Cold water immersion attenuates anabolic signalling and skeletal muscle fiber hypertrophy, but not strength gain, following whole-body resistance training

1. Title page

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2. Abstract

Purpose: We determined the effects of CWI on long-term adaptations and post-exercise molecular responses in skeletal muscle before and after resistance training. **Methods:** Sixteen males (22.9 ± 4.6 y; 85.1 ± 17.9 kg; mean ± SD) performed resistance training (3 $d \cdot wk^{-1}$) for 7 wk, with each session followed by either CWI (15 min at 10°C, COLD group, *n* = 8) or passive recovery (15 min at 23°C, CON group, *n* = 8). Exercise performance [one-repetition maximum (1-RM) leg press and bench press, countermovement jump, squat jump and ballistic push-up], body composition (dual x-ray absorptiometry), and post-exercise (i.e., +1 and +48 h) molecular responses were assessed before and after training. **Results:** Improvements in 1-RM leg press were similar between groups [130 ±69 kg, pooled effect size (ES): 1.53; ±90% confidence interval (CI) 0.49], while increases in type II muscle fiber cross-sectional area were attenuated with CWI (-1959 μM^2; ±1675; ES: -1.37; ±0.99). Post-exercise mTORC1 signalling (rps6 phosphorylation) was blunted for COLD at POST +1 h (-0.4-fold, ES: -0.69; ±0.86) and POST +48 h (-0.2-fold, ES: -1.33; ±0.82), while basal protein degradation markers (FOX-O1 protein content) were increased (1.3-fold, ES: 2.17; ±2.22). Training-induced increases in HSP27 protein content were attenuated for COLD (-0.8-fold, ES, -0.94 ±0.82), which also reduced total HSP72 protein content (-0.7-fold, ES: -0.79, ±0.57). **Conclusion:** CWI blunted resistance training-induced muscle fiber hypertrophy, but not maximal strength, potentially via reduced skeletal muscle protein anabolism and increased catabolism. Post-exercise CWI should therefore be avoided if muscle hypertrophy is desired.

New and noteworthy: This study adds to existing evidence that post-exercise cold water immersion attenuates muscle fiber growth with resistance training, which is potentially mediated by attenuated post-exercise increases in markers of skeletal muscle anabolism coupled with increased catabolism, and suggests blunted muscle fiber growth with cold water immersion does not necessarily translate to impaired strength development.

3. Introduction

Cold water immersion (CWI) is a popular recovery technique aimed at limiting, and accelerating recovery from, short-term exercise-induced decrements in exercise performance (72). Reported benefits of CWI include faster recovery of muscle strength (4, 62, 69), muscle soreness (4, 32, 57, 66, 69), perceptions of fatigue (9, 48, 57, 65, 66), markers of inflammation (39, 50, 53, 65) and muscle damage (19, 62). Improved recovery from single exercise sessions, mediated by CWI, is theorized to improve long-term adaptations to exercise training by enhancing subsequent training load and/or quality (72). However, as some of the post-exercise effects purportedly blunted by CWI also stimulate exercise-induced adaptations (8), CWI may actually hinder exercise training adaptations in some circumstances. Indeed, regular post-exercise CWI during resistance training can attenuate improvements in both maximal strength and muscle mass (56, 77).

Skeletal muscle hypertrophy consequent to resistance training is mediated by the dynamic changes in protein synthesis and breakdown stimulated by single exercise sessions (52, 55). Application of CWI in the post-exercise recovery period may influence post-exercise muscle protein synthesis and/or breakdown rates via a variety of mechanisms. For example, cold-induced vasoconstriction reduces muscle blood flow (26, 37, 38), which is positively associated with post-exercise muscle protein synthesis (MPS) rates (23, 67). Increased MPS following exercise also appears partially dependent upon the post-exercise inflammatory response (68), which is blunted following CWI application according to some (39, 50, 53, 65), but not all (51, 77), studies. As well as influencing MPS, animal studies suggest cold application may promote protein degradation (10).

Any influence of CWI application on post-exercise MPS or breakdown is likely mediated via the molecular pathways governing these responses. Rates of MPS are controlled by the mechanistic target of rapamycin complex 1 (mTORC1) signalling pathway, which includes the downstream targets p70S6K (p70 kDa ribosomal protein subunit kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E binding protein 1) (25). Rates of muscle protein breakdown are primarily controlled via the ubiquitin proteasome pathway (24). Key members of this pathway include muscle-specific E3 ubiquitin ligases MuRF-1 (muscle RING finger-1) and MaFbx/Atrogin-1 (muscle atrophy F-box), and the FOX-O subfamily of transcription factors that include FOX-O1 and FOX-O3a (33, 60). Modulation of heat shock proteins (HSP) may also influence muscle mass regulation, since several HSPs interact with key components of the mTORC1 and ubiquitin proteasome pathways (1, 5, 15, 16, 35, 61, 71, 79), and may also stabilise disrupted muscle contractile elements and assist in post-exercise regeneration and remodelling (34, 49).

Evidence has emerged suggesting CWI application after a single session of resistance exercise influences some of the molecular responses mediating hypertrophic adaptation in human skeletal muscle. In one study (56), CWI (10 min at 10°C) attenuated post-exercise mTORC1 signalling (specifically, p70S6K phosphorylation) and satellite cell activation after a single session of lower-body resistance training. Conversely, the expression and localisation of HSP72 and αβ-crystallin were unchanged by CWI (51). Continuing this protocol for 12 weeks blunted the increases in type II muscle fiber cross-sectional area (CSA), myonuclear accretion, and one-repetition maximum (1-RM) leg press and leg extension strength (56). These data suggest the negative effects of CWI on resistance training adaptations may be underpinned by modulation of the early post-exercise anabolic profile in skeletal muscle. Whether CWI also influences post-exercise markers of protein degradation in human skeletal muscle has, however, not been investigated. Moreover, since post-exercise molecular responses are modulated by periods of training (73, 76), it is unclear whether the influence of CWI on these responses are attenuated over time, which has implications for longer-term effects on training adaptation.

The inherent limitations of existing evidence showing attenuated resistance training adaptations with CWI may also compromise the applicability of their findings to athletic populations. For example, some studies have applied CWI to only a single limb (3, 22, 30, 31, 47, 77, 78), and/or used training protocols incorporating either a single exercise (22, 47, 77, 78) or lower-body exercises only (56), all of which are uncommon training practices. We therefore aimed to examine whether post-exercise CWI application modulates key adaptations following seven weeks of whole-body resistance training. In addition, we investigated the effects of CWI on post-exercise anabolic and catabolic molecular responses to a single session of whole-body resistance training, and compared these responses before and after the training intervention.

4. Methodology

Participants

Sixteen recreationally-active males (see Table 1 for participant characteristics) who had not been involved in regular resistance training for at least six months completed the study. Participants were fully informed of the study procedures, screened for cardiovascular or musculoskeletal conditions, and gave written informed consent before participation. All protocols and procedures were approved by the Human Research Ethics Committee at Victoria University and conformed to the Declaration of Helsinki.

*****INSERT TABLE 1 ABOUT HERE*****

Study overview

An overview of the study procedures is shown in Figure 1. Before preliminary testing, participants were familiarized with all performance assessments, including leg press one-repetition maximum (1-RM), bench press 1-RM, and ballistic exercise performance [counter-movement jump (CMJ), squat jump, and ballistic push-up] tests. Participants were also familiarized with all resistance training exercises to ensure appropriate technique and to determine loads for their first training session. One week following the familiarisation session, participants underwent a dual energy x-ray absorptiometry (DXA) scan and repeated the performance assessments, which served as pre-training (PRE) data. After preliminary testing, participants were pair-matched for leg press 1-RM, and one of each pair was randomly allocated to either the CWI (COLD; $n = 8$) or control (CON; $n = 8$) groups. At least 72 h after preliminary testing, participants performed a biopsy trial that doubled as the first session of a seven-week, whole-body, resistance training program. Post-training performance tests (POST) were performed during the last training session, followed by a second DXA scan and second biopsy trial conducted between 72 and 96 h later.

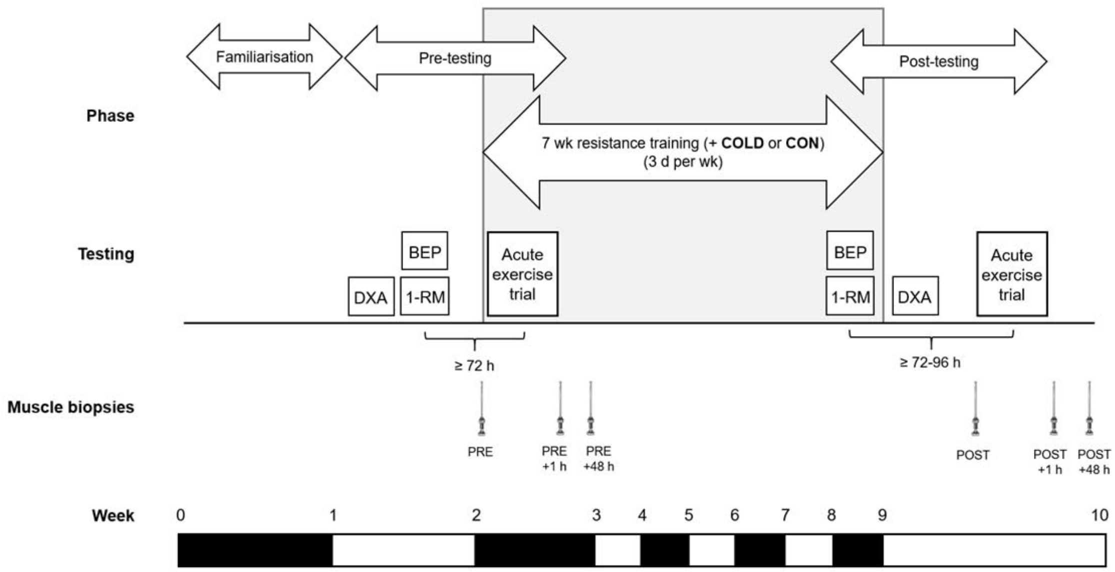

Figure 1. Study overview. DXA, dual x-ray absorptiometry scan; BEP, ballistic exercise performance (countermovement jump, squat jump, ballistic push-up) testing; 1-RM, one-repetition maximum (leg press and bench press) testing.

*****INSERT FIGURE 1 ABOUT HERE*****

Ballistic exercise performance

Countermovement jump (CMJ) performance

Before testing, participants performed a warm-up consisting of 5 min of stationary cycling at 1W/kg body mass. Countermovement jump (CMJ) performance was assessed using a force plate (Fitness Technology, Skye, SA). Jumps began from a standing starting position, with the feet approximately shoulder-width apart and hands placed on the hips throughout. Participants then lowered themselves to a self-selected depth and jumped for maximal height without pausing between the eccentric and concentric phases. Participants were encouraged to be as explosive as possible during the movement to achieve maximal jump height. Three maximal CMJs were performed by each participant, with one min of rest between each jump. The jump whereby the highest peak force was achieved was chosen for analysis.

Squat jump performance

Squat jump performance was assessed in the same manner as for CMJ; however, participants were required to remain static in the bottom position of the jump for 3 s before performing the concentric phase of the jump. The jump whereby the highest peak force was achieved was chosen for analysis.

Ballistic push-up performance

Participants adopted a push-up position with their hands in the centre of the force plate and elbows at full extension. They then lowered themselves to 90° elbow flexion, remained static for 2 s, and then pushed up as explosively as possible to achieve maximal height from the force plate. Participants were required to keep their body straight throughout the procedure. The trial whereby the highest peak force was achieved was chosen for analysis.

Maximal strength

Maximal strength was assessed via one-repetition maximum (1-RM) leg press and bench press exercises using a plate-loaded 45° incline leg press (Hammer Strength Linear, Schiller Park, IL) and standard bench press, respectively. Following a standardized warm-up of 6, 4 and 2 repetitions at 50, 70 and 90% estimated 1-RM, respectively, single repetitions of increasing load were attempted until the maximal load for one repetition was determined. Three minutes of recovery was given between attempts. Leg press repetitions began with the knee fully extended and the heel placed at the bottom edge of the foot plate. The foot plate was lowered until the knee angle reached 90° and was then returned to full extension. Bench press repetitions started from full elbow extension, after which the barbell was lowered to the chest and then lifted to full elbow extension.

Body composition

Body composition was assessed via Dual X-ray Absorptiometry (DXA) (Discovery W, Hologic Inc.,Bedford, MA) both pre- and post-training. Participants were scanned in the fasted state and were instructed not to perform any exercise for 12 h prior to each scan. The scanner was calibrated daily, and the same certified densitometry technician performed and analysed both the pre- and post-training scans for each participant.

Resistance training (RT) intervention

The resistance training (RT) program was performed three times per week on non-consecutive days (see Table 2), for seven weeks. Training intensity was set at 12-RM for all exercises except for dips and abdominal curls, which were set at 20-RM. Once a participant could perform all sets of a particular exercise at the target number of repetitions at the prescribed load, the load for that exercise was then increased by ~5% for the next session. Two minutes of recovery was allowed between sets. At the start of the third session for each week, both leg press and bench press 1-RM were assessed (as described previously).

