

1 **1. Title page**

2
3 **Title:** Cold water immersion attenuates anabolic signalling and skeletal muscle fiber
4 hypertrophy, but not strength gain, following whole-body resistance training.

5
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27 **2. Abstract**

28 **Purpose:** We determined the effects of CWI on long-term adaptations and post-exercise
29 molecular responses in skeletal muscle before and after resistance training. **Methods:** Sixteen
30 males (22.9 ± 4.6 y; 85.1 ± 17.9 kg; mean \pm SD) performed resistance training ($3 \text{ d}\cdot\text{wk}^{-1}$) for
31 7 wk, with each session followed by either CWI (15 min at 10°C , COLD group, $n = 8$) or
32 passive recovery (15 min at 23°C , CON group, $n = 8$). Exercise performance [one-repetition
33 maximum (1-RM) leg press and bench press, countermovement jump, squat jump and
34 ballistic push-up], body composition (dual x-ray absorptiometry), and post-exercise (i.e., +1
35 and +48 h) molecular responses were assessed before and after training. **Results:**
36 Improvements in 1-RM leg press were similar between groups [130 ± 69 kg, pooled effect
37 size (ES): 1.53; $\pm 90\%$ confidence interval (CI) 0.49], while increases in type II muscle fiber
38 cross-sectional area were attenuated with CWI ($-1959 \mu\text{M}^2$; ± 1675 ; ES: -1.37; ± 0.99). Post-
39 exercise mTORC1 signalling (rps6 phosphorylation) was blunted for COLD at POST +1 h (-
40 0.4-fold, ES: -0.69; ± 0.86) and POST +48 h (-0.2-fold, ES: -1.33; ± 0.82), while basal protein
41 degradation markers (FOX-O1 protein content) were increased (1.3-fold, ES: 2.17; ± 2.22).
42 Training-induced increases in HSP27 protein content were attenuated for COLD (-0.8-fold,
43 ES, -0.94 ± 0.82), which also reduced total HSP72 protein content (-0.7-fold, ES: -0.79,
44 ± 0.57). **Conclusion:** CWI blunted resistance training-induced muscle fiber hypertrophy, but
45 not maximal strength, potentially via reduced skeletal muscle protein anabolism and
46 increased catabolism. Post-exercise CWI should therefore be avoided if muscle hypertrophy
47 is desired.

48

49 **New and noteworthy:** This study adds to existing evidence that post-exercise cold water
50 immersion attenuates muscle fiber growth with resistance training, which is potentially
51 mediated by attenuated post-exercise increases in markers of skeletal muscle anabolism

- 52 coupled with increased catabolism, and suggests blunted muscle fiber growth with cold water
- 53 immersion does not necessarily translate to impaired strength development.

54 **3. Introduction**

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

66

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

77

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

91

92 Evidence has emerged suggesting CWI application after a single session of resistance
93 exercise influences some of the molecular responses mediating hypertrophic adaptation in
94 human skeletal muscle. In one study (56), CWI (10 min at 10°C) attenuated post-exercise
95 mTORC1 signalling (specifically, p70S6K phosphorylation) and satellite cell activation after
96 a single session of lower-body resistance training. Conversely, the expression and localisation
97 of HSP72 and $\alpha\beta$ -crystallin were unchanged by CWI (51). Continuing this protocol for 12
98 weeks blunted the increases in type II muscle fiber cross-sectional area (CSA), myonuclear
99 accretion, and one-repetition maximum (1-RM) leg press and leg extension strength (56).
100 These data suggest the negative effects of CWI on resistance training adaptations may be
101 underpinned by modulation of the early post-exercise anabolic profile in skeletal muscle.
102 Whether CWI also influences post-exercise markers of protein degradation in human skeletal

103 muscle has, however, not been investigated. Moreover, since post-exercise molecular
104 responses are modulated by periods of training (73, 76), it is unclear whether the influence of
105 CWI on these responses are attenuated over time, which has implications for longer-term
106 effects on training adaptation.

