesis (9, 28), including mitochondrial transcription factor (Tfam) (59), apoptosis inducing factor (AIF) (64), mitofusin-2 (Mfn2) (68), dynamin-related protein 1 (Drp1) (39), and synthesis of cytochrome *c* oxidase 2 (SCO2)

(44). Should cold stress increase p53 activity, this would provide an alternate explanation by which post-exercise CWI might augment exercise-induced mitochondrial biogenesis.

Two studies have investigated the effects of regular post-exercise CWI on skeletal muscle adaptations to high-intensity endurance training (1, 33). Consistent with a greater increase in *PGC-1α* mRNA after a single session of high-intensity running followed by CWI (34), regular post-exercise CWI (administered during four weeks of training) was reported to increase the abundance of a number of proteins associated with mitochondrial biogenesis (e.g., PGC-1α, subunits of respiratory complexes I and III, and β-HAD) (33). In contrast, a recent study by Aguiar et al (1) reported that regular CWI administered during 4 weeks of high-intensity cycle training had no additional effect on training-induced increases in PGC-1α protein content. However, although one study has observed a significant increase in p53 protein content following four weeks of sprint interval training (SIT) (28), no study has investigated the effects of regular CWI on the content of this transcription factor. Another major gap in the literature is that no study has investigated the effect of regular post-exercise CWI on markers of mitochondrial function (e.g., mass-specific mitochondrial respiration) and content (e.g., citrate synthase activity).

We investigated, for the first time in the same experiment, the effects of post-exercise CWI on mitochondrial content and function and some of the underlying molecular mechanisms. The present investigation was divided into two studies (a single exercise trial with biopsies and a training intervention). The aim of the biopsy trial was to investigate the response of the p53 pathway, and other molecular mechanisms, following a single exposure to post-exercise CWI. The aim of the training intervention was to investigate the effects of regular post-exercise CWI on the protein content of these transcription factors, as well as mitochondrial content and function. It was hypothesized that CWI administered after SIT would augment

these molecular responses (independent of shivering, i.e., non-shivering thermogenesis), consistent with the up-regulation of PGC-1α gene expression and protein accumulation reported when CWI was administered following high-intensity running (33, 34). It was also hypothesized that a CWI-induced increase in p53 signaling and protein accumulation would provide a basis for improved aerobic phenotype adaptations, including mitochondrial respiration, maximal oxygen consumption ($\dot{V}O_{2peak}$), and endurance exercise performance. Considering the widespread use and acceptance by athletes of CWI as a recovery modality following training, the overall aim of this research was to provide mechanistic insights into its effects on skeletal muscle adaptations to training.

METHODS

Participants

Recruited participants were recreationally-active, performing some form of aerobic exercise (e.g. running, cycling) for ~30-60 min per day, ~2-3 days per week. Participants were not involved in a structured training regime or specifically trained to compete in any one sport. Informed consent was obtained prior to participation, and all participants were screened for cardiovascular risk factors associated with exercise. All procedures were approved by the Institution's Human Research Ethics Committee. The study followed a two-group parallel design, in which participants were assigned to one of two recovery conditions in a randomized, counter-balanced fashion. These conditions were a passive control (CON) or cold-water immersion (COLD).

Briefly, the experimental protocol consisted of i) familiarization and baseline testing; ii) a single exercise trial with biopsies, iii) a six-week SIT intervention, and iv) post-training testing. Nineteen healthy males began the study and completed the biopsy trial (CON, n=10; COLD, n=9). Of these nineteen participants, sixteen completed the six-week training

intervention and all post-training measures (CON, *n*=8; COLD, *n*=8). Participant characteristics are detailed in Table 1.

Table 1: Participant characteristics

	CON Group	COLD Group
Biopsy Trial		
Age (years)	25 ± 6	23 ± 4
Mass (kg)	81.3 ± 13.2	77.4 ± 7.7
Height (cm)	181.7 ± 8.9	179.2 ± 11.4
Body mass index	24.6 ± 3.4	24.2 ± 2.7
Training Intervention		
Age (years)	26 ± 7	24 ± 4
Mass (kg)	82.9 ± 14.3	77.1 ± 8.1
Height (cm)	181.8 ± 10.0	179.8 ± 12.1
Body mass index	25.1 ± 3.7	24.0 ± 2.8

Familiarization

Prior to baseline testing, participants made several familiarization visits to the laboratory to become accustomed with the equipment and testing procedures. These sessions included a graded exercise test (GXT), a 2-km cycling time trial (TT_2), and 20-km cycling time trial (TT_{20}), all performed on isolated days, and separated by at least 24 h. Participants also completed a familiarization session of the SIT, and their assigned recovery condition.

Baseline Testing

Participants completed baseline trials of the TT_2, TT_{20}, and GXT protocols. All baseline trials were separated by at least 24 h, and performed in the morning (0700-1000) following an overnight fast. Participants were asked to refrain from exercise and alcohol consumption for 24 h prior to each trial.

Biopsy Trial

Following an overnight fast and abstinence from exercise and alcohol for the preceding twenty-four hours, participants reported to the laboratory in the morning (0730) for the biopsy trial. Upon arrival, the lateral aspect of the right thigh (dominant leg for all participants) was anaesthetized (1% Xylocaine). Three separate incisions were made through the skin and underlying fascia, corresponding to the three muscle biopsies taken during the experiment (pre-exercise, post-recovery and 3 h post-recovery). The incisions were made approximately one-third of the distance from the knee to the hip, parallel to the long axis of the leg and spaced 1-2 cm apart. Using a 5-mm Bergström needle modified with suction (21), muscle biopsies were taken from the belly of the *vastus lateralis*. The first biopsy was taken at rest, immediately before the SIT session (Pre). Participants then performed the single SIT session and their designated recovery condition. Exactly two minutes post-recovery, a second muscle biopsy was taken (Post-Rec). Participants remained in the laboratory, fasted and at rest, until a third biopsy was taken 3 h post-recovery (3 h). The Post-Rec and 3 h biopsy incision sites were covered with sterile gauze and a water-proof dressing (Tegaderm, 3M, North Ryde, Australia) during exercise and recovery. Following each biopsy, the corresponding incision site was closed with a suture. Samples were blotted on filter paper to remove blood and immediately snap-frozen in liquid nitrogen, then stored at -80°C until subsequent analyses. Prior to freezing, a small piece (~15-20 mg) of fresh muscle was sectioned from the pre-training biopsy for immediate analysis (within 5 min) of mitochondrial respiration. Participants were allowed to consume water *ad libitum* during the trial. This session doubled as the first training session for the sixteen participants who subsequently completed the SIT intervention.