*****INSERT TABLE 2 ABOUT HERE*****

Recovery interventions

Five minutes after completing each RT session, participants underwent their assigned recovery intervention for 15 min. Participants in the COLD group were seated (with legs fully extended) in an inflatable bath (iBody, iCool Sport, Australia), and immersed in water up to their sternum. Water temperature was maintained at 10°C with a cooling/heating unit (Dual Temp Unit, iCool Sport, Australia). Participants in the CON group instead sat in a chair in a room maintained at 23°C for the 15 min period.

Muscle biopsy trial

Participants were asked to refrain from exercise and alcohol in the 24 h preceding the muscle biopsy trial, and reported to the lab in a fasted state after ingesting a standardized dinner (containing 53.1 g carbohydrate, 41 g protein and 10.9 g fat) the night before. After sitting quietly for ~10 min, a resting *vastus lateralis* muscle biopsy was taken (described below). Participants rested for a further 10 min before performing the first session of their RT program, followed by their allocated recovery intervention. Participants then rested for 1 h before a second muscle biopsy was taken. Participants were then given a post-exercise snack (containing 61.2 g carbohydrate, 13.2 g protein and 13.4 g fat) before leaving the laboratory. Participants returned to the laboratory for a third biopsy sample 48 h after completing the exercise session. Participants were also asked to refrain from exercise and alcohol in the 24 h preceding this biopsy and reported to the lab in a fasted state following a standardized dinner (equivalent to the pre-trial dinner) the night before. The biopsy trial was repeated 72 to 96 h after the final resistance training session.

Muscle biopsy procedure

During the pre- and post-training biopsy trials, a needle muscle biopsy was taken from the middle third of the *vastus lateralis* muscle at rest, and 1 and 48 h after exercise. After injection of a local anaesthetic into the skin and fascia [1% lidocaine (xylocaine)], a small incision was made and a muscle sample taken using a Stille biopsy needle modified with suction (20). Each biopsy was taken from the participant's dominant leg via a separate incision, 1 to 2 cm proximal from the previous biopsy. Muscle samples were blotted on filter paper to remove excess blood, immediately frozen in liquid nitrogen, and stored at −80 °C until subsequent analysis. A small portion of each biopsy sample (~20 mg) was embedded in Tissue-Tek (Sakura, Finetek, NL), frozen in liquid nitrogen-cooled isopentane, and stored at −80 °C for subsequent immunofluorescence analysis.

Muscle temperature assessment

Muscle temperature responses to the exercise and recovery protocols were assessed immediately following the fourth session of the RT program. This session was chosen as it involved the same RT protocol as the muscle biopsy trial, thereby providing a representation of muscle temperature responses during this trial, while limiting the number of invasive measures obtained. Immediately after completion of the RT protocol, a thermistor was inserted at a site ~5 cm lateral to the mid-point between the participant's anterior superior iliac spine and head of the patella, on the dominant leg (9). An 18 gauge needle (Optiva IV Catheter 18GX1.75", Smiths Medical, USA) was inserted at the marked site, after which it was subsequently removed whilst leaving the catheter in the quadriceps muscle. A needle thermistor probe (Model T-204A, Physitemp Instruments, USA) was inserted through the catheter, to a depth of ~4 cm below the skin. The thermistor probe and catheter were securely covered and fastened to the leg, allowing for movement and continual measurement (2 Hz) of muscle temperature during the recovery intervention.

Immunohistochemistry

Muscle cross-sections (10 μM) were cut at -20°C using a cryostat (CM 1950, Leica Biosystems, Buffalo Grove, IL), mounted on uncoated glass slides, and frozen at -80°C until subsequent analysis. After thawing for 10 min at room temperature, sections were rinsed briefly with 1×PBS (phosphate buffered saline; 0.1M; Sigma Aldrich, St Louis, MO), fixed with cold paraformaldehyde (4% v/v in 1×PBS) for 10 min at room temperature, rinsed three times with 1×PBS, and then blocked for 1 h at room temperature in a 3% w/v BSA solution in 1×PBS. After blocking, sections were then incubated with a primary antibody for myosin heavy chain type I (cat no. M8421, Sigma Aldrich, St Louis, MO), diluted 1:25 in 3% w/v BSA/PBS, for 2 h at room temperature. Slides were then washed three times in 1×PBS for 5 min each before incubation with a secondary antibody (Alexa Fluor® 568 conjugate Goat anti-mouse IgG1, cat. no. A-21124, Thermo Fisher Scientific, Waltham, MA) diluted 1:500 in 3% w/v BSA/PBS for 1 h in the dark at room temperature. Sections were again washed three times in 1×PBS for 5 min each, before incubation with Wheat Germ Agglutinin (WGA) (Alexa Fluor® 488 Conjugate; cat. no. W11261, Thermo Fisher Scientific, Waltham, MA), diluted to 1:100 in 1×PBS (from a 1.25 mg/mL stock solution), for 15 min at room temperature. Sections were washed again twice with 1×PBS for 3 min each, blotted dry with a Kim-Wipe, and anti-fade solution (Prolong™ Gold AntiFade Mountant; cat. no. P36930; Thermo Fisher Scientific, Waltham, MA) added to each section before the coverslip was mounted. Stained muscle sections were air-dried overnight and viewed with a confocal microscope (Olympus FV10i, Shinjuku, Japan). Images were captured with a 10× objective and analysed using MyoVision Basic software (version 1.0) (74). Analysis was completed by an investigator blinded to all groups and time points. For each subject, muscle fiber CSA was determined for both type I and type II muscle fibers. For the COLD and CON groups, a total of 59 ± 19, and 50 ± 24 (mean ± SD) type I fibers and 87 ± 40, and 75 ± 42 (mean ± SD) type II fibers were analysed per subject (and per timepoint), respectively. Representative immunohistochemistry images for both training groups at pre- and post-training are shown in Figure 2.

Western blotting

The abundance of target proteins in muscle samples were determined with all constituents present (i.e., without centrifugation) (42). Frozen muscle was cut into 20 μm sections (Cryostat HM550, Thermo Scientific, Australia), and approximately 20 sections were dissolved in 200 μL homogenising buffer [125 mM Tris-HCl, 4% SDS, 10% Glycerol, 10 mM EGTA, 100 mM DTT, with 0.1 % v/v protease and phosphatase inhibitor cocktail (#P8340 and #P5726, Sigma Aldrich, Castle Hill, NSW, Australia)], which were vortexed and then freeze-thawed. The protein concentration of each sample was then determined using a commercially-available assay with SDS neutralizer (Red 660, G-Biosciences, Astral Scientific, Gymea NSW, Australia) and samples were diluted to equivalent concentrations (1 $\mu g \cdot \mu L^{-1}$) in homogenising buffer. Bromophenol blue (1% v/v) was added to samples and pooled samples, and aliquots of each sample were made to avoid multiple freeze-thaw cycles. Samples were heated at 95 °C for 5 min before 6 to 8 μg protein was loaded per lane into pre-cast 26-well 4 to 20% gradient gels (Criterion™ TGX Stain-Free™ Precast, BioRad, Gladesville NSW, Australia). A molecular weight ladder (PageRuler® Plus, Thermo Scientific, Australia) and a five-point calibration curve (4 to 24 μg) consisting of a pooled sample were also loaded on each gel to allow direct comparison of blot intensities via linear regression (42). Samples from both the CON and CWI groups were loaded into each gel. Optimal loading volumes were determined for each protein target to ensure that blot intensities were within the linear range of the standard curve (i.e., to avoid primary antibody saturation) (42). After separation by SDS PAGE, stain-free gels were activated by UV light (ChemiDoc™ MP, BioRad, Gladesville NSW, Australia) and imaged prior to antibody incubation to visualise the total protein of each lane, both for confirmation of sample loading and for subsequent loading control normalisation. Proteins were then transferred to PVDF membranes (Trans-Blot® Turbo™, BioRad, Gladesville NSW, Australia), which were then blocked in 20 mM Tris, 150 mM NaCl, and 0.1% Tween 20 (TBST) containing 5% nonfat milk for 1 h at room temperature, washed with TBST, and then incubated with primary antibody overnight at 4ºC. To determine protein expression and phosphorylation, membranes were incubated with the following antibodies diluted 1:1000 in TBST containing 5% w/v BSA and 0.1% w/v sodium azide. Primary antibodies for phosphorylated (p-) p-mTOR^{Ser2448} (#5536), mTOR (#2972), p-p70S6K1^{Thr389} (#9234), p70S6K1 (#2708), p-4E-BP1$^{Thr37/46}$ (#2855), 4E-BP1 (#9644), p-rps6$^{Ser235/236}$ (#2211), rps6 (#2217), p-FOXO1^{Ser256} (#9461), FOXO1 (#2880), p-FOXO3a^{Ser253} (#13129), and FOXO3a (#12829) were from Cell Signalling Technology (Danvers, MA), p-HSP27^{Ser82} (#ALX-804-588), p-HSP27^{Ser15} (#ADI-SPA-525), HSP27 (#ADI-SPA-800), p-αB-crystallin^{Ser59} (#ADI-SPA-227), αβ-crystallin (#ADI-SPA-222), HSP72 (#ADI-SPA-810) was from Enzo Life Sciences (Farmingdale, NY), and MuRF1 (#MP3401) was from ECM Biosciences (Versailles, KY). Membranes were washed 5 times with TBST, before probing with appropriate horseradish peroxidase-conjugated secondary antibody (PerkinElmer, Glen Waverley, Victoria, Australia), at a dilution of 1:50,000 – 100,000 in 5% non-fat milk TBST for 1 h at room temperature. Protein-antibody-HRP conjugates were incubated in ECL (SuperSignal® West Femto, Thermo Scientific, Australia) and imaged with a high sensitivity CCD camera (ChemiDoc™ MP, BioRad, Gladesville NSW, Australia) for subsequent analysis (ImageLab v 5.1, BioRad, Gladesville NSW, Australia). Total protein loading of each sample was determined from stain-free images of each gel, and these values were then used to normalise each protein of interest after normalisation to its respective standard curve. Representative western blot images for each measured protein are shown in Figure 6.

Statistical analyses

To reduce bias from non-uniformity of error, heteroscedastic data were logarithmically transformed before analysis (e.g., for Western blot data) (45). For these data, geometric mean and SD (geometric mean × / ÷ SD) are reported. All other data are reported as mean ± SD unless otherwise specified. Linear mixed models were used to determine the influence of recovery condition (i.e., COLD or CON) on outcome variables, with "time" (repeated measure across all timepoints), "training status" (i.e., pre- vs. post-training), "group" and "group × time" as fixed factors, and "subject" as a random factor. First-order autoregressive covariance structures were used for all models, and model fit was assessed by ⁻2 log likelihood (21). In the absence of a statistically significant ($P < 0.05$) group × time interaction, effects over time are reported on pooled group data (i.e., for both groups combined). The magnitude of within-group changes in dependent variables (and between-group differences in these changes) were quantified as Cohen's *d* (effect size, ES), applying thresholds of < 0.2 = trivial, 0.2-0.6 = small, 0.6-1.2 = moderate, 1.2-2.0 = large, 2.0-4.0 = very large and > 4.0 = extremely large (29). Effects were considered substantial if there was a >75% probability of being positive relative to the smallest worthwhile change (ES = 0.2), and effects with a >5% probability of being either substantially positive or negative were deemed unclear (29). Uncertainty of effects were determined as 90% confidence intervals (CI) and precise P values (unless $P < 0.001$) (13). Linear mixed models were analysed using IBM SPSS Statistics Version 25 (IBM, Somers, NY) and ES and CI values were determined via custom Excel spreadsheets (28). Percent compliance between groups was compared using an independent samples *t*-test (IBM SPSS Statistics Version 25, Somers, NY) and ES and CI values were determined using a custom Excel spreadsheet (27).

5. Results

For a detailed summary of statistical data for all within- and between-group effects considered substantial in magnitude, see Tables 3 and 4, respectively.

Training compliance

Training compliance was not different between CON (92.3 ± 6.2%) and COLD (91.1 ± 4.7%) (P = 0.676, ES: 0.20; ±90% CI 0.83).

Muscle temperature assessment

Between the completion of the fourth training session and end of the post-exercise recovery intervention, muscle temperature decreased more for COLD (-3.5°C ± 3.5) vs. CON (-0.5°C ± 0.5) (group × time interaction: P = 0.031, ES: 2.27; ±1.27).

<u>Basal responses to training</u>

Performance measures

Maximal strength

There was no group × time interaction (P = 0.959, ES: 0.04; ±0.78) for one-repetition maximum (1-RM) leg press (Table 2), which increased at POST for both groups combined (time main effect: P < 0.001, Table 3).