107

108 The inherent limitations of existing evidence showing attenuated resistance training
109 adaptations with CWI may also compromise the applicability of their findings to athletic
110 populations. For example, some studies have applied CWI to only a single limb (3, 22, 30,
111 31, 47, 77, 78), and/or used training protocols incorporating either a single exercise (22, 47,
112 77, 78) or lower-body exercises only (56), all of which are uncommon training practices. We
113 therefore aimed to examine whether post-exercise CWI application modulates key
114 adaptations following seven weeks of whole-body resistance training. In addition, we
115 investigated the effects of CWI on post-exercise anabolic and catabolic molecular responses
116 to a single session of whole-body resistance training, and compared these responses before
117 and after the training intervention.

118 **4. Methodology**

119 *Participants*

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

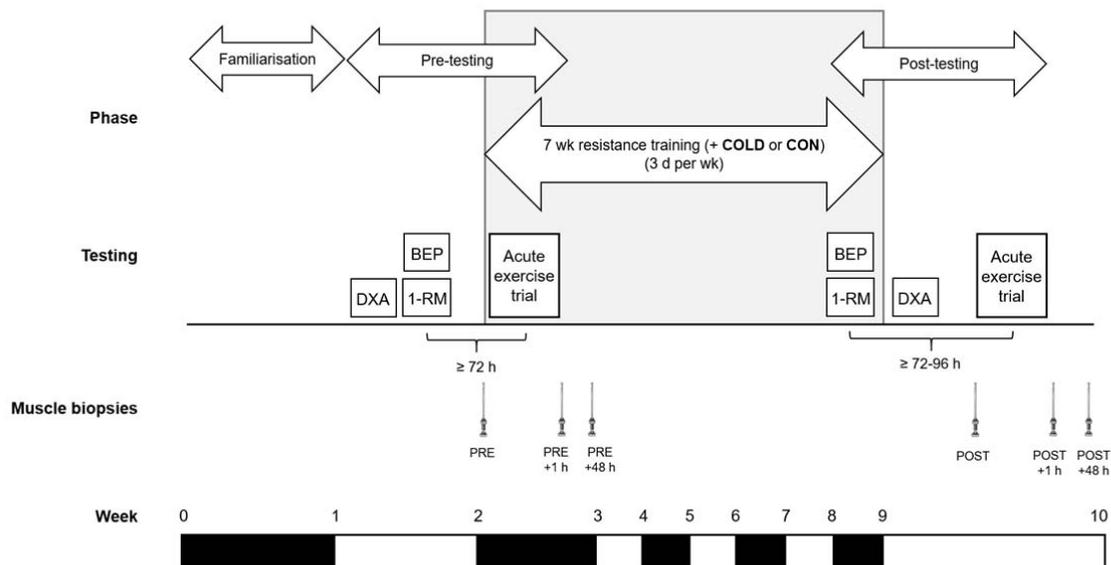
126

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

128

129 *Study overview*

130 An overview of the study procedures is shown in Figure 1. Before preliminary testing,
131 participants were familiarized with all performance assessments, including leg press one-
132 repetition maximum (1-RM), bench press 1-RM, and ballistic exercise performance [counter-
133 movement jump (CMJ), squat jump, and ballistic push-up] tests. Participants were also
134 familiarized with all resistance training exercises to ensure appropriate technique and to
135 determine loads for their first training session. One week following the familiarisation
136 session, participants underwent a dual energy x-ray absorptiometry (DXA) scan and repeated
137 the performance assessments, which served as pre-training (PRE) data. After preliminary
138 testing, participants were pair-matched for leg press 1-RM, and one of each pair was
139 randomly allocated to either the CWI (COLD; $n = 8$) or control (CON; $n = 8$) groups. At least
140 72 h after preliminary testing, participants performed a biopsy trial that doubled as the first
141 session of a seven-week, whole-body, resistance training program. Post-training performance
142 tests (POST) were performed during the last training session, followed by a second DXA
143 scan and second biopsy trial conducted between 72 and 96 h later.