174 *Sprint Interval Training Intervention*

175 Sixteen participants completed three training sessions per week for six weeks. On an
176 electronically-braked cycle ergometer (Velotron, Racer-Mate, Seattle, WA), participants first
177 completed a 5-min warm-up at 75 W. This was immediately followed by 4-6 x 30-s 'all-out'
178 efforts at a constant resistance corresponding to 7.5-9.5% of body mass, separated by 4 min
179 of rest. To ensure a progressive training stimulus during the six-week training period,
180 participants performed 4 repetitions in weeks 1-2, five in weeks 3-4, and six in weeks 5-6.
181 Resistance ranged from 7.5-9.5% of body mass, so that the fatigue-induced reduction in
182 power during the 30-s bout was at least 20 $W.s^{-1}$. To eliminate individual variance with self-
183 administered 'speeding up' of the flywheel, participants began each effort from a rolling start
184 corresponding to 120 rpm. During each effort, participants were given extensive verbal
185 encouragement and asked to remain seated in the saddle. This protocol has been shown to
186 rapidly upregulate the oxidative phenotype, comparable to long-duration continuous
187 endurance exercise (22, 23). Participants were asked to refrain from all other types of
188 exercise during the training intervention.

189 *Post-Training Testing*

190 A resting muscle biopsy was taken ~48-72 h after the final training session from the same leg
191 used during the baseline testing (Post). Participants also completed post-training trials for the
192 TT_2, TT_{20}, and GXT protocols. The timing and nature of post-training testing was identical to
193 baseline testing.

Exercise Performance Tests

Time Trials

On the same electronically-braked cycle ergometer used during training, participants were instructed to complete TT_2 and TT_{20} self-paced time-trials as quickly as possible. The only feedback given to participants during the trials was an update of distance covered: the half-way mark (1 km) during the TT_2 and every 2 km during the TT_{20}. Participants were allowed to control the gear ratio throughout the entire time trial, corresponding to three gearings on the chain-ring (28, 39 and 52) and ten on the rear sprocket (23, 21, 19, and 17-11). Heart rate (RS800sd, Polar Electro Oy, Finland), exercise duration and average power were recorded during the trial.

Graded Exercise Test

Participants performed a discontinuous GXT on an electronically-braked cycle ergometer (Lode, Groningen, The Netherlands) to determine their lactate threshold (LT) and peak aerobic power (W_{peak}). An intermittent protocol was used, with 4-min exercise stages and 30-s rest stages. Following a 5-min steady state warm-up at 75 W, the workload was increased by 30 W every 4.5 min until the participant reached volitional fatigue. The starting workload varied between 60-120 W, ascertained from the familiarization GXT, capping the number of stages to a maximum of ten. Participants were instructed to maintain a pedaling cadence of 70 rpm and wore a heart rate monitor during the test. The test was stopped when pedaling cadence dropped below 60 rpm. Blood samples for the GXT were taken from a venous catheter (Optiva IV Catheter 20G x 1.75", Smiths Medical, USA) inserted into an antecubital vein 10 min prior to the first blood draw. The LT was determined as the workload at which venous blood lactate increased 1 mM above baseline (10), and was calculated using Lactate-E version 2.0 software (47). W_{peak} was calculated as previously reported (30):

$W_{peak} = W_{final} + (\frac{t}{240} \cdot 30)$; where W_{final} was the power output of the last completed stage and *t* was the time in seconds of any final uncompleted stage.

Peak Oxygen Uptake

After volitional fatigue in the GXT, participants performed 5 min of passive rest before performing a $\dot{V}O_{2peak}$ test. This comprised of a steady-state cycle to fatigue at a supra-maximal power output corresponding to 105% of W_{peak}, previously reported to elicit $\dot{V}O_{2peak}$ values no different to that determined during a ramp incremental test performed 5 min previously (55). Participants were asked to maintain a pedaling cadence of 90-100 rpm until volitional fatigue, with the test terminated when cadence dropped below 80 rpm. Expired gases were analyzed every 15 s using a custom-made metabolic cart. The gas analyzers (S-3lA/II and CD-3A analyzers, Ametek, PA, USA) were calibrated using known gas concentrations prior to each test (20.93% O_2, 0.04% CO_2 and 16.10% O_2, 4.17% CO_2; BOC Gases, Australia). $\dot{V}O_{2peak}$ was defined as the average of the two highest consecutive values reached during the test.

Recovery Interventions

Exactly five minutes after completing each training session, participants performed their assigned recovery intervention for 15 min. Seated (with legs fully extended), participants were immersed in water up to their umbilicus (COLD: 10°C), or rested on a laboratory bed (CON: 23°C). The COLD condition was performed in an inflatable bath (iBody, iCool Sport, Australia), and water temperature was maintained with a cooling/heating unit (Dual Temp Unit, iCool Sport, Australia) as previously described (12). We have previously reported the application of this CWI protocol after the same exercise protocol to reduce intramuscular (4 cm) temperature from 37.1°C to 33.6°C following an identical exercise session (12). Due to

the nature of water immersion *per se*, a hydrostatic pressure of ~20-30 mmHg (71) would have been exerted on the COLD participants during recovery. There were no observed or reported signs of shivering during CWI for all participants, at all time points.

Muscle Analyses - Biopsy Trial (Pre vs Post-Rec vs 3h)

Preparation of Whole-Muscle Lysates for Western Blotting

A 10-15 mg piece of frozen muscle was added to ice-cold homogenizing buffer (37.5 µL.mg^{-1} tissue; 20 mM Tris, 137 mM NaCl, 1% Triton-X, 10% Glycerol, 2.7 mM KCl, 1 mM MgCl, 1 mM EDTA, 1 µg.mL^{-1} aprotinin, 1 µg.mL^{-1} leupeptin, 1 mM benzamedine, 1 mM Na_3VO_4, 1 mM NaF, 5 mM sodium pyrophosphate, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride – adjusted to pH 7.4), and homogenized on ice using a motorized pestle. The whole muscle lysate was used for Western blotting without centrifugation (46). Protein concentration of the lysate was determined using a commercially-available colorimetric assay (Bio-Rad, Hercules, CA), using bovine serum albumin (BSA) as the standard. All samples were diluted with distilled water to a standard concentration (1.5 µg. µL^{-1}), and further diluted with 2 x Laemmli buffer (125 mmol.L^{-1} Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.015% bromophenol blue, 10% β-mercaptoethanol). The resultant lysate was split equally into two parts: one unboiled and the other boiled at 95°C for 5 min. Both lysate fractions were subsequently stored at -80°C for future analyses.