Similar to lower-body strength, there was no group × time interaction (P = 0.582, ES: 0.08; ±0.35) for 1-RM bench press (Table 2), which increased at POST for both groups combined (time main effect: P = 0.001, Table 3).

Countermovement jump (CMJ), squat jump, and ballistic push-up performance

There was a group × time interaction (P = 0.006) for peak CMJ force (Table 2), which increased at POST only for CON (Table 3) and with a greater change vs. COLD (Table 4).

There was no group × time interaction for neither peak squat jump force (P = 0.249, ES: 0.33; ±0.51) nor ballistic push-up force (P = 0.898, ES: 0.05; ±0.30), neither of which changed over time for both groups combined (time main effect: P = 0.355, ES: 0.13; ±0.36 and P = 0.898, ES: 0.03; ±0.23, respectively, see Table 2).

Body composition

There was no group × time interaction (P = 0.867, ES: 0.02; ±0.22) for total lean mass (Table 2), which increased at POST for both groups combined (time main effect: P < 0.001, Table 3).

There was no group × time interaction for lower-body lean mass (P = 0.935, ES: 0.22; ±0.37) or upper-body lean mass (P = 0.669, ES: 0.06; ±0.30, Table 2). For both groups combined, both lower-body and upper-body lean mass were increased at POST (time main effect: P = 0.002 and P < 0.001, respectively, Table 3).

There was no group × time interaction (P = 0.423, ES: 0.09; ±0.15) for fat mass (Table 2), which decreased at POST for both groups combined (time main effect: P = 0.005, Table 3).

Muscle fiber CSA

There was no group × time interaction ($P = 0.568$, ES: 0.52; ±1.38) for type I muscle fiber CSA (Figure 2A), which was unchanged at POST for both groups combined (time main effect: $P = 0.175$, ES: 0.42; ±0.92).

There was no group × time interaction ($P = 0.062$) for type II muscle fiber CSA (Figure 2B); however, there was a greater PRE-POST change for CON vs. COLD (Table 4). Representative immunohistochemical images for changes in muscle fiber CSA are shown in Figure 2 (C-F).

*****INSERT TABLE 3 ABOUT HERE*****

*****INSERT TABLE 4 ABOUT HERE*****

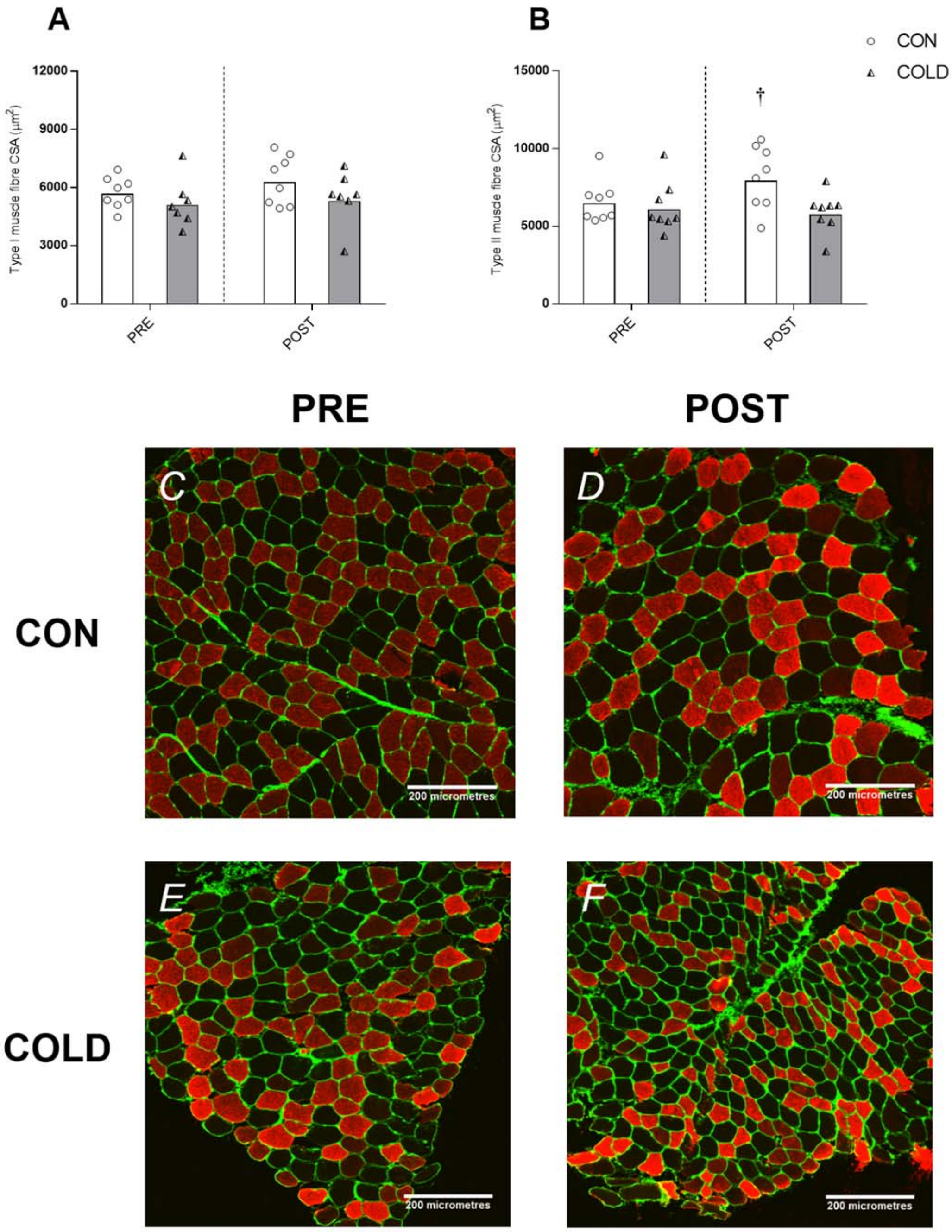

Figure 2. Type I (A) and type II (B) muscle fiber cross-sectional area (CSA) before (PRE), and after (POST) seven weeks of resistance training with either cold-water immersion (COLD) or passive control (CON) applied after each training session. Data are mean values ± SD.

Representative confocal microscope immunofluorescence images of muscle cross-sections obtained before (PRE) and after (POST) seven weeks of resistance training with application of either control (CON; images C and D, respectively) or cold-water immersion (COLD;

images E and F, respectively) or after each training session. Muscle fiber membranes are visualized green, type I muscle fibers are visualized red, and type II muscle fibers are unstained. Scale bar = 200 μm.
† = Substantially greater change for CON vs. COLD.

*****INSERT FIGURE 2 ABOUT HERE*****

Total protein content

Total p70S6K protein

There was no group × time interaction (P = 0.152, ES: 0.67, ±0.70) for total p70S6K protein (Figure 3B), which was unchanged at POST for both groups combined (time main effect: P = 0.888, ES: 0.03; ±0.74).

Total rps6 protein

There was no group × time interaction (P = 0.577, ES: 0.51, ±1.33) for total rps6 protein (Figure 3D), which increased at POST for both groups combined (time main effect: P = 0.009, Table 3).

Total 4E-BP1 protein

There was no group × time interaction (P = 0.128, ES: 0.33, ±0.43) nor main effect of time (P = 0.061, ES: 0.26; ±0.35) for total 4E-BP1 protein (Figure 3F).

Total FOX-O1 protein

There was no group × time interaction (P = 0.108) for total FOX-O1 protein (Figure 4B), which increased at POST for both groups combined (time main effect: P = 0.007, Table 3). There was, however, a greater PRE-POST change in total FOX-O1 protein for COLD vs. CON (Table 4).

Total FOX-O3a protein

There was no group × time interaction (*P* = 0.644, ES: 1.50, ±1.97) for total FOX-O3a protein (Figure 4D), which was unchanged at POST for both groups combined (time main effect: *P* = 0.195, ES: 0.54; ±1.34).

Total MuRF-1 protein

There was no group × time interaction (*P* = 0.596, ES: 0.10, ±0.36) for total MuRF-1 protein (Figure 4E), which was unchanged at POST for both groups combined (time main effect: *P* = 0.313, ES: 0.10, ±0.25).

Total HSP27 protein

There was no group × time interaction (*P* = 0.113) for total HSP27 protein (Figure 5B), which increased at POST for both groups combined (time main effect: *P* < 0.001, Table 3), with a greater PRE-POST change for CON vs. COLD (Table 4).

Total HSP72 protein

There was no group × time interaction (*P* = 0.465) for total HSP72 protein (Figure 5D), which decreased at POST for both groups combined (time main effect: *P* < 0.013, Table 3), due to a reduction for COLD (Table 3) and not for CON (-0.8-fold × / ÷ 1.4, ES: -0.33, ±0.65).

Total αβ crystallin protein

There was no group × time interaction (*P* = 0.488, ES: 0.29, ±0.88) for total αβ crystallin protein (Figure 5F), which increased at POST for both groups combined (time main effect: *P* = 0.004, Table 3).

Responses to single exercise sessions before and after training

mTORC1 signalling responses

p-p70S6K ^{Thr389}

There was no group × time interaction (P = 0.411), nor influence of training status (P = 0.369), for p70S6K^{Thr389} phosphorylation (Figure 3A). p70S6K^{Thr389} phosphorylation was, however, increased for both groups combined at PRE +1 h, PRE +48 h, and POST +48 h (time main effect: P = 0.001, Table 3).

p-rps6 $^{Ser235/236}$

There was no group × time interaction (P = 0.154), nor influence of training status (P = 0.707), for rps6$^{Ser235/236}$ phosphorylation (Figure 3C), which was increased for both groups combined at PRE +1 h, POST +1 h, and POST +48 h (time main effect: P < 0.001, Table 3). There were also greater increases in rps6$^{Ser235/236}$ phosphorylation for CON vs. COLD at both POST +1 h and POST +48 h (Table 4).

p-4E-BP1 $^{Thr36/47}$

There was no group × time interaction (P = 0.440) nor main effects of training status (P = 0.94) or time (P = 0.395) for 4E-BP1$^{Thr36/47}$ phosphorylation (Figure 3E). There was, however, a greater increase in 4E-BP1$^{Thr36/47}$ phosphorylation for CON vs. COLD from PRE-PRE +1 h (Table 4).

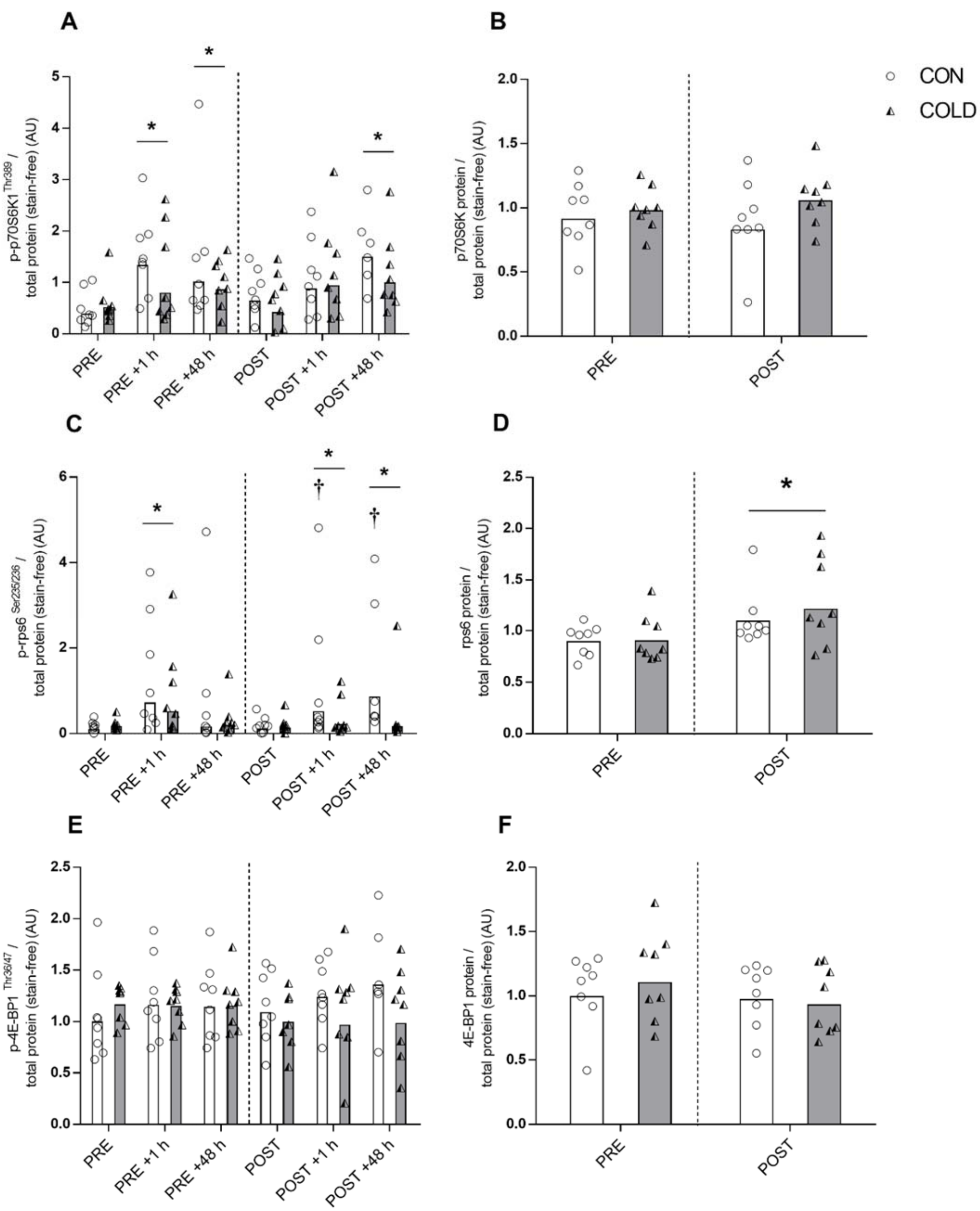

Figure 3. **mTORC1 signalling responses**. Phosphorylation and total proteins levels of p70S6K^{Thr389} (A, B respectively), rps6$^{Ser235/236}$ (C, D respectively), and 4E-BP1$^{Thr36/47}$ (E, F respectively) before (PRE) and after (POST) seven weeks of resistance training with either cold-water immersion (COLD) or passive control (CON) applied after each training session, as well as 1 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h) and after (POST +1 h, POST +48 h) the training period (phosphorylated proteins only). Data shown are back-transformed individual participant values and geometric means.
* = $P < 0.05$ vs. PRE, † = substantially greater change vs. COLD.