145

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

149

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

151

152 *Ballistic exercise performance*

153 Countermovement jump (CMJ) performance

154 Before testing, participants performed a warm-up consisting of 5 min of stationary cycling at
 155 1W/kg body mass. Countermovement jump (CMJ) performance was assessed using a force
 156 plate (Fitness Technology, Skye, SA). Jumps began from a standing starting position, with
 157 the feet approximately shoulder-width apart and hands placed on the hips throughout.
 158 Participants then lowered themselves to a self-selected depth and jumped for maximal height
 159 without pausing between the eccentric and concentric phases. Participants were encouraged
 160 to be as explosive as possible during the movement to achieve maximal jump height. Three

161 maximal CMJs were performed by each participant, with one min of rest between each jump.
162 The jump whereby the highest peak force was achieved was chosen for analysis.

163

164 Squat jump performance

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

169

170 Ballistic push-up performance

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

176

177 *Maximal strength*

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

186 press repetitions started from full elbow extension, after which the barbell was lowered to the
187 chest and then lifted to full elbow extension.

188

189 *Body composition*

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

195

196 *Resistance training (RT) intervention*

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

204

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

206

207 *Recovery interventions*

208 Five minutes after completing each RT session, participants underwent their assigned
209 recovery intervention for 15 min. Participants in the COLD group were seated (with legs
210 fully extended) in an inflatable bath (iBody, iCool Sport, Australia), and immersed in water

211 up to their sternum. Water temperature was maintained at 10°C with a cooling/heating unit
212 (Dual Temp Unit, iCool Sport, Australia). Participants in the CON group instead sat in a chair
213 in a room maintained at 23°C for the 15 min period.

214

215 *Muscle biopsy trial*

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

229

230 *Muscle biopsy procedure*

231 During the pre- and post-training biopsy trials, a needle muscle biopsy was taken from the
232 middle third of the *vastus lateralis* muscle at rest, and 1 and 48 h after exercise. After
233 injection of a local anaesthetic into the skin and fascia [1% lidocaine (xylocaine)], a small
234 incision was made and a muscle sample taken using a Stille biopsy needle modified with
235 suction (20). Each biopsy was taken from the participant's dominant leg via a separate

236 incision, 1 to 2 cm proximal from the previous biopsy. Muscle samples were blotted on filter
237 paper to remove excess blood, immediately frozen in liquid nitrogen, and stored at -80°C
238 until subsequent analysis. A small portion of each biopsy sample (~ 20 mg) was embedded in
239 Tissue-Tek (Sakura, Finetek, NL), frozen in liquid nitrogen-cooled isopentane, and stored at
240 -80°C for subsequent immunofluorescence analysis.

241

242 *Muscle temperature assessment*

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

256

257 *Immunohistochemistry*

258 Muscle cross-sections ($10\ \mu\text{M}$) were cut at -20°C using a cryostat (CM 1950, Leica
259 Biosystems, Buffalo Grove, IL), mounted on uncoated glass slides, and frozen at -80°C until
260 subsequent analysis. After thawing for 10 min at room temperature, sections were rinsed