Western Blot Analysis

Optimization gels were run for all antibodies to determine optimal conditions for western blot detection (i.e. loading volume and boiled vs un-boiled lysate). Muscle lysates were loaded equally (15 µg) and separated on 10% self-cast SDS-PAGE gels by means of electrophoresis, at 100 V for 1.5-2 h (Bio-Rad, Mississauga, ON, Canada). Unboiled muscle lysate was used

for all proteins except p-p53^{Ser15}, in which samples were re-boiled at 95°C for 3 min prior to loading. Once resolved, gels were transferred wet onto polyvinylidene fluoride (PVDF) membranes in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, 0.01% SDS) at 100 V for 90 min. Membranes were then blocked with 5% skim milk in Tris-buffered saline (TBST: 150 mM NaCl, 20 mM Tris, 0.1% Tween-20, pH 7.4) for 1 h at room temperature. Following 5 x 5 min washes in TBST, the membranes were incubated overnight (4°C with gentle agitation) with monoclonal primary antibodies (Cell Signaling Technology, MA, USA; CST), diluted 1:1000 in 5% BSA or skim milk, against phosphorylated-AMPK^{Thr172} (Product Number 2531 CST), p38 mitogen-activated protein kinase (p38 MAPK$^{Thr180/Tyr182}$; 9211 CST), and p53^{Ser15} (9284 CST). The following morning, membranes were again washed 5 x 5-min in TBST, and incubated in the appropriate species-specific horseradish peroxidase-conjugated secondary antibodies (Goat anti-rabbit IgG, NEF812001EA; Perkin Elmer, Waltham, MA, USA) at room temperature for 90 min, diluted 1:10000 (1:5000 for p-AMPK only) in 5% skim milk and TBST. After a further 5 x 5-min washes in TBST, membranes were exposed to a chemiluminescent liquid (Clarity^{TM} Western ECL Substrate, Bio-Rad, Hercules, CA, USA) and visualized using a VersaDoc 4000 MP imaging system (Bio-Rad, Hercules, CA, USA). Band densities were determined using Image Lab 5.1 software (Bio-Rad, Hercules, CA, USA). Membranes were then stained with 0.1% Coomassie R-350 (PhastGelTM Blue R, GE Healthcare) as previously described (69), thereby providing a loading and transfer control. Comparative samples for each participant were loaded into the same gel, and one lane for each gel was reserved for an internal standard to account for inter-gel variability. Raw blot density was normalized to the internal standard and coomassie stain prior to analysis.

Total RNA Isolation

Approximately 20-30 mg of frozen muscle was added to 1 g of zirconia/silica beads (1.0 mm: Daintree Scientific, Tasmania, Australia) and 800 µL of commercially available TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and homogenized using an electronic homogenizer (FastPrep FP120 Homogenizer, Thermo Savant). Samples were centrifuged at 13000 rpm for 15 min (4°C) to pellet cell debris. The upper homogenate was then removed and pipetted into 250 µL chloroform (Sigma Aldrich, St Louis, MO), and again centrifuged at 13000 rpm for 15 min (4°C). Without disturbing the interphase, the top phase was pipetted into a fresh Eppendorf containing 400 µL of 2-isopropanol alcohol (Sigma-Aldrich, St Louis, MO, USA) and 10 µL of 5 M NaCl, and stored at -20°C overnight to allow for RNA precipitation. The following morning the sample was centrifuged at 13000 rpm for 20 min (4°C), following which the majority of the isopropanol was aspirated. The remaining RNA pellet was washed once with 75% ethanol made with diethylpyrocarbonate-treated (DEPC) H_2O (Invitrogen Life Sciences), and centrifuged at 9000 rpm for 8 min (4°C). Ethanol was aspirated off, and the pellet was re-suspended in 5 uL of heated DEPC-treated H_2O. RNA concentration was quantified spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA) at 230 (A_{230}), 260 (A_{260}) and 280 (A_{280}) nm, with an average yield of 1251.9 ± 466.7 ng.µL^{-1}, an $A_{260/280}$ ratio of 1.78 ± 0.11, and an $A_{260/230}$ ratio of 1.14 ± 0.38. RNA was stored at -80°C until further analysis.

Real-Time RT-PCR

One microgram of RNA was reverse transcribed into cDNA using a thermal cycler (S1000™ Thermal Cycler, Bio-Rad, Hercules, CA, USA) and a commercially available kit (iScript™ cDNA Synthesis Kit, Bio-Rad, Melbourne, Australia) with random hexamers and oligo dTs, according to the manufacturer's instructions. All samples and RT negative controls were run

311 together to prevent technical variation. 'Real-time' PCR reactions (total volume 10 µL) were
312 performed with iTaq^{TM} Universal SYBR$^{®}$ Green Supermix (Bio-Rad, Hercules, CA, USA) as
313 the fluorescent agent. The following cycling patterns were used: initial denaturation at 95°C
314 for 3 min, 40 cycles of 95°C for 15 s and 60°C for 60 s (Mastercycler® RealPlex^{2},
315 Eppendorf, Hamburg, Germany). Forward and reverse primers for target genes were designed
316 using sequences obtained from GenBank (Table 2). All samples were run in duplicate with
317 negative controls, using an automated pipetting system (epMotion 5070, Eppendorf,
318 Hamburg, Germany). To account for the efficiency of RT and initial RNA concentration, the
319 mRNA abundance of the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase
320 (*GAPDH*), TATA-binding protein (*TBP*) and β_2 microglobulin (*β2M*) were quantified, and
321 target genes were normalized to the geometric mean of all three. Relative changes in mRNA
322 abundance were calculated as described previously (31).