*****INSERT FIGURE 3 ABOUT HERE*****

Protein degradation responses

p-FOX-O1 ^{Ser256}

There was no group × time interaction (*P* = 0.311) nor influence of training status (*P* = 0.202) for FOX-O1^{Ser256} phosphorylation (Figure 4A), which was unchanged over time for both groups combined (*P* = 0.302). There was, however, a greater increase for CON vs. COLD at both POST +1 h and POST +48 h (Table 4).

p-FOX-O3a ^{Ser253}

There was no group × time interaction (*P* = 0.414) nor influence of training status (*P* = 0.688) for FOX-O3a^{Ser253} phosphorylation (Figure 4C), which decreased at POST +1 h for both groups combined (time main effect: *P* = 0.010, Table 3).

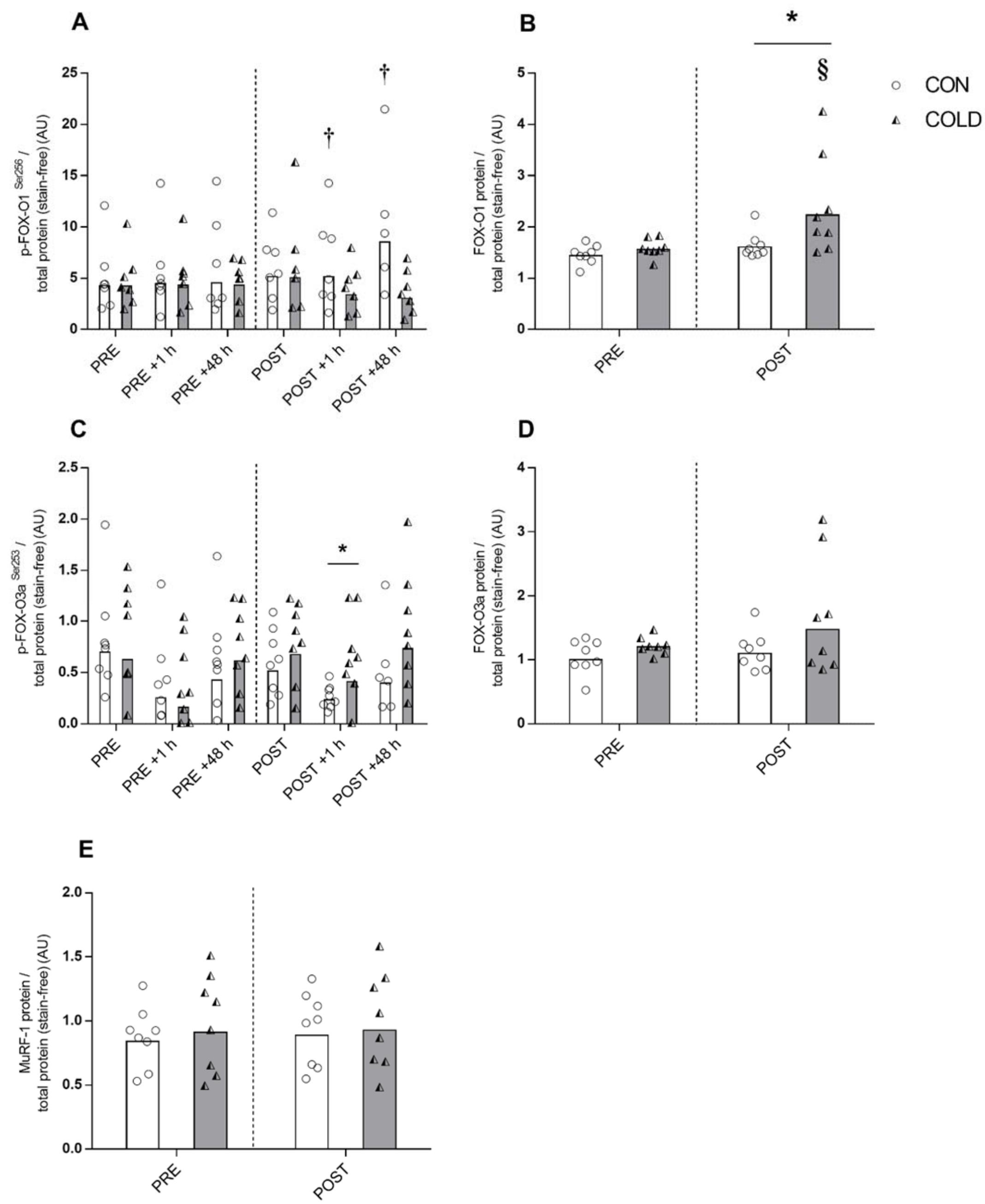

Figure 4. **Protein degradation-related responses**. Phosphorylation and total proteins levels of FOX-O1^{Ser256} (A, B respectively), FOX-O3a ^{Ser253} (C, D respectively) and MuRF-1 (E) before (PRE) and after (POST) seven weeks of resistance training with either cold-water immersion (COLD) or passive control (CON) applied after each training session, as well as 1 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h) and after

(POST +1 h, POST +48 h) the training period (phosphorylated proteins only). Data shown are back-transformed individual participant values and geometric means.
* = $P < 0.05$ vs. PRE, † = substantially greater change vs. COLD, § = substantially greater change vs. CON.

*****INSERT FIGURE 4 ABOUT HERE*****

Heat shock protein responses

p-HSP27 ^{Ser15}

There was no group × time interaction ($P = 0.804$) nor influence of training status ($P = 0.110$) for HSP27^{Ser15} phosphorylation (Figure 5A), which increased for both groups combined at PRE +1 h and POST +1 h (time main effect: $P < 0.001$, Table 3). The increase in HSP27^{Ser15} phosphorylation at PRE +1 h was also greater for COLD vs. CON (Table 4).

p-HSP27 ^{Ser82}

There was no group × time interaction ($P = 0.377$) nor influence of training status ($P = 0.354$) for HSP27^{Ser82} phosphorylation (Figure 5C), which increased for both groups combined at PRE +1 h and POST +1 h (time main effect: $P < 0.001$, Table 3).

p-αβ crystallin ^{Ser59}

There was no group × time interaction ($P = 0.900$) nor influence of training status ($P = 0.483$) for αβ crystallin^{Ser59} phosphorylation (Figure 5E), which increased for both groups combined at PRE +1 h, PRE +48 h, and POST +1 h (time main effect: $P < 0.001$, Table 3).

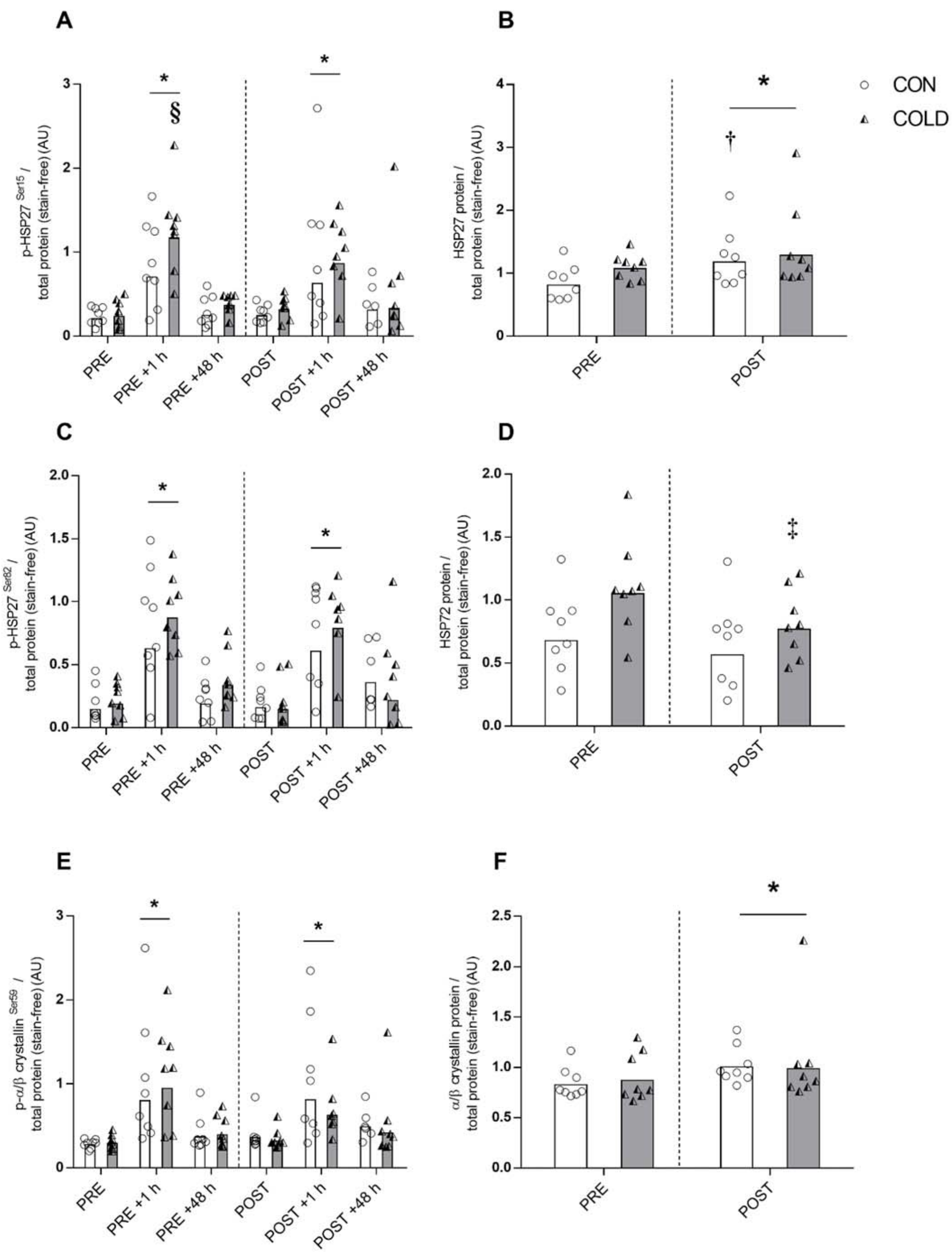

Figure 5. Heat shock protein responses. Phosphorylation of HSP27^{Ser15} (A), HSP27^{Ser82} (C) and αβ crystallin^{Ser59} (E), and total protein levels of HSP27 (B), HSP72 (D), and αβ crystallin (F) before (PRE) and after (POST) seven weeks of resistance training with either cold-water immersion (COLD) or passive control (CON) applied after each training session, as well as 1 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h) and after (POST +1 h, POST +48 h) the training period (phosphorylated proteins only). Data shown are back-transformed individual participant values and geometric means.

* = P < 0.05 vs. PRE, ‡ = substantial change vs. PRE. † = substantially greater change vs. COLD, § = substantially greater change vs. CON.