261 briefly with 1×PBS (phosphate buffered saline; 0.1M; Sigma Aldrich, St Louis, MO), fixed
262 with cold paraformaldehyde (4% v/v in 1×PBS) for 10 min at room temperature, rinsed three
263 times with 1×PBS, and then blocked for 1 h at room temperature in a 3% w/v BSA solution
264 in 1×PBS. After blocking, sections were then incubated with a primary antibody for myosin
265 heavy chain type I (cat no. M8421, Sigma Aldrich, St Louis, MO), diluted 1:25 in 3% w/v
266 BSA/PBS, for 2 h at room temperature. Slides were then washed three times in 1×PBS for 5
267 min each before incubation with a secondary antibody (Alexa Fluor® 568 conjugate Goat
268 anti-mouse IgG1, cat. no. A-21124, Thermo Fisher Scientific, Waltham, MA) diluted 1:500
269 in 3% w/v BSA/PBS for 1 h in the dark at room temperature. Sections were again washed
270 three times in 1×PBS for 5 min each, before incubation with Wheat Germ Agglutinin (WGA)
271 (Alexa Fluor® 488 Conjugate; cat. no. W11261, Thermo Fisher Scientific, Waltham, MA),
272 diluted to 1:100 in 1×PBS (from a 1.25 mg/mL stock solution), for 15 min at room
273 temperature. Sections were washed again twice with 1×PBS for 3 min each, blotted dry with
274 a Kim-Wipe, and anti-fade solution (Prolong™ Gold AntiFade Mountant; cat. no. P36930;
275 Thermo Fisher Scientific, Waltham, MA) added to each section before the coverslip was
276 mounted. Stained muscle sections were air-dried overnight and viewed with a confocal
277 microscope (Olympus FV10i, Shinjuku, Japan). Images were captured with a 10× objective
278 and analysed using MyoVision Basic software (version 1.0) (74). Analysis was completed by
279 an investigator blinded to all groups and time points. For each subject, muscle fiber CSA was
280 determined for both type I and type II muscle fibers. For the COLD and CON groups, a total
281 of 59 ± 19 , and 50 ± 24 (mean \pm SD) type I fibers and 87 ± 40 , and 75 ± 42 (mean \pm SD) type
282 II fibers were analysed per subject (and per timepoint), respectively. Representative
283 immunohistochemistry images for both training groups at pre- and post-training are shown in
284 Figure 2.

285

286

287 *Western blotting*

288 The abundance of target proteins in muscle samples were determined with all constituents
289 present (i.e., without centrifugation) (42). Frozen muscle was cut into 20 μm sections
290 (Cryostat HM550, Thermo Scientific, Australia), and approximately 20 sections were
291 dissolved in 200 μL homogenising buffer [125 mM Tris-HCl, 4% SDS, 10% Glycerol, 10
292 mM EGTA, 100 mM DTT, with 0.1 % v/v protease and phosphatase inhibitor cocktail
293 (#P8340 and #P5726, Sigma Aldrich, Castle Hill, NSW, Australia)], which were vortexed
294 and then freeze-thawed. The protein concentration of each sample was then determined using
295 a commercially-available assay with SDS neutralizer (Red 660, G-Biosciences, Astral
296 Scientific, Gynea NSW, Australia) and samples were diluted to equivalent concentrations (1
297 $\mu\text{g}\cdot\mu\text{L}^{-1}$) in homogenising buffer. Bromophenol blue (1% v/v) was added to samples and
298 pooled samples, and aliquots of each sample were made to avoid multiple freeze-thaw cycles.
299 Samples were heated at 95 °C for 5 min before 6 to 8 μg protein was loaded per lane into pre-
300 cast 26-well 4 to 20% gradient gels (Criterion™ TGX Stain-Free™ Precast, BioRad,
301 Gladesville NSW, Australia). A molecular weight ladder (PageRuler® Plus, Thermo
302 Scientific, Australia) and a five-point calibration curve (4 to 24 μg) consisting of a pooled
303 sample were also loaded on each gel to allow direct comparison of blot intensities via linear
304 regression (42). Samples from both the CON and CWI groups were loaded into each gel.
305 Optimal loading volumes were determined for each protein target to ensure that blot
306 intensities were within the linear range of the standard curve (i.e., to avoid primary antibody
307 saturation) (42). After separation by SDS PAGE, stain-free gels were activated by UV light
308 (ChemiDoc™ MP, BioRad, Gladesville NSW, Australia) and imaged prior to antibody
309 incubation to visualise the total protein of each lane, both for confirmation of sample loading
310 and for subsequent loading control normalisation. Proteins were then transferred to PVDF