Table 2: Primer sequences used for real-time PCR

Gene (protein)	Forward Primer	Reverse Primer
*GAPDH (*glyceraldehyde 3-phosphate dehydrogenase)	AAAGCCTGCCGGTGACTAAC	CGCCCAATACGACCAAATCAGA
TBP (TATA-box binding protein)	CAGTGACCCAGCAGCATCACT	AGGCCAAGCCCTGAGCGTAA
β2M (β_2-microglobin)	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
PPARGC1A (PGC-1α)	GGCAGAAGGCAATTGAAGAG	TCAAAACGGTCCCTCAGTTC
NRF1 (Nuclear respiratory factor 1)	CTACTCGTGTGGGACAGCAA	AGCAGACTCCAGGTCTTCCA
NFE2L2 (NRF2)	AAGTGACAAGATGGGCTGCT	TGGACCACTGTATGGGATCA
TFAM (Mitochondrial transcription factor A)	CCGAGGTGGTTTTCATCTGT	GCATCTGGGTTCTGAGCTTT
TP53 (P53)	GTTCCGAGAGCTGAATGAGG	TTATGGCGGGAGGTAGACTG
MFN2 (Mitofusin-2)	CCCCCTTGTCTTTATGCTGATGTT	TTTTGGGAGAGGTGTTGCTTATTTC
AIFM1 (AIF)	GATTGCAACAGGAGGTACTCCAAGA	GATTTGACTTCCCGTGAAATCTTCTC
SCO2 (synthesis of cytochrome c oxidase)	CTTCACTCACTGCCCTGACA	CGGTCAGACCCAACAGCTT
DNM1L (DRP1)	CACCCGGAGACCTCTCATTC	CCCCATTCTTCTGCTTCCAC
CIRBP (CIRP)	CCCGACTCAGTGGCCG	AGCGACTGCTCATTGGTGTC
RBM3 (Putative RNA-binding protein 3)	GGACGTTCTCGCTACGTACTC	ACATGGCAGTTCAAGTCCTGG
UCP3 (Uncoupling protein 3)	CCACAGCCTTCTACAAGGGATTTA	ACGAACATCACCACGTTCCA

PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; NRF2, Nuclear respiratory factor 2; AIF; Apoptosis-inducing factor; DRP1, dynamin-related protein 1; CIRP, Cold-inducible RNA-binding protein.

Muscle Analyses - SIT Intervention (Pre vs Post)

Preparation of Permeabilized Skeletal Muscle Fibers.

A small section (~15-20 mg) of fresh muscle was analyzed for mitochondrial respiration pre- and post-training. The sample was immediately placed into a cold biopsy preservation solution (BIOPS; 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$, 20 mM taurine, 50 mM K^+-4-morpholinoethanesulfonic acid (MES), 15 mM Na_2phosphocreatine, 20 mM imidazole and 0.5 mM dithiothreitol (DTT) - pH 7.1 (52)), and subsequently mechanically separated using forceps. Fibers were permeabilized by gentle agitation (30 min at 4°C) in a solution of BIOPS containing 50 µg/mL of saponin, and washed (3 x 7 min at 4°C) by gentle agitation in the respiration medium solution (MiR05; 0.5mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO4, 20 mM HEPES, 110 mM sucrose and 1% bovine serum albumin (BSA) - pH 7.1 at 37°C (52)). This method selectively permeabilizes the cellular membrane leaving the mitochondria intact, allowing for "*in-situ*" measurements of mitochondrial respiration.

High-Resolution Respirometry

After washing, fibers were weighed (~3-4 mg wet weight) and assayed in duplicate in a high-resolution respirometer (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria), with each chamber containing 2 mL of MiR05. Oxygen concentration (nmol/mL) and oxygen flux ($pmol \cdot s^{-1} \cdot mg^{-1}$) were measured at 37°C and recorded using DatLab software (Version 5, Oroboros Instruments), corrected to instrumental background oxygen flux. Each chamber was continually re-oxygenated by direct syringe injection of O_2 into the chamber to allow for maintenance of O_2 concentration (275-450 nmol/mL) to avoid a potential oxygen diffusion limitation.

Mitochondrial respiration measurements were taking using a substrate-uncoupler-inhibitor titration (SUIT) protocol. Firstly, the substrates pyruvate (final chamber concentration; 2 mM) and malate (5 mM) were added in the absence of adenylates for measurement of leak respiration (L) with electron entry through Complex I (CI) (CI_L). Next, adenosine diphosphate (ADP) was added (5 mM) for measurement of maximal oxidative phosphorylation (OXPHOS) capacity (P) with electron input through CI (CI_P), followed by addition of succinate (10 mM) for measurement of P with electron supply through CI and Complex II (CII) combined ($CI\&II_P$). This state provides convergent electron input to the Q-junction through CI (NADH provided by malate and pyruvate) and CII ($FADH_2$ provided by succinate) and supports maximal mitochondrial respiration by reconstruction of the citric acid cycle function. Cytochrome *c* (10 μM) was next added to assess outer mitochondrial membrane integrity – increases in O_2 flux > 10% after cytochrome *c* addition indicated compromised membrane integrity, in which data was excluded. A series of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone titrations (FCCP, 0.75-1.5 μM) were performed, to determine maximal uncoupled respiration and electron transport system capacity (E), with convergent electron input through CI and CII ($CI\&II_E$). Rotenone (0.5 μM), an inhibitor of CI, was added next to determine E with electron input through CII alone (CII_E). Antimycin A (2.5 μM), an inhibitor of Complex III (CIII), was then added to measure residual oxygen consumption capacity (ROX); this was used to correct all respiration values. Mitochondrial respiration measures represent an average of the technical duplicates (i.e. average of both chambers), expressed in both absolute oxygen flux (mass-specific mitochondrial respiration) and corrected to citrate synthase activity (mitochondria-specific respiration). Complete mitochondrial respiration data sets were available for 12 participants (CON, n=6; COLD n=6) as a result of malfunctions with the respirometer and/or poor muscle sample quality.

Preparation of Whole-Muscle Lysates and Western Blot Analyses

Preparation of the whole-muscle lysate was performed in the same manner as that for the biopsy trial. The whole muscle lysate was used for both enzyme activity assays and Western blotting, without centrifugation. Western blots were performed with unboiled muscle lysate for all proteins except p53, in which samples were re-boiled at 95°C for 3 min prior to loading. Membranes were incubated overnight with monoclonal primary antibodies (1:500-5000 in 5% skim milk) against p53 (2527 CST), and PGC-1α (ST1202 Merck Millipore, Billerica, MA, USA). Secondary antibodies (Goat anti-mouse IgG (NEF822001EA Perkin Elmer, Waltham, MA, USA) for PGC-1α) diluted 1:7000-30000 in 5% skim milk and TBST. All other western blot procedures were identical to those detailed in the biopsy trial methodology.

Citrate Synthase Activity

The maximal enzyme activity of citrate synthase (CS) was determined using the lysate prepared for western blot analyses, and analyzed on a spectrophotometer (Bio-Rad, Hercules, CA). Each well contained a 50-fold dilution of lysate (2mg/mL), 3mM Acetyl CoA, 1mM dystrobrevin beta (DTNB), and 10 mM oxaloacetate (OAA), made to volume (250 µL) with Tris buffer (100 mM). Reactions were analyzed over a 3 min period at 30°C, with absorbance (412 nM) recorded every 15 s. Samples were analyzed in triplicate, with enzyme activity expressed as moles per hour per kilogram of protein ($\mu M.h^{-1}.kg^{-1}$ protein).