INSERT FIGURE 5 ABOUT HERE

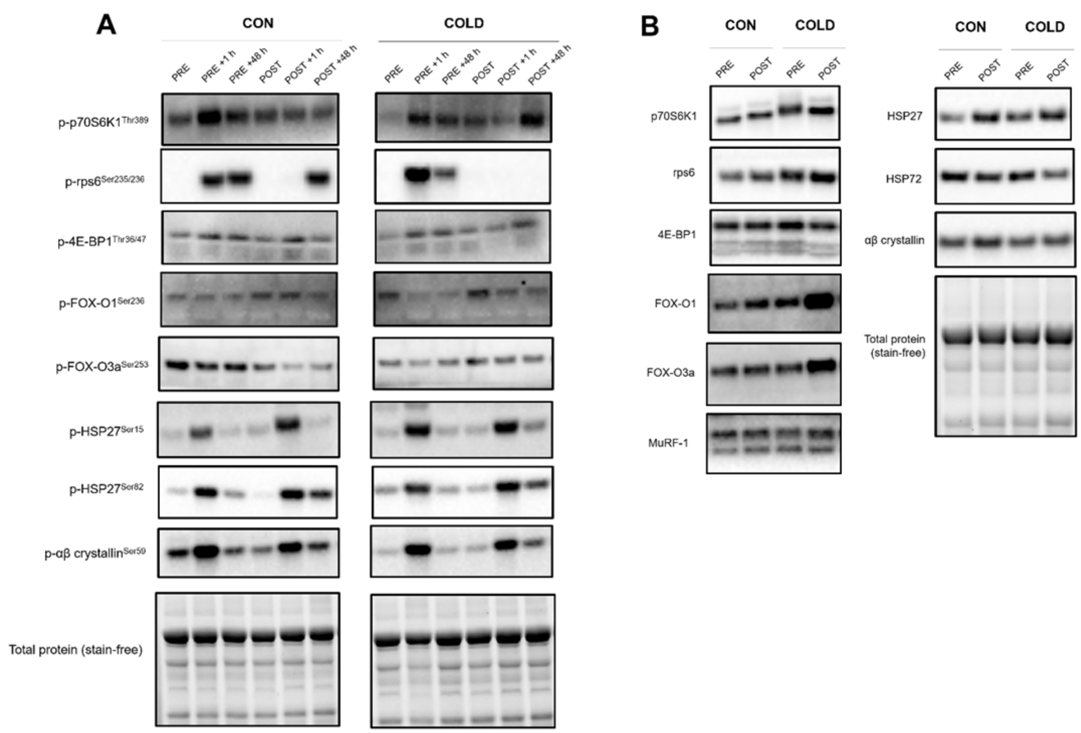

Figure 6. Representative Western blot images for analysed phosphorylated proteins (A) and total protein content (B) before (PRE) and after (POST) seven weeks of resistance training with either cold-water immersion (COLD) or passive control (CON) applied after each training session, as well as 1 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h) and after (POST +1 h, POST +48 h) the training period.

INSERT FIGURE 6 ABOUT HERE

6. Discussion

This study provides novel insights on the influence of post-exercise CWI on adaptations to whole-body resistance training, and the potential underlying mechanisms in skeletal muscle. Repeated post-exercise CWI blunted the training-induced increase in type II muscle fiber CSA following seven weeks of resistance training, which coincided with attenuated post-exercise mTORC1 signalling (i.e., rps6 phosphorylation) after the training period. Repeated post-exercise CWI also increased basal levels of protein degradation markers (e.g., FOX-O1 protein content) in skeletal muscle after the training period. Taken together, these observations suggest CWI may shift post-exercise muscle protein balance towards reduced protein synthesis and increased breakdown, culminating in blunted muscle fiber hypertrophy. However, the negative influence of CWI on muscle fiber hypertrophy did not translate to impeded maximal strength development. These data further highlight the negative influence of post-exercise CWI on muscle fiber hypertrophy, and suggest post-exercise CWI should be avoided if muscle hypertrophy is desired.

The findings that CWI attenuated post-exercise anabolic signalling responses to single resistance training sessions, together with blunted type II muscle hypertrophy, are in agreement with previous work (56). Roberts et al. (56) also reported an attenuated increase in *vastus lateralis* type II fiber size following resistance training coupled with post-exercise CWI compared with an active recovery. In a separate sub-study (56), these responses occurred alongside a blunted increase in p70S6K phosphorylation after the first training session (at both 2 and 24 hours post-exercise) and attenuated myonuclei accretion after the training period. This blunting of p70S6K phosphorylation did not, however, influence the phosphorylation response of rps6, a key downstream target of p70S6K (54), nor other key proteins that regulate MPS, such as 4E-BP1 (eIF4E binding protein 1) (56). In contrast to these findings (56), we noted similar post-exercise p70S6K phosphorylation with CWI application compared with passive recovery, which was elevated for both conditions before (at +1 and +48 h) and after the training period (at +48 h), and instead saw blunted post-exercise phosphorylation of rps6, a key downstream target of p70S6K, after the training period.

A novel aspect of this study was assessment of post-exercise molecular responses to single resistance training sessions, combined with either CWI or passive recovery, both before and after the training intervention. This allowed insight into the potential modulation of any CWI-mediated effects on post-exercise molecular responses following a training period. Using this approach, we observed blunted mTORC1 signalling (i.e., rps6 phosphorylation) for the CWI group compared with CON after (i.e., at both POST +1 h and POST +48 h), but not before, the training period. This observation highlights the discordance between molecular responses to exercise performed in untrained and trained states, and suggests the blunting of anabolic responses by CWI may be exacerbated with repeated sessions of resistance training. Since these responses coincided with the timepoint whereby attenuated type II muscle fiber CSA was observed, this suggests muscle growth may be even further compromised with longer period of resistance training and CWI. From a mechanistic perspective, the negative influence of CWI on post-exercise anabolic responses may be mediated by the influence of cold exposure and thermogenesis on energy metabolism. For example, enhanced thermogenesis and associated increases in myoplasmic AMP during cold exposure (64) may have influenced AMPK activity, which would potentially inhibit mTORC1 signalling (7). However, as direct measures of AMPK activity were unfortunately not possible in the present study, this mechanism remains speculative. Evidence of increased thermogenesis with CWI is perhaps further supported by the greater loss of fat mass experienced by the COLD group, which may have resulted from a lower net energy balance (stimulated by shivering and non-shivering thermogenesis) (63) following each CWI session.

Despite the blunted improvement in type II muscle fiber CSA seen following resistance training with CWI, we did not observe any influence of CWI in lower-body lean mass assessed via DXA. This apparent discordance may be explained by the limitations of each measurement as indices of changes in whole muscle size, and because changes in whole-muscle size do not always reflect changes in muscle fiber CSA (44). The reliability and sensitivity of DXA-derived measures of lean mass is highly dependent on levels of hydration and prior exercise (43). Although we attempted to control for both of these factors, the sensitivity of DXA for detecting small changes in muscle size is relatively poor compared to more sophisticated imaging techniques, such as MRI (magnetic resonance imaging) (36) or CT (computed tomography) (14). Indeed, previous studies (56) have reported attenuated increases in thigh muscle volume following resistance training coupled with CWI when assessed via MRI, which was consistent with their observations of blunted muscle fiber size. We therefore cannot exclude the possibility that DXA was not sensitive enough to detect changes in whole-muscle size that may have been underpinned by the responses seen at the muscle fiber level. In addition to differences in the sensitivity of DXA-derived lean mass versus direct measurements of muscle fiber CSA, differences in the region-specificity of each measure may also explain the discordant responses observed. For example, DXA provides an estimate of lean mass in the entire lower extremities, whereas muscle biopsies can only reflect a specific site in the *vastus lateralis*. As hypertrophy of the quadriceps femoris musculature occurs heterogeneously following resistance training (17), these region-specific differences may explain the observation of increased muscle fiber size in the absence of changes in total lower-body lean mass.

Unlike previous work (56), attenuated muscle fiber hypertrophy with lower-body CWI did not occur alongside blunted maximal lower-body strength gain. Although muscle hypertrophy has traditionally been associated with muscle strength gain (40), recent work has questioned the role of training-induced muscle hypertrophy in improved maximal strength (11). From this perspective, any influence of CWI on muscle hypertrophy may have little influence on strength, particularly when assessed during complex, dynamic tasks. Since strength is a highly task-specific phenomenon (41), it is also possible our findings were influenced by the particular measure of strength chosen. Since the contribution of neural factors (i.e., learning and coordination) to strength gain is larger during higher-complexity tasks (58), any attenuation of muscle hypertrophy may have less influence on strength gain when assessed during higher- versus lower-complexity tasks. It is interesting to note the magnitude of attenuated strength gain with CWI application in a previous study (56) appeared greater when assessed during lower- versus higher-complexity strength tasks (i.e., 1-RM leg extension vs. leg press). As we employed a relatively high-complexity task (1-RM leg press) as the only strength outcome measure, this may explain why we did not observe any influence of blunted hypertrophy on maximal strength gain. Nevertheless, our results are in agreement with others showing relatively weak relationships between training-induced muscle hypertrophy and strength (2, 12, 18), and suggest blunted muscle hypertrophy with application of CWI can occur without any influence on dynamic strength development. However, although we did not observe impaired 1-RM strength gains with CWI application, we did observe a blunting of peak force during the CMJ. While not directly assessed in this study, this finding aligns with previous observations of blunted improvement in rate of force development after resistance training with CWI application (56) and suggests improvement in force-generating capacity during rapid, dynamic movements may be compromized with CWI.

Since these tasks are likely more relevant to athletic performance situations compared with maximal strength *per se*, the influence of CWI on these variables warrants further attention.

Another novel aspect of this study was analysis of molecular mediators of protein degradation following resistance training coupled with regular CWI. The transcription of muscle-specific E3 ubiquitin ligases that mediate protein degradation, including MuRF-1, is regulated by the FOX-O family of transcription factors (59). After training, we observed a greater basal increase in total FOX-O1 protein content with CWI, but no change in MuRF-1 protein content for either group. We also noted discordant between-group FOX-O1 and FOX-O3a phosphorylation responses to the single exercise sessions performed before and after the intervention period. For example, post-exercise phosphorylation of FOX-O3a^{Ser253} was acutely decreased before the training period (at PRE +1 h) for both groups (although this was not statistically significant), yet FOX-O1^{Ser256} phosphorylation was unchanged. Conversely, post-exercise increases in FOX-O1^{Ser256} phosphorylation were attenuated following CWI at both +1 h and +48 after the training period, whereas there were little changes noted for FOX-O3a^{Ser253} phosphorylation (although pooled data showed a decrease at POST +1 h). Based solely on these discordant FOX-O1 and FOX-O3a phosphorylation responses, it is unclear whether CWI induced a shift towards increased protein degradation, although the increased basal FOX-O1 protein content after the training period provides support for this occurring with CWI. Nonetheless, although increases in markers of protein degradation may be seen as counteractive to muscle anabolism, these responses are in fact necessary to facilitate exercise-induced skeletal muscle remodelling by removing damaged proteins and/or providing amino acid substrates for synthesising new proteins (70). Because it is difficult to infer the balance between skeletal muscle anabolism and catabolism from these data, the contribution of these responses to the observed changes in muscle fiber size remains unclear.

The heat-shock family of proteins are important for cellular homeostasis, protein preservation and degradation (46), and play key roles in several processes involved in exercise adaptations. For example, HSP72 regulates mRNA elongation rate (35) and inhibits several steps involved in protein degradation (5, 16, 61, 79). HSP27 and αβ-crystallin also inhibit protein degradation pathways (1, 15, 71) and bind to cytoskeletal and myofibrillar proteins following muscle damaging exercise, where they are thought to stabilise disrupted elements and assist in regeneration and remodelling (34, 49). Our data suggested a single session of resistance exercise, performed before the training period, induced similar increases in $HSP27^{Ser15}$ phosphorylation at PRE +1 h for both conditions, although this change was further enhanced for COLD (ES: 0.82; ±1.01). Similar post-exercise changes in $HSP27^{Ser15}$ phosphorylation were however noted between groups after the training period. A similar pattern of response was also observed for $HSP27^{Ser82}$ phosphorylation, with robust increases during the early post-exercise period both before and after the intervention (i.e., at PRE +1 h and POST +1 h), which was also not different between groups. Basal levels of HSP27 protein were elevated after the training intervention for both groups, although this effect was greater for CON (ES: 0.94; ±0.82). Total protein levels of αβ-crystallin were similarly increased at POST for both groups, while similar effects of a single exercise session on p-αβ-$crystallin^{Ser59}$ were observed for both groups both before and after training, although there was a more prolonged increased in p-αβ-$crystallin^{Ser59}$ before training for both CON and COLD. Taken together, these data suggest repeated CWI blunts the chronic, but not acute, HSP27 response to resistance exercise. These responses may have contributed to the blunted fiber hypertrophy for COLD, given these small HSPs appear to be important for muscle remodelling (34, 49). Moreover, while basal HSP72 protein levels were unchanged for CON, they were reduced (0.7-fold) for COLD (ES: 0.79; ±0.57). Since HSP72 inhibits protein degradation (5, 16, 61,

79) and promotes protein synthesis (35), the downregulation of HSP72 may have contributed to the blunted increase in muscle fiber size observed for COLD.