311 membranes (Trans-Blot® Turbo™, BioRad, Gladesville NSW, Australia), which were then
312 blocked in 20 mM Tris, 150 mM NaCl, and 0.1% Tween 20 (TBST) containing 5% nonfat
313 milk for 1 h at room temperature, washed with TBST, and then incubated with primary
314 antibody overnight at 4°C. To determine protein expression and phosphorylation, membranes
315 were incubated with the following antibodies diluted 1:1000 in TBST containing 5% w/v
316 BSA and 0.1% w/v sodium azide. Primary antibodies for phosphorylated (p-) p-mTOR^{Ser2448}
317 (#5536), mTOR (#2972), p-p70S6K1^{Thr389} (#9234), p70S6K1 (#2708), p-4E-BP1^{Thr37/46}
318 (#2855), 4E-BP1 (#9644), p-rps6^{Ser235/236} (#2211), rps6 (#2217), p-FOXO1^{Ser256} (#9461),
319 FOXO1 (#2880), p-FOXO3a^{Ser253} (#13129), and FOXO3a (#12829) were from Cell
320 Signalling Technology (Danvers, MA), p-HSP27^{Ser82} (#ALX-804-588), p-HSP27^{Ser15} (#ADI-
321 SPA-525), HSP27 (#ADI-SPA-800), p- α B-crystallin^{Ser59} (#ADI-SPA-227), α β -crystallin
322 (#ADI-SPA-222), HSP72 (#ADI-SPA-810) was from Enzo Life Sciences (Farmingdale, NY),
323 and MuRF1 (#MP3401) was from ECM Biosciences (Versailles, KY). Membranes were
324 washed 5 times with TBST, before probing with appropriate horseradish peroxidase-
325 conjugated secondary antibody (PerkinElmer, Glen Waverley, Victoria, Australia), at a
326 dilution of 1:50,000 – 100,000 in 5% non-fat milk TBST for 1 h at room temperature.
327 Protein-antibody-HRP conjugates were incubated in ECL (SuperSignal® West Femto,
328 Thermo Scientific, Australia) and imaged with a high sensitivity CCD camera (ChemiDoc™
329 MP, BioRad, Gladesville NSW, Australia) for subsequent analysis (ImageLab v 5.1, BioRad,
330 Gladesville NSW, Australia). Total protein loading of each sample was determined from
331 stain-free images of each gel, and these values were then used to normalise each protein of
332 interest after normalisation to its respective standard curve. Representative western blot
333 images for each measured protein are shown in Figure 6.

334

335 *Statistical analyses*

336 To reduce bias from non-uniformity of error, heteroscedastic data were logarithmically
337 transformed before analysis (e.g., for Western blot data) (45). For these data, geometric mean
338 and SD (geometric mean \times / \div SD) are reported. All other data are reported as mean \pm SD
339 unless otherwise specified. Linear mixed models were used to determine the influence of
340 recovery condition (i.e., COLD or CON) on outcome variables, with “time” (repeated
341 measure across all timepoints), “training status” (i.e., pre- vs. post-training), “group” and
342 “group \times time” as fixed factors, and “subject” as a random factor. First-order autoregressive
343 covariance structures were used for all models, and model fit was assessed by $-2 \log$
344 likelihood (21). In the absence of a statistically significant ($P < 0.05$) group \times time
345 interaction, effects over time are reported on pooled group data (i.e., for both groups
346 combined). The magnitude of within-group changes in dependent variables (and between-
347 group differences in these changes) were quantified as Cohen’s d (effect size, ES), applying
348 thresholds of $< 0.2 =$ trivial, $0.2-0.6 =$ small, $0.6-1.2 =$ moderate, $1.2-2.0 =$ large, $2.0-4.0 =$
349 very large and $> 4.0 =$ extremely large (29). Effects were considered substantial if there was a
350 $>75\%$ probability of being positive relative to the smallest worthwhile change (ES = 0.2), and
351 effects with a $>5\%$ probability of being either substantially positive or negative were deemed
352 unclear (29). Uncertainty of effects were determined as 90% confidence intervals (CI) and
353 precise P values (unless $P < 0.001$) (13). Linear mixed models were analysed using IBM
354 SPSS Statistics Version 25 (IBM, Somers, NY) and ES and CI values were determined via
355 custom Excel spreadsheets (28). Percent compliance between groups was compared using an
356 independent samples t -test (IBM SPSS Statistics Version 25, Somers, NY) and ES and CI
357 values were determined using a custom Excel spreadsheet (27).