Statistical Analyses

Data are reported in the text as means and standard deviation (mean ± SD), unless otherwise stated. Comparisons between conditions were analyzed using a two-way general linear model (ANOVA) with repeated measures for time, where the within-subject factor was time (Biopsy

Trial - Pre vs Post-Rec vs 3 h; Training Intervention – Pre vs Post) and the between-subject factor was condition (CON vs COLD). Where significant time or interaction (time x condition) effects were found, multiple pairwise comparisons were evaluated by Welch's *t* test, corrected to the false discovery rate method (19). The level of significance for all data was set at $P < 0.05$. The above analyses were performed using IBM SPSS Statistics V20 (IBM Corporation, USA). To compliment the statistical hypothesis testing, effect sizes (ES) were calculated to assess the magnitude of observed effects. Cohen's conventions for effect size (Cohen's $d \pm$ 90% confidence intervals) were used for interpretation, where ES = 0.2, 0.5, and 0.8 are considered as small, medium and large, respectively. Raw western blot densitometry data (corrected to internal standard and coomassie stain) and changes in mRNA abundance (RT-PCR data) were used for statistical analyses. For graphical purposes, baseline western blot and RT-PCR values were normalized to 1.0, such that Post-Rec and 3 h values correspond to fold change from Pre values.

RESULTS

SIT Session and Training

Both groups performed similar volumes of total work during the single session of SIT, 67.5 ± 11.8 kJ (CON) and 65.3 ± 10.1 kJ (COLD) ($P > 0.05$). Similarly, both groups performed similar volumes of training over the six weeks, 1518.7 ± 180.9 kJ and 1487.0 ± 242.4 kJ for CON and COLD respectively ($P > 0.05$). There was 98% adherence to the training program, with 2 participants in the CON group and 3 participants in COLD group missing one training session each due to illness. No differences were observed for the characteristics listed in Table 1 ($P > 0.05$), demonstrating effective randomization of participants into the two conditions.

Muscle Analyses –Biopsy Trial

Western Blots

Significant effects of time were observed for the phosphorylation (p-) of p38 MAPK$^{Thr180/182}$ (P = 0.029) and p-AMPK^{Thr172} (P = 0.002). Specifically, p-p38 MAPK$^{Thr180/182}$ was elevated post-recovery (P = 0.029, ES = 1.00 ± 0.70), but returned to basal levels by 3 h post-recovery (P = 0.487). There was a similar increase in p-AMPK^{Thr172} post-recovery (P = 0.002, ES = 0.78 ± 0.51), which remained significantly elevated 3 h post-recovery (P = 0.006, ES = 0.77 ± 0.40). There were no interaction effects for either p-p38 MAPK$^{Thr180/182}$ (P = 0.901) or p-AMPK^{Thr172} (P = 0.472) (Fig. 1).

There was a significant effect of time for p-p53^{Ser15} (P = 0.007). Specifically, p-p53^{Ser15} was elevated post-recovery (P = 0.011, ES 1.54 ± 1.15) and remained significantly elevated 3 h post-recovery (P = 0.011, ES 2.39 ± 1.70). There was no interaction effect for p-p53^{Ser15} (P = 0.142). However, there were large effects for the change in p-p53^{Ser15} content between conditions (COLD *vs* CON) at both post-recovery (2.7-fold *vs* 1.5-fold; ES = 1.68 ± 1.79) and 3 h post-recovery (3.7-fold *vs* 1.7-fold; ES = 2.69 ± 2.78) (Fig. 1).

mRNA

There were no effects of time for the housekeeping genes *GAPDH* (P = 0.668), *TBP* (P = 0.748), and *β2M* (P = 0.193). A significant effect of time (P < 0.001) was observed for *PGC-1α* mRNA, with post-hoc analysis revealing it was significantly elevated 3 h post-recovery (P < 0.001, ES = 8.49 ± 1.77). However, no interaction effect was observed for *PGC-1α* mRNA (P = 0.738). There were no time or interaction effects for the mRNA content of nuclear respiratory factor-1 (*NRF-1*; P = 0.212 and 0.812, respectively), nuclear respiratory factor-2 (*NRF-2*; P = 0.492 and 0.645, respectively) or mitochondrial transcription factor (*TFAM*; P =

0.318 and 0.467, respectively) (Fig. 2A).

There were no main effects of time for the mRNA content of *P53* (P = 0.062), or the p53 downstream targets mitofusin-2 (*MFN2*; P = 0.335), dynamin-related protein 1 (*DRP1*; P = 0.994), synthesis of cytochrome *c* oxidase (*SCO2*; P = 0.274) or apoptosis inducing factor (*AIF*; P = 0.517). An interaction effect was demonstrated for *MFN2* mRNA (P = 0.033), but when corrected to the false discovery rate the critical significance level was not achieved. There were no interaction effects for *P53* (P = 0.707), *DRP1* (P = 0.279), *SCO2* (P = 0.214) or *AIF* (P = 0.078) mRNA. At the 3 h post-recovery time-point, there were moderate effects for the change in mRNA content of *MFN2* (1.2-fold *vs* 0.8-fold; ES = 0.53 ± 0.40), *AIF* (1.3-fold *vs* 0.9-fold; ES = 0.63 ± 0.65) and *SCO2* (1.3-fold *vs* no change; ES = 0.70 ± 0.80) between conditions (Fig. 2B).

No time or interaction effects were observed for the mRNA content of cold-inducible RNA-binding protein (*CIRP*; P = 0.475 and 0.168, respectively), RNA-binding motif protein 3 (*RBM3*; P = 0.151 and 0.262, respectively), or uncoupling protein 3 (*UCP3; P* = 0.245 and 0.490, respectively). However, there was a moderate effect (ES = 0.50 ± 0.57) for the change in *RBM3* mRNA content between conditions 3 h post-recovery (1.3-fold *vs* no change) (Fig. 2C).

Muscle Analyses – Training Intervention

Western Blots

There were no time or interaction effects for PGC-1α (P = 0.437 and 0.751, respectively) or p53 (P = 0.304 and 0.446, respectively) content (Fig. 3).

CS Activity and Mitochondrial Respiration

There were no time (10.0 ± 23.8% increase; $P = 0.232$) or interaction ($P = 0.656$) effects for changes in maximal citrate synthase activity as a result of training (Fig. 4).

There were no interaction effects for all mass-specific respiration measures ($P > 0.05$). However, there was a significant time effect for $CI\&II_E$ ($P = 0.013$, ES = 0.82 ±0.48), which increased 21.6 ± 24.8% following six weeks of SIT. Similar mean percentage changes were observed for the change in CI_P (23.8 ± 47.5%; $P = 0.181$, ES = 0.37 ± 0.49), $CI\&II_P$ (13.4 ± 19.8%; $P = 0.172$, ES = 0.43 ± 0.35) and CII_E (30.6 ± 47.5%; $P = 0.546$, ES = 0.61 ± 0.53) as a result of training, but these were not significant due to the considerable individual variability observed (Fig. 5A).