While the present data suggest CWI application after individual resistance training sessions blunts muscle fiber hypertrophy (but not strength gain), these responses were observed in previously untrained individuals. It is unclear, therefore, whether similar findings would occur in resistance-trained individuals, whose relative improvements in both strength and muscle growth would likely be less compared with untrained individuals. Our data suggest that blunted muscle fiber hypertrophy with CWI may be mediated via modulation of molecular pathways regulating muscle protein synthesis and degradation. However, our findings do not elucidate the specific upstream factors directly influenced by CWI that mediated the observed effects on post-exercise molecular responses and muscle fiber hypertrophy. While a number of CWI-mediated factors could have influenced these responses (e.g., post-exercise inflammation, satellite cell activation, reactive oxygen species generation, hormonal responses, changes in muscle blood flow), none of these factors were measured in the present study. It is possible that if the resistance training protocol were altered to exacerbate residual neuromuscular fatigue and potentially inflammation (e.g., by increasing the frequency and/or volume of training), CWI might have been beneficial for hastening recovery and maintaining training intensity, and therefore may have differentially influenced long-term adaptation. Higher frequencies and/or volumes of resistance training are more likely to be completed by more highly-trained individuals, further suggesting the applicability of the present findings to these populations may be limited.

Conclusions

The present study provides novel insights into the modulation of key adaptations to whole-body resistance training combined with lower-body CWI. We provide additional evidence of blunted muscle fiber hypertrophy following resistance training coupled with post-exercise CWI. We provide evidence that CWI attenuates post-exercise anabolic responses both before and after seven weeks of resistance training, and increases basal levels of protein degradation markers post-training. The observation that the CWI-mediated blunting of anabolic responses to single resistance exercise bouts persists after a period of training has implications for muscle growth following longer-term training periods when coupled with CWI. Importantly, the attenuation of muscle fiber hypertrophy with CWI did not impair maximal strength, which potentially reflects the discordance between training-induced changes in muscle mass and strength. Together, these data further highlight the ability of CWI to blunt resistance training-induced muscle growth, but not strength, and suggest avoidance of post-exercise CWI when muscle hypertrophy is a desired resistance training outcome.

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7. Author contributions

A.C.P., S.L.H., D.J.B., R.C.P., and C.K.A. conceived and designed research; J.J.F., A.J.T and A.C.P. performed experiments; J.J.F and A.C.P. analyzed data; J.J.F., J.R.B., A.J.T., E.D.H., D.J.B. and A.C.P interpreted results; J.J.F prepared figures; J.J.F. and A.C.P. drafted manuscript; J.R.B., A.J.T., A.P.G., S.L.H., C.K.A., R.C.P., E.D.H. and D.J.B. edited and revised manuscript; all authors approved final version of manuscript.

All data collection and aspects of data analysis were performed in the exercise physiology and biochemistry laboratories at Victoria University (Footscray Park campus), Melbourne, Australia. Aspects of data analysis were also performed in the exercise biochemistry laboratory at Deakin University (Burwood campus), Melbourne, Australia.

8. Disclosures

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9. Figure legends

Figure 1. Study overview. DXA, dual x-ray absorptiometry scan; BEP, ballistic exercise performance (countermovement jump, squat jump, ballistic push-up) testing; 1-RM, one-repetition maximum (leg press and bench press) testing.

Figure 2. Type I (A) and type II (B) muscle fiber cross-sectional area (CSA) before (PRE), and after (POST) seven weeks of resistance training with either cold-water immersion (COLD) or passive control (CON) applied after each training session. Data are mean values ± SD. Representative confocal microscope immunofluorescence images of muscle cross-sections obtained before (PRE) and after (POST) seven weeks of resistance training with application of either control (CON; images C and D, respectively) or cold-water immersion (COLD; images E and F, respectively) or after each training session. Muscle fiber membranes are visualized green, type I muscle fibers are visualized red, and type II muscle fibers are unstained. Scale bar = 200 μm. † = Substantially greater change for CON vs. COLD.

Figure 3. **mTORC1 signalling responses**. Phosphorylation and total proteins levels of p70S6K^{Thr389} (A, B respectively), rps6$^{Ser235/236}$ (C, D respectively), and 4E-BP1$^{Thr36/47}$ (E, F respectively) before (PRE) and after (POST) seven weeks of resistance training with either cold-water immersion (COLD) or passive control (CON) applied after each training session, as well as 1 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h) and after (POST +1 h, POST +48 h) the training period (phosphorylated proteins only). Data shown are back-transformed individual participant values and geometric means. * = $P < 0.05$ vs. PRE, † = substantially greater change vs. COLD.

Figure 4. **Protein degradation-related responses**. Phosphorylation and total proteins levels of FOX-O1^{Ser256} (A, B respectively), FOX-O3a ^{Ser253} (C, D respectively) and MuRF-1 (E) before (PRE) and after (POST) seven weeks of resistance training with either cold-water immersion (COLD) or passive control (CON) applied after each training session, as well as 1 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h) and after (POST +1 h, POST +48 h) the training period (phosphorylated proteins only). Data shown are back-transformed individual participant values and geometric means. * = $P < 0.05$ vs. PRE, † = substantially greater change vs. COLD, § = substantially greater change vs. CON.

Figure 5. Heat shock protein responses. Phosphorylation of HSP27^{Ser15} (A), HSP27^{Ser82} (C) and αβ crystallin^{Ser59} (E), and total protein levels of HSP27 (B), HSP72 (D), and αβ crystallin (F) before (PRE) and after (POST) seven weeks of resistance training with either cold-water immersion (COLD) or passive control (CON) applied after each training session, as well as 1 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h) and after (POST +1 h, POST +48 h) the training period (phosphorylated proteins only). Data shown are back-transformed individual participant values and geometric means. * = $P < 0.05$ vs.

PRE, ‡ = substantial change vs. PRE. † = substantially greater change vs. COLD, § = substantially greater change vs. CON.

Figure 6. Representative Western blot images for analysed phosphorylated proteins (A) and total protein content (B) before (PRE) and after (POST) seven weeks of resistance training with either cold-water immersion (COLD) or passive control (CON) applied after each training session, as well as 1 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h) and after (POST +1 h, POST +48 h) the training period.

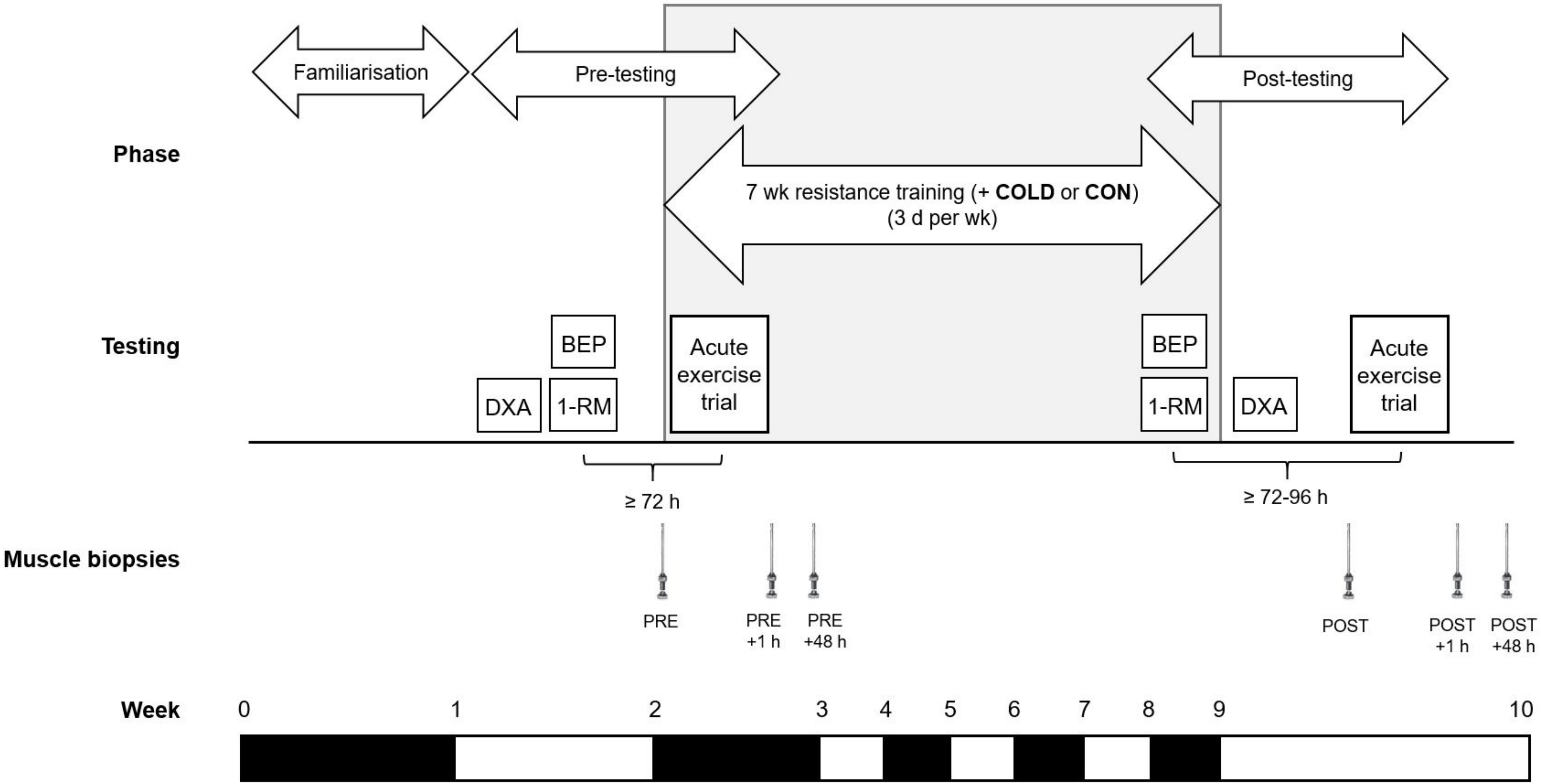
10. Tables

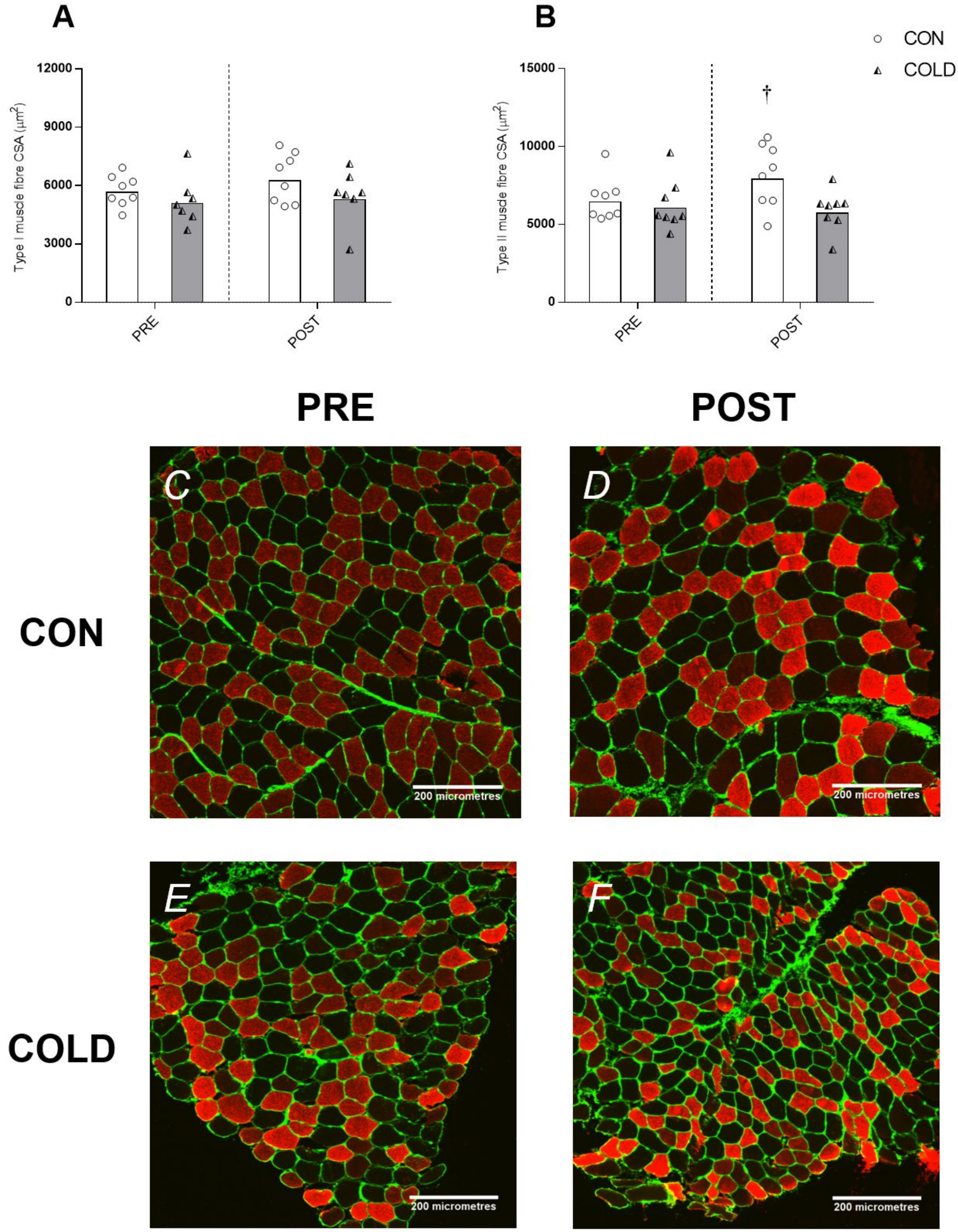
Table 1. Participant physical characteristics, exercise performance and body composition data for the control (CON) and cold water immersion (COLD) training groups. Data shown are group means ± SD. * = $P < 0.05$ vs. PRE.