358

359

360 **5. Results**

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

363

364 **Training compliance**

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

367

368 **Muscle temperature assessment**

369 Between the completion of the fourth training session and end of the post-exercise recovery
370 intervention, muscle temperature decreased more for COLD ($-3.5^\circ\text{C} \pm 3.5$) vs. CON ($-0.5^\circ\text{C} \pm$
371 0.5) (group \times time interaction: $P = 0.031$, ES: 2.27; ± 1.27).

372

373 **Basal responses to training**

374 **Performance measures**

375 *Maximal strength*

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

379

380 Similar to lower-body strength, there was no group \times time interaction ($P = 0.582$, ES: 0.08;
381 ± 0.35) for 1-RM bench press (Table 2), which increased at POST for both groups combined
382 (time main effect: $P = 0.001$, Table 3).

383

384

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

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

388

389 There was no group \times time interaction for neither peak squat jump force ($P = 0.249$, ES:
390 0.33 ; ± 0.51) nor ballistic push-up force ($P = 0.898$, ES: 0.05 ; ± 0.30), neither of which
391 changed over time for both groups combined (time main effect: $P = 0.355$, ES: 0.13 ; ± 0.36
392 and $P = 0.898$, ES: 0.03 ; ± 0.23 , respectively, see Table 2).

393

394 **Body composition**

395 There was no group \times time interaction ($P = 0.867$, ES: 0.02 ; ± 0.22) for total lean mass (Table
396 2), which increased at POST for both groups combined (time main effect: $P < 0.001$, Table
397 3).

398

399 There was no group \times time interaction for lower-body lean mass ($P = 0.935$, ES: 0.22 ; ± 0.37)
400 or upper-body lean mass ($P = 0.669$, ES: 0.06 ; ± 0.30 , Table 2). For both groups combined,
401 both lower-body and upper-body lean mass were increased at POST (time main effect: $P =$
402 0.002 and $P < 0.001$, respectively, Table 3).

403

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

406

407 **Muscle fiber CSA**

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

411 There was no group \times time interaction ($P = 0.062$) for type II muscle fiber CSA (Figure 2B);
412 however, there was a greater PRE-POST change for CON vs. COLD (Table 4).