When normalized to CS activity (mitochondrial-specific respiration), there were no interaction effects for all measures ($P > 0.05$). There were no time effects for CI_L ($P = 0.648$), CI_P ($P = 0.453$) or $CI\&CII_P$ ($P = 0.656$), and small effects (with no effect of time) for the change in $CI\&II_E$ (11.4 ± 24.1%; $P = 0.121$, ES = 0.33 ± 0.47) and CII_E (21.8 ± 49.7%; $P = 0.775$, ES = 0.31 ± 0.58) as a result of training (Fig. 5B).

Performance Measures

GXT and VO_{2peak}

Peak power output during the GXT increased by 5.8 ± 5.7 % and 6.1 ± 5.6% in the CON and COLD groups, respectively (time effect; $P = 0.001$, ES = 0.31 ± 0.12). Similarly, $\dot{V}O_{2peak}$ increased significantly as a result of training (main effect of time; $P = 0.002$, ES = 0.61 ± 0.28), with a 9.6 ± 6.7% increase in the CON group and a 7.6 ± 11.0% increase in the COLD group. There were no main interaction effects for peak power output ($P = 0.979$) or $\dot{V}O_{2peak}$ ($P = 0.633$) (Fig. 6).

As a result of training, the lactate threshold increased by 162.1 ± 26.7 W to 171.3 ± 36.6 W for the CON group, and from 155.8 ± 54.8 W to 160.2 ± 59.8 W for the COLD group, with no main effect of time (P = 0.074) or interaction (P = 0.494).

Time Trials

The time required to finish the TT_2 decreased after training by 3.3 ± 4.5% and 2.4 ± 3.1% in the CON and COLD groups, respectively (time effect; P = 0.010, ES = 0.36 ± 0.21). TT_2 mean power correspondingly increased from 261.3 ± 45.0 to 283.5 ± 46.1 W for the CON group, and from 248.1 ± 66.4 to 263.7 ± 59.2 W for the COLD group (time effect; P = 0.023, ES = 0.34 ± 0.21). There were no effects of time for TT_{20} duration (P = 0.224) or mean power (P = 0.208). Furthermore, there were no interaction effects for TT_2 duration (P = 0.699) and mean power (P = 0.666), or TT_{20} duration (P = 0.889) and mean power (P = 0.703) (Fig. 7).

DISCUSSION:

The main finding of this study was that, contrary to expectations, CWI administered after sprint-interval exercise had limited effects on exercise-induced mitochondrial biogenesis, changes in mitochondrial content or function, and measures of endurance exercise performance. A novel finding of this study was that p53 phosphorylation is increased following a single session of SIT; however, this response did not translate into training-induced increases in the content of this protein after six weeks of SIT. In contrast to previous reports of greater increases in *PGC-1α* mRNA following a single exposure to post-exercise CWI (34), and greater increases in PGC-1α protein content following regular post-exercise CWI (33), we observed no differences between conditions in the present study. Six weeks of SIT significantly increased peak aerobic power, $\dot{V}O_{2peak}$, maximal uncoupled respiration (through complexes I and II), and 2-km time-trial performance. However, regular CWI

performed after each training session had no effect on changes in these parameters, consistent with the lack of an effect of CWI on markers of mitochondrial biogenesis following a single exercise session. While these observations suggest CWI is not detrimental to endurance adaptations in response to six weeks of SIT, they question whether CWI following SIT is an effective strategy to promote mitochondrial biogenesis and improvements in endurance performance.

Consistent with previous work following high-intensity interval and continuous running (7), this study provides the observation that $p53^{Ser15}$ phosphorylation is increased in human skeletal muscle following a single session of SIT (4 x 30-s all-out cycling efforts) (Fig. 1). Additionally, we observed that this response is unaltered by CWI. As shown in a number of different cell and tissue types, p53 plays an important role in regulating mitochondrial content and oxidative capacity (5). In contrast to the response to other non-genotoxic stresses, such as fluctuations in oxygen or carbohydrate availability (8, 9), the cold stress imposed by post-exercise CWI had no significant effect on p53 phosphorylation. Given the paucity of research investigating the acute effects of exercise on the p53 pathway, it is possible that the 3 h post-recovery time point may not have captured the maximal increase in the phosphorylation of this protein. Nonetheless, the large difference in p53-phosphorylation between conditions 3 h post-recovery (increase of 3.7-fold for COLD versus 1.7-fold for CON) suggests further research is warranted to investigate the effects of CWI on the p53 pathway. To further test the hypothesis that CWI may augment exercise-induced p53 phosphorylation, and consequently stability (18), a number of p53-target genes were also investigated (Fig. 2B). Two proteins central to mitochondrial remodeling are the fusion protein Mfn2, and the fission protein Drp-1, both of which are activated by p53 (39, 68). Mfn2 is a mitochondrial GTPase that plays an important role in mitochondrial fusion, therefore contributing to the maintenance of the mitochondrial network (4). In contrast to the increase in *MFN2* mRNA following a single

bout of sub-maximal intensity continuous cycling (1 h at 60% W_{max}) (36, 62), there was no change in both conditions in the present study. Similarly, there was no change in *DRP1* mRNA in both conditions in the present study, consistent with previous research demonstrating unaltered protein content following two to four weeks of high-intensity interval training (HIT) (27, 28, 51). Other downstream targets of p53, *AIF* and *SCO2* mRNA, were unaltered in the CON condition in the present study, consistent with the few published studies available (42, 57). We add that this response was not influenced by post-exercise CWI.

Post-exercise CWI also had no additional effect on the exercise-induced increases in AMPK and p38 MAPK phosphorylation, which has previously been reported following a single session of high-intensity cycling (24, 40). The prolonged increase in AMPK phosphorylation (up to 3 h post-recovery) observed in the current study was most likely due to glucose deprivation following an extended fast (~12 h by the 3 h post-recovery biopsy) and the completion of an intense exercise session (15). Contrary to expectations, CWI did not augment p38 MAPK phosphorylation, despite hypothermic stress being a well-accepted upstream activator (26). This can possibly be attributed, in part, to the already large exercise-induced increase in the level of p38 MAPK phosphorylation observed (~250-300% compared with previous reports of an ~140% increase (24)); this may have meant there was no augmented response to the additional stress of CWI. Alternatively, the 3 h post-recovery biopsy may not have adequately captured the time course of changes in the AMPK and p38 MAPK signaling pathways following post-exercise CWI. However, consistent with links between activation of p38 MAPK and AMPK and downstream $p53^{Ser15}$ phosphorylation (7), CWI had no additional effect on the phosphorylation of these kinases following a single session of SIT.