Table 2. Details of the resistance training (RT) intervention performed by both the control (CON) and cold water immersion (COLD) groups.

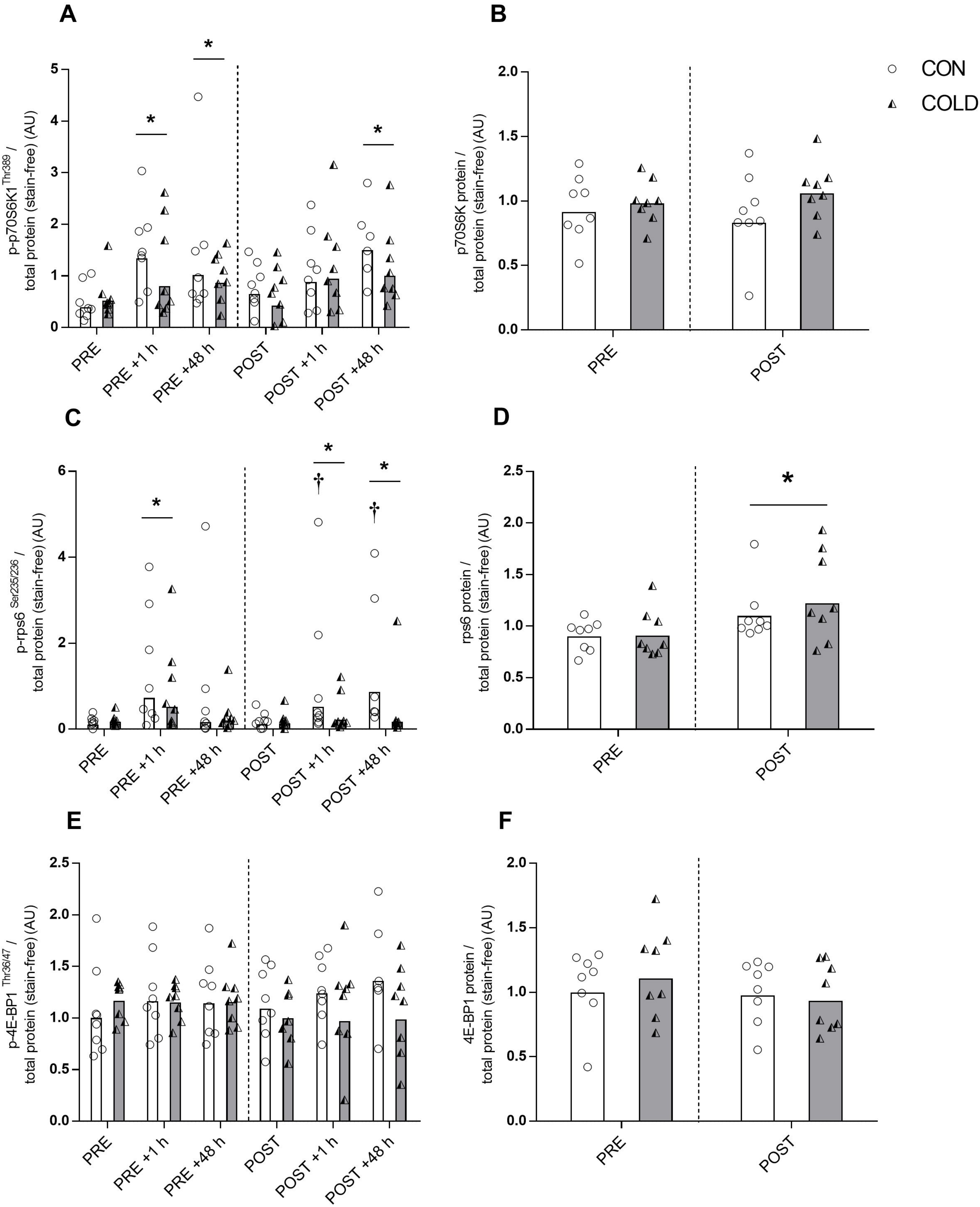
Table 3. Summary of all within-group effects considered substantial in magnitude.

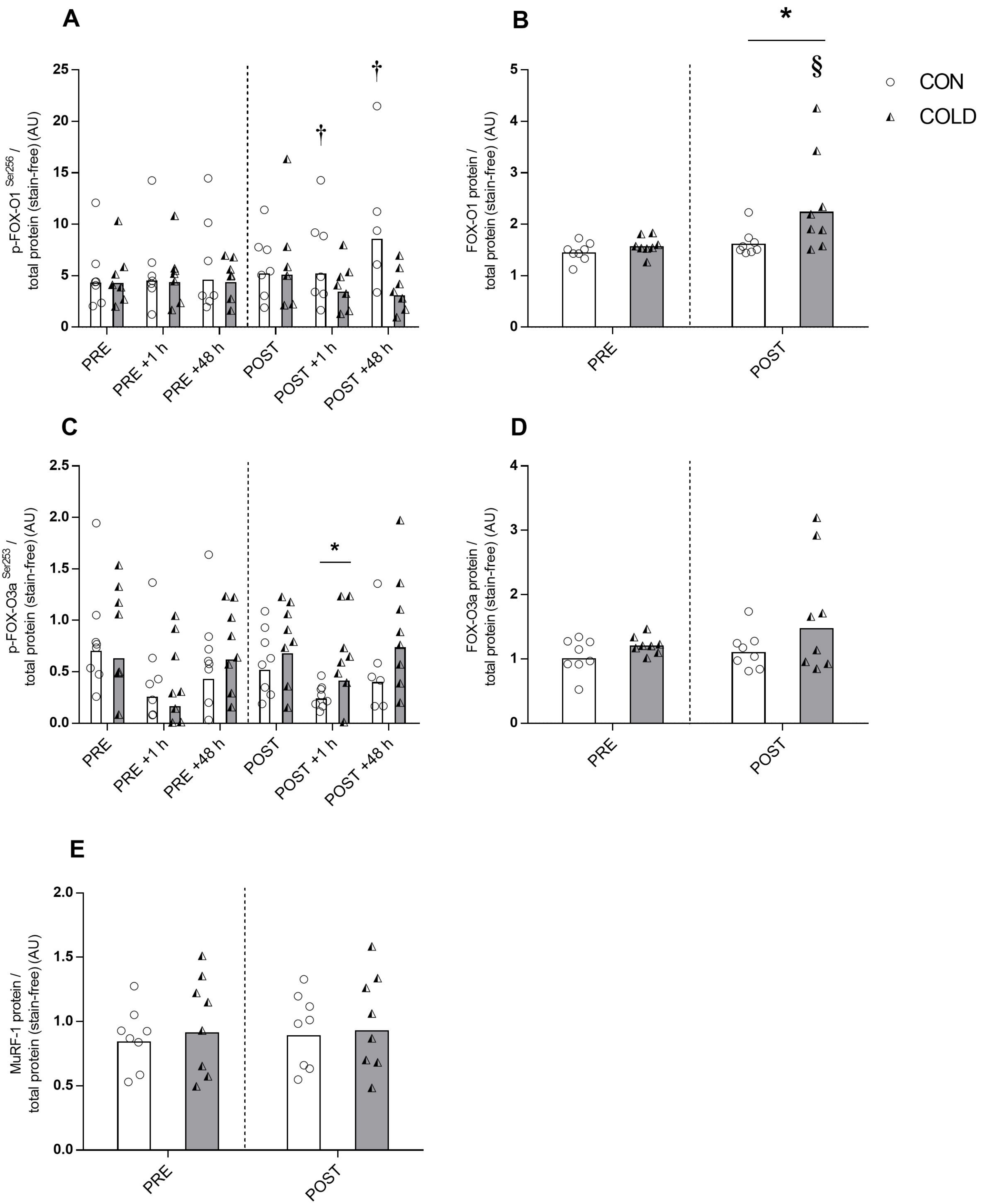
Table 4. Summary of all between-group effects considered substantial in magnitude.


Familiarisation
Pre-testing
Post-testing
Phase
7 wk resistance training (+ COLD or CON)
(3 d per wk)
Testing
BEP
DXA
1-RM
Acute exercise trial
BEP
1-RM
DXA
Acute exercise trial
≥ 72 h
≥ 72-96 h
Muscle biopsies
PRE
PRE +1 h
PRE +48 h
POST
POST +1 h
POST +48 h
Week
0
1
2
3
4
5
6
7
8
9
10

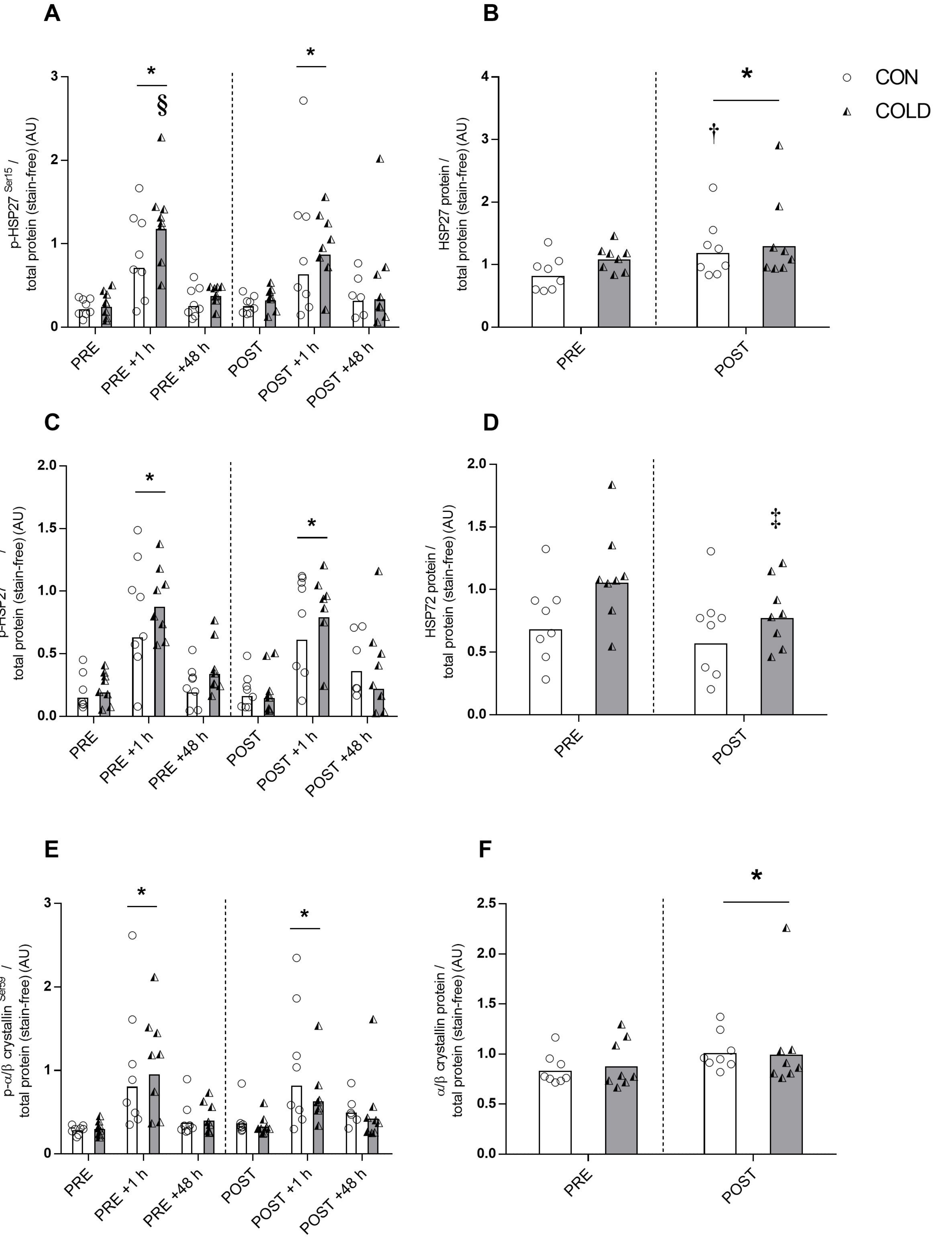


A
B
CON
COLD
Type I muscle fibre CSA (μm2)
Type II muscle fibre CSA (μm2)
0
3000
6000
9000
12000
0
5000
10000
15000
†
PRE
POST
PRE
POST
CON
COLD
C
D
E
F
200 micrometres
200 micrometres
200 micrometres
200 micrometres