Both energy- (AMPK) and stress-dependent (p38 MAPK) kinase signaling (2, 35) has been implicated in PGC-1α activation following HIT (41), a response that was supported in the present study. Similar to previous research (40), we observed an increase in *PGC-1α* mRNA following a single session of SIT (Fig. 2A). However, post-exercise CWI had no additional influence on the increase in *PGC-1α* mRNA, consistent with the lack of an influence on its upstream activators p38 MAPK and AMPK. This contrasts with previous reports that post-exercise CWI (8-10°C for 10-15 min) (34, 37) and cold ambient exposure (4°C for 3-4 h) (61, 62) augment *PGC-1α* mRNA levels, compared with a passive room temperature control. However, certain methodological differences may explain the inconsistencies between these studies and the current study. For example, it is unlikely that 15 min of CWI would have elicited a shivering response and/or a reduction in core temperature of the same magnitude as the 3-4 h cold exposure used in the two studies from Slivka (61, 62). In regards to the studies by Ihsan (34) and Joo (37), these inconsistencies could be explained by the mode and intensity of exercise (and thus the metabolic stimulus) performed prior to CWI, the temperature and duration of CWI, differences in nutritional status of participants between studies, and/or differences in RT-PCR methodology. This may be further compounded by the fact that our participants were fasted, which may similarly enhance AMPK and p38 MAPK phosphorylation and subsequent PGC-1α activation (6).

To provide further insight into the effects of regular post-exercise CWI on potential cellular mechanisms modulating adaptation to SIT, the protein content of PGC-1α following the training period was also measured. No difference was observed between the CON and COLD conditions, consistent with the similar changes in *PGC-1α* mRNA observed after a single session of SIT with and without CWI. This is consistent with the non-significant ~1.2-fold increase in PGC-1α protein content observed when CWI was used regularly after a high-intensity running training period (33). While PGC-1α protein has been reported to increase

(~1.5 fold) following cold-air exposure (4°C for as little as 4 d) in rodent skeletal muscle (13, 49), these increases were likely the result of shivering thermogenesis (49) and extended cold-exposure (i.e., 4°C, 24 h/d for 4-5 wk) (13) - which were not characteristic of the CWI protocol used in the current study. The present data therefore suggest that CWI has no effect on SIT-induced PGC-1α protein accumulation in humans, but continued work in this area (including potential CWI-induced alterations in PGC-1α post-translational modification and nuclear content) will help clarify the above-mentioned inconsistencies.

A novel aspect of this study was to directly assess for the first time the effect of regular post-exercise CWI on training-induced improvements in mitochondrial respiration in permeabilized muscle fibers. Despite a smaller than expected increase for the CON group in the current study, there was a ~13% increase in maximal mass-specific respiration ($CI\&II_P$) when participants were pooled, comparable to previous reports in participants of a similar training status (28). The smaller improvements reported in the current study, as compared with Granata et al. (28), may be explained by the number of 'all-out' bouts performed, differences in age and training status, and the large variability typically associated with respiration measures. However, consistent with the similar response for markers of mitochondrial adaptation (i.e., PGC-1α and p53 protein content), regular CWI had no influence on training-induced changes in mass-specific mitochondrial respiration.

To determine if the increases in mass-specific respiration were qualitative or quantitative, mass-specific respiration was corrected to CS activity (to calculate mitochondria-specific respiration). Located exclusively in the mitochondria (65), CS is an enzyme commonly used in exercise training studies as a biomarker of mitochondrial content (11, 38). Similar to the observations for mass-specific respiration, and consistent with similar training-induced increases PGC-1α and p53 protein content, CWI had no effect on changes in mitochondria-

specific respiration. As such, and contrary to our hypothesis, these data suggest CWI does not serve to augment increases in mitochondrial respiration in response to SIT. Further research is warranted to investigate whether CWI can augment training-induced mitochondrial adaptations, especially when combined with a larger training volume that will induce more robust increases in CS activity/mitochondrial content (11).

Another novel aspect of this study was to investigate changes in the mRNA content of the cold-shock proteins (CSPs) CIRP and RBM3, and UCP3, following post-exercise CWI (Fig. 2C). It is well accepted that transcription and translation is suppressed in response to cold stress, accompanied by a reduction in RNA degradation and alternate splicing of pre-mRNA (3, 63, 73). Despite their integral roles in mRNA stabilization and global protein synthesis under cold stress (20, 63), *CIRP* and *RBM3* mRNA were unchanged following post-exercise CWI. Previously reported to reduce intramuscular temperature by ~3°C (12), the CWI protocol used in the current study induced a small (~30%) yet non-significant increase in *RBM3* mRNA 3 h after CWI. However, it is possible that a more drastic reduction in muscle temperature (~5°C) is needed for a significant increase in *RBM3* mRNA (20). Similarly, *CIRP* mRNA was unchanged 3 h following post-exercise CWI. The expression of *CIRP* mRNA however, has been reported to peak between 6 and 24 h following mild (32°C) cold exposure in mouse fibroblasts (48), which may explain the lack of response 3 h after post-exercise CWI. UCP3, an uncoupling protein expressed predominantly in skeletal muscle (60), has recently been implicated as an integral mediator of mammalian thermogenesis during cold exposure (54). In the current study, *UCP3* mRNA was unchanged 3 h following post-exercise CWI (Fig. 2C), consistent with the response reported by Slivka et al (62). It is possible that the CWI protocol used in the current study did not provide an adequate cold-stress to elicit an increase in *UCP3* mRNA. Another potential explanation for the subtle effect of post-exercise CWI on *CIRP*, *RBM3* and *UCP3* mRNA is the exercise stimuli itself,

as a more pronounced increase in gene expression may have occurred without a preceding exercise stimulus.

An important applied aspect of this research was to determine if regular CWI had any effect on exercise performance. Although it has previously been suggested that CWI may be detrimental to training-induced changes in exercise performance (74), this study provides evidence that regular CWI following sprint-interval cycling has no detrimental (or advantageous) influence on physiological and performance markers of endurance adaptations, including time-trial performance, the lactate threshold, $\dot{V}O_{2peak}$, and peak aerobic power. These findings support the absence of a significant difference between conditions for the measured markers of mitochondrial biogenesis (i.e., p53, PGC-1α, CS activity etc.). It is important to note that $\dot{V}O_{2peak}$, peak aerobic power, and time-trial performance all increased following SIT, without concurrent increases in molecular markers (i.e. PGC-1α protein content, mitochondrial respiration, CS activity). Although these molecular markers are have been associated with improved $\dot{V}O_{2max}$ (41), many other factors could have contributed to the increase in performance markers as a result of SIT, including alterations in hemodynamic (16, 17) and cardiorespiratory (14, 45, 66) factors, as well as improvements in substrate transport and utilisation (14, 17, 25). Nonetheless, this performance data supports the only other published study investigating the effects of regular CWI following cycling training, whereby CWI performed four times per week during three weeks of cycling training did not impair performance adaptations in competitive cyclists (29). Given that the participants in the current study were considerably less trained in comparison, this suggests that training status does not influence these effects. Finally, this data, along with that of Halson et al. (29), does not support concerns of CWI being detrimental to adaptations to endurance training (32, 74).

Perspective and Significance

The most important finding from the present study was that regular CWI following sprint-interval cycling has no advantageous (or detrimental) influence on markers of endurance-related training adaptations and cycling performance. This study provides evidence that p53 phosphorylation is increased following a single session of SIT; however, this did not translate into a greater increase in its content following six weeks of SIT, suggesting that this signaling response does not accurately predict training responses. Contrary to our hypothesis, a number of molecular markers associated with skeletal muscle metabolic adaptations and mitochondrial biogenesis were not altered by post-exercise CWI when compared with a passive control. For example, CWI had no additional influence on the exercise-induced increases in p38 MAPK or AMPK activation, consistent with the lack of response of their downstream targets PGC-1α and p53. Given the inconsistencies in molecular responses between the current study and those reported with CWI following high-intensity interval exercise [12, 15], future research incorporating larger sample sizes and/or additional biopsy time points may help clarify the effects of CWI on the signaling pathways and training adaptations associated with SIT. These findings refute previous suggestions that exercise-induced training benefits may be counteracted by possible CWI-induced long-term detrimental effects on muscular adaptation, but also question whether post-exercise CWI following sprint-interval cycling is an effective strategy to promote mitochondrial biogenesis and improvements in endurance performance.

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FIGURE CAPTIONS:

Figure 1: Phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK$^{Thr180/Tyr182}$) (A),adenosine monophosphate-activated protein kinase (AMPK^{Thr172}) (B), and p53^{Ser15} (C) from muscle sampled pre-exercise (Pre), post-recovery (Post-Rec), and 3 h post recovery (3 h) for control (CON; *n*=10) and cold-water immersion (COLD; *n*=9) conditions. Thr, threonine; Tyr, tyrosine; Ser, serine. *Significant difference compared with pre ($P < 0.05$). ‡ Large effect (ES > 0.8) compared with CON**.** Data are presented as mean ± S.D. Representative western blots are displayed above each graph. The vertical dividing line represents non-adjacent lanes from separate gels. All samples were derived at the same time and processed in parallel.

Figure 2: Gene expression from muscle sampled 3 h post-recovery for control (CON; *n*=10) and cold-water immersion (COLD; *n*=9) conditions for *PGC-1α* and downstream targets (A), *P53* and downstream targets (B), and cold-shock/uncoupling proteins (C). *NRF-1/2*, Nuclear respiratory factors 1/2; *TFAM*, Mitochondrial transcription factor; *PGC-1α*, Peroxisome proliferator-activated receptor gamma coactivator 1-α; *DRP1*, dynamin-related protein 1; *MFN2*, mitofusion 2; *AIF*, apoptosis-inducing factor; *SCO2*, synthesis of cytochrome c oxidase 2; *CIRP*, cold-inducible RNA-binding protein; *RBM3*, putative RNA-binding protein 3; *UCP3*, uncoupling protein 3. Corrected to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), TATA-binding protein (*TBP*) and β_2 microglobulin (*β2M*) housekeeping genes. *Significant difference compared with pre ($P < 0.05$). † Moderate effect (ES = 0.5-0.8) compared with CON. Data are presented as mean ± S.D.

Figure 3: Total PGC-1α and p53 protein content before (Pre) and after (Post) six weeks of sprint interval training (SIT) for the control (CON; *n*=8) and cold-water immersion (COLD; *n*=8) conditions. Data are presented as fold change (mean ± S.D.) from pre values.

Representative western blots are displayed above each graph. All samples were derived at the same time and processed in parallel.

Figure 4: Maximal activity of the mitochondrial enzyme citrate synthase (CS) before (Pre) and after (Post) six weeks of sprint interval training (SIT) for the control (CON; *n*=8) and cold-water immersion (COLD; *n*=8) conditions. Data are presented as mean ± S.D.

Figure 5: Mitochondrial respiration measurements at different coupling control states. A: mass-specific mitochondrial respiration, and B: mitochondria-specific respiration (mass-specific respiration normalized to citrate synthase activity). Respiration was measured in permeabilized muscle fibers (*vastus lateralis)* obtained before and after six weeks of sprint interval training (SIT) for control (CON, *n*=6) and cold-water immersion (COLD, *n*=6) conditions. Data are presented as percent change (mean ± S.E.M.) from pre values. CI_L: Leak respiration state (L) in the absence of adenylates and limitation of flux by electron input through Complex I (CI); CI_P; maximal oxidative phosphorylation state (P) with saturating levels of ADP and limitation of flux by electron input through CI; $CI\&II_P$: P with saturating levels of ADP and limitation of flux by convergent electron input through CI and Complex II (CII); $CI\&II_E$: maximal electron transport system capacity (E) with saturating levels of ADP and limitation of flux by convergent electron input through CI and CII; CII_E: E with saturating levels of ADP and limitation of flux by electron input through CII. * Significant difference compared with pre-training ($P < 0.05$). † Moderate effect compared with pre-training. ^ Small effect compared with pre-training.

Figure 6: Peak power output (W) achieved during the graded exercise test (GXT), and peak oxygen uptake ($\dot{V}O_{2peak}$; $mL.kg^{-1}.min^{-1}$) achieved during the steady-state cycle to fatigue bout, before (Pre) and after (Post) six weeks of sprint interval training (SIT) for the control

940 (CON; *n*=8) and cold-water immersion (COLD; *n*=8) conditions. * Significant effect of
941 training ($p < 0.05$). Data are presented as mean ± S.D.

942 Figure 7: 2-km (TT_2) and 20-km (TT_{20}) cycling time-trial performance before (Pre) and after
943 (Post) six weeks of sprint interval training (SIT) for the control (CON; *n*=8) and cold-water
944 immersion (COLD; *n*=8) conditions. * Significant effect of training ($p < 0.05$). Data are
945 presented as mean ± S.D.