A
B
C
D
E
F
CON
COLD
p-p70S6K1 Thr389 / total protein (stain-free) (AU)
p70S6K protein / total protein (stain-free) (AU)
p-rps6 Ser235/236 / total protein (stain-free) (AU)
rps6 protein / total protein (stain-free) (AU)
p-4E-BP1 Thr36/47 / total protein (stain-free) (AU)
4E-BP1 protein / total protein (stain-free) (AU)
PRE
PRE +1 h
PRE +48 h
POST
POST +1 h
POST +48 h
*
†



A
B
C
D
E
CON
COLD
p-FOX-O1 Ser256 / total protein (stain-free) (AU)
FOX-O1 protein / total protein (stain-free) (AU)
p-FOX-O3a Ser253 / total protein (stain-free) (AU)
FOX-O3a protein / total protein (stain-free) (AU)
MuRF-1 protein / total protein (stain-free) (AU)
PRE
PRE +1 h
PRE +48 h
POST
POST +1 h
POST +48 h
†
*
§



A
p-HSP27 Ser15 / total protein (stain-free) (AU)
PRE
PRE +1 h
PRE +48 h
POST
POST +1 h
POST +48 h
B
HSP27 protein / total protein (stain-free) (AU)
CON
COLD
C
p-HSP27 Ser82 / total protein (stain-free) (AU)
D
HSP72 protein / total protein (stain-free) (AU)
E
p-α/β crystallin Ser59 / total protein (stain-free) (AU)
F
α/β crystallin protein / total protein (stain-free) (AU)

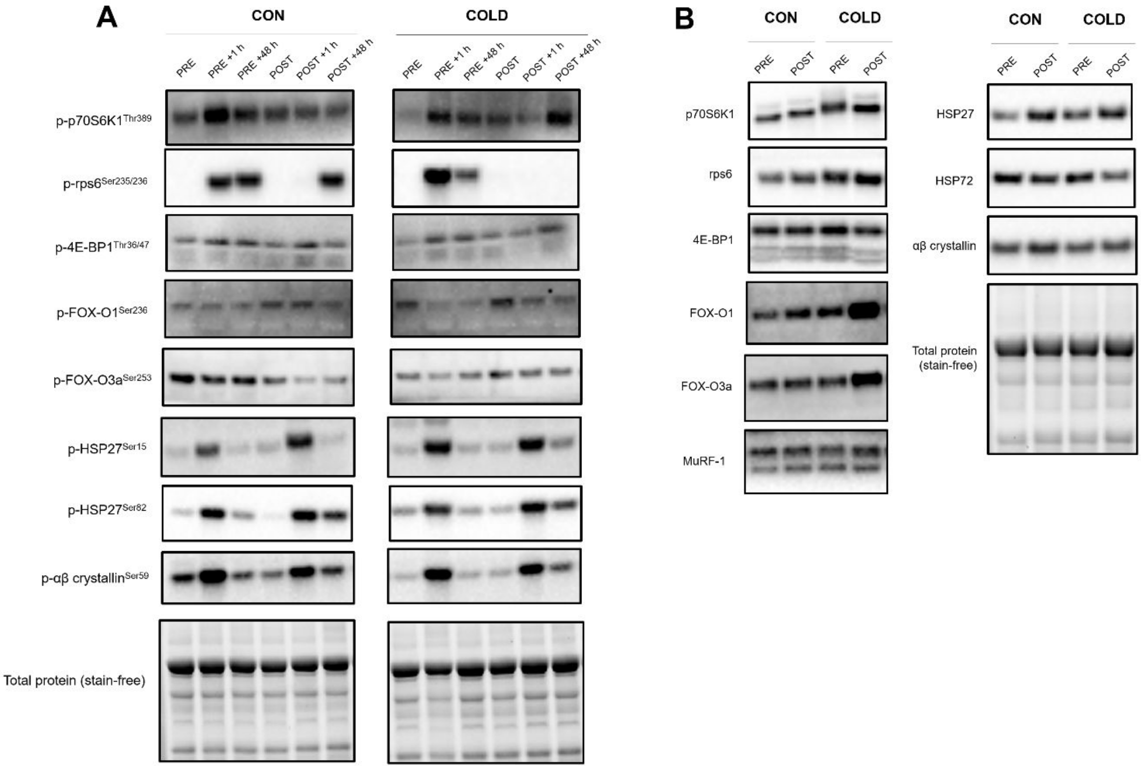

A
CON
COLD
PRE
PRE +1 h
PRE +48 h
POST
POST +1 h
POST +48 h
p-p70S6K1Thr389
p-rps6Ser235/236
p-4E-BP1Thr36/47
p-FOX-O1Ser236
p-FOX-O3aSer253
p-HSP27Ser15
p-HSP27Ser82
p-αβ crystallinSer59
Total protein (stain-free)
B
CON
COLD
PRE
POST
p70S6K1
rps6
4E-BP1
FOX-O1
FOX-O3a
MuRF-1
HSP27
HSP72
αβ crystallin
Total protein (stain-free)

Table 1. Participant physical characteristics, exercise performance and body composition data for the control (CON) and cold water immersion (COLD) training groups. Data shown are group means ± SD. * = $P < 0.05$ vs. PRE.

	CON		COLD	
	PRE	POST	PRE	POST
Physical characteristics				
Age (y)	25.0 ± 4.9	-	20.9 ± 3.4	-
Height (m)	1.84 ± 0.06	-	1.80 ± 0.08	-
Body mass (kg)	88.5 ± 22.3	90.3 ± 22.5*	80.4 ± 10.7	81.2 ± 11
Maximal strength				
1-RM leg press (kg)	338 ± 78	464 ± 111*	346 ± 55	480 ± 108*
1-RM bench press (kg)	79.5 ± 17.2	86.4 ± 20.6*	75.6 ± 16	83.4 ± 14.5*
Ballistic exercise performance				
CMJ peak force (N)	1850 ± 380	1948 ± 425*†	1908 ± 324	1846 ± 318
Squat jump peak force (N)	1997 ± 451	2129 ± 495	2008 ± 372	1987 ± 323
Ballistic push-up peak force (N)	881 ± 188	884 ± 176	855 ± 102	856 ± 74
Body composition				
Upper-body lean mass (kg)	38.9 ± 7.0	40.8 ± 7.1*	36.3 ± 3.4	37.7 ± 4.0*
Lower-body lean mass (kg)	21.6 ± 2.0	22.5 ± 3.3*	20.5 ± 2.1	21.4 ± 2.4*
Total lean mass (kg)	60.6 ± 8.9	63.3 ± 10.3*	55.7 ± 5.3	59.1 ± 6.2*
Body fat (%)	19.6 ± 12.4	18.5 ± 11.4*	15.6 ± 6.8	13.9 ± 6.7*

Table 2. Details of the resistance training (RT) intervention performed by both the control (CON) and cold water immersion (COLD) groups.

Session	Exercise	Sets x repetitions
Session 1	Back squat	3 x 12
	Barbell bench press	3 x 12
	Lat pulldown	3 x 12
	Walking lunges	3 x 12 each leg
	Shoulder press	3 x 12
	Dumbbell bicep curl	3 x 12
	Tricep extension	3 x 12
	Lying leg raise	3 x 12
Session 2	45° Leg press	3 x 12
	Dumbbell bench press	3 x 12
	Bent-over row	3 x 12
	Stiff-leg deadlift	3 x 12
	Upright row	3 x 12
	Barbell bicep curl	3 x 12
	Tricep dips	3 x 20
	Abdominal curls	3 x 20
Session 3	45° Leg press 1-RM	
	Bench press 1-RM	
	Back squat	5 x 12
	Barbell bench press	5 x 12

Table 3. Summary of all within-group effects considered substantial in magnitude.

Measure	Group	Change between	Mean change		Standardised effect size (ES)		Effect magnitude
			Absolute or fold change	90% CI	ES (*d*)	±90% CI	
Performance measures							
1-RM leg press	Pooled	PRE-POST	130 kg	± 69	1.53	0.49	large
1-RM bench press	Pooled	PRE-POST	7.3 kg	± 6.8	0.40	0.26	small
Peak CMJ force	CON	PRE-POST	98 N	± 101	0.24	0.16	small
Body composition							
Total lean mass	Pooled	PRE-POST	2.6 kg	± 1.9	0.31	0.14	small
Upper-body lean mass	Pooled	PRE-POST	0.4 kg	± 0.3	0.36	0.18	small
Lower-body lean mass	Pooled	PRE-POST	0.9 kg	± 1.2	0.37	0.27	small
Fat mass	Pooled	PRE-POST	-1.4 %	± 1.7	-0.13	0.11	trivial
Total protein content							
Total rps6 protein	Pooled	PRE-POST	1.3-fold	×/÷ 1.2	1.13	1.25	moderate
Total FOX-O1 protein	Pooled	PRE-POST	1.3-fold	×/÷ 1.3	1.62	1.75	large
Total HSP27 protein	Pooled	PRE-POST	1.3-fold	×/÷ 1.2	0.85	0.60	moderate

Measure	Group	Change between	Mean change		Standardised effect size (ES)		Effect magnitude
			Absolute or fold change	90% CI	ES (*d*)	±90% CI	
Total HSP72 protein	Pooled	PRE-POST	0.8-fold	×/÷ 1.3	0.50	0.48	small
	COLD	PRE-POST	-0.7-fold	×/÷ 1.2	-0.79	0.57	moderate
Total αβ crystallin protein	Pooled	PRE-POST	1.2-fold	×/÷ 1.1	0.66	0.53	moderate
Protein phosphorylation							
p-p70S6K Thr389	Pooled	PRE-PRE+1 h	2.3-fold	×/÷ 2.1	1.29	1.13	large
		PRE-PRE+48 h	2.1-fold	×/÷ 1.7	1.14	0.84	moderate
		POST-POST+48 h	2.4-fold	×/÷ 2.6	0.77	0.84	moderate
p-rps6 Ser235/236	Pooled	PRE-PRE+1 h	4.7-fold	×/÷ 2.3	1.45	0.77	large
		POST-POST+1 h	2.7-fold	×/÷ 2.9	1.77	0.84	large
		POST-POST+48 h	2.6-fold	×/÷ 2.9	0.75	0.84	moderate
p-FOX-O3a Ser253	Pooled	POST-POST+1 h	-0.5-fold	×/÷ 1.8	-0.9	0.8	moderate
p-HSP27 Ser15	Pooled	PRE-PRE+1 h	4.0-fold	×/÷ 1.7	2.3	0.9	very large
		POST-POST+1 h	2.6-fold	×/÷ 1.5	2.1	0.8	very large
p-HSP27 Ser82	Pooled	PRE-PRE+1 h	4.4-fold	×/÷ 1.5	2.0	0.50	very large
		POST-POST+1 h	4.5-fold	×/÷ 1.7	1.8	0.60	large
p-αβ crystallin Ser59	Pooled	PRE-PRE+1 h	3.0-fold	×/÷ 1.5	4.5	1.8	extremely large
		PRE-PRE+48 h	1.3-fold	×/÷ 1.3	1.2	1.0	large
		POST-POST+1 h	2.1-fold	×/÷ 1.4	2.2	1.1	very large

Table 4. Summary of all between-group effects considered substantial in magnitude.

Measure	Group comparison	Change between	Mean difference in change		Standardised effect size (ES)		Effect magnitude
			Absolute or fold difference	90% CI	ES (*d*)	±90% CI	
Performance measures							
Peak CMJ force	CON vs. COLD	PRE-POST	160 N	± 73	0.44	0.27	small
Muscle fiber CSA							
Type II muscle fiber CSA	CON vs. COLD	PRE-POST	1915 μM^2	± 1675	1.37	0.99	large
Total protein content							
Total FOX-O1 protein	CON vs. COLD	PRE-POST	-1.3-fold	×/÷ 1.4	-2.17	2.22	very large
Total HSP27 protein	CON vs. COLD	PRE-POST	0.8-fold	×/÷ 1.3	0.94	0.82	moderate
Protein phosphorylation							
p-rps6 Ser235/236	CON vs. COLD	POST-POST+1 h	0.4-fold	×/÷ 3.0	0.69	0.86	moderate
		POST-POST+48 h	0.2-fold	×/÷ 2.9	1.33	0.82	large
p-4E-BP1 Thr36/47	CON vs. COLD	PRE-PRE+1 h	0.9-fold	×/÷ 1.2	0.40	0.45	small
p-FOX-O1 Ser256	CON vs. COLD	POST-POST+1 h	0.5-fold	×/÷ 2.1	1.03	1.11	moderate
		POST-POST+48 h	0.5-fold	×/÷ 1.6	1.13	0.72	moderate
p-HSP27 Ser15	CON vs. COLD	PRE-PRE+1 h	-1.6-fold	×/÷ 1.8	-0.82	1.01	moderate