413 Representative immunohistochemical images for changes in muscle fiber CSA are shown in
414 Figure 2 (C-F).

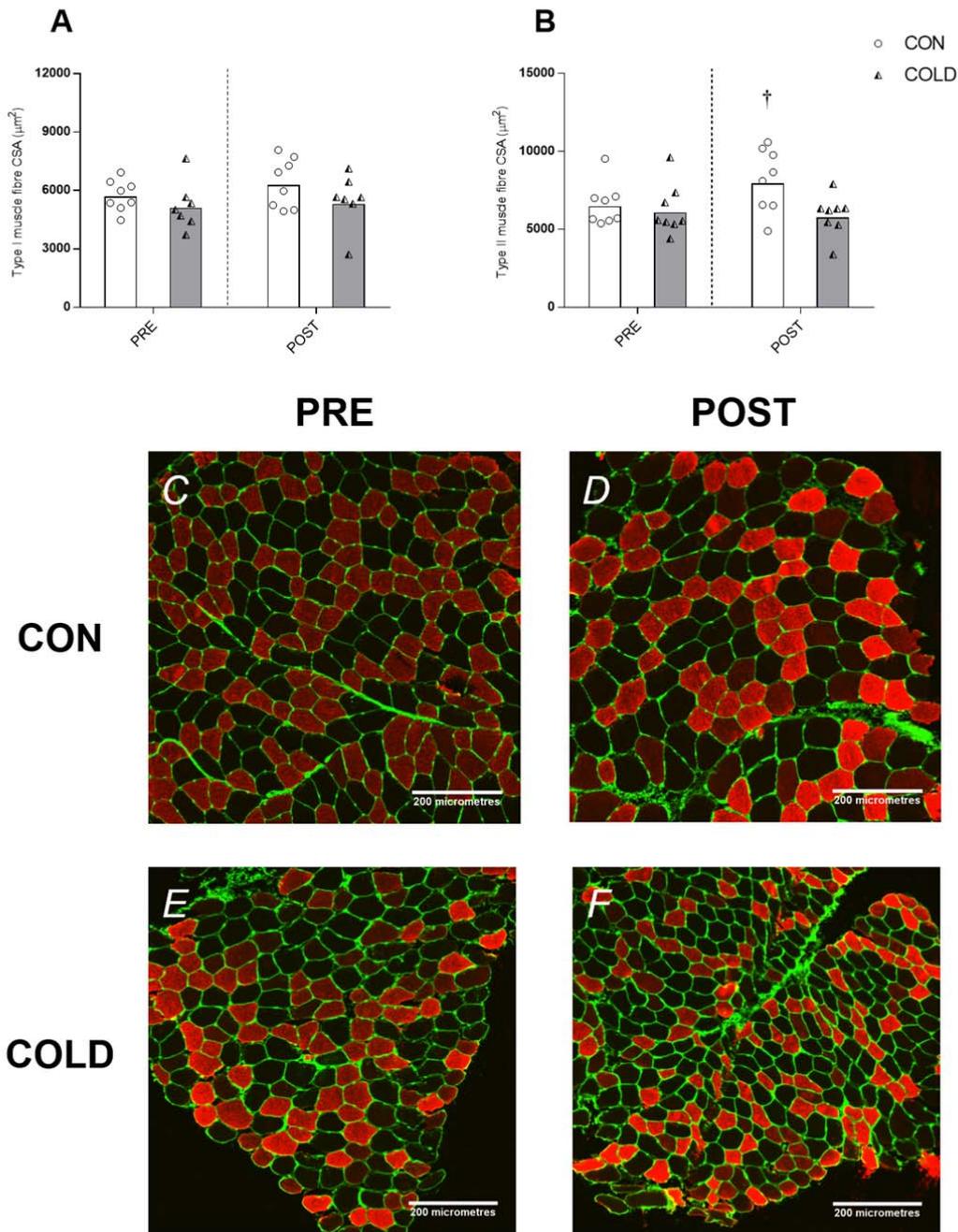
415

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

417

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

419



420
 421 **Figure 2.** Type I (A) and type II (B) muscle fiber cross-sectional area (CSA) before (PRE),
 422 and after (POST) seven weeks of resistance training with either cold-water immersion
 423 (COLD) or passive control (CON) applied after each training session. Data are mean values \pm
 424 SD.

425
 426 Representative confocal microscope immunofluorescence images of muscle cross-sections
 427 obtained before (PRE) and after (POST) seven weeks of resistance training with application
 428 of either control (CON; images C and D, respectively) or cold-water immersion (COLD;

429 images E and F, respectively) or after each training session. Muscle fiber membranes are
430 visualized green, type I muscle fibers are visualized red, and type II muscle fibers are
431 unstained. Scale bar = 200 μm .

432 † = Substantially greater change for CON vs. COLD.

433

434

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

436

437 **Total protein content**

438 *Total p70S6K protein*

439 There was no group \times time interaction ($P = 0.152$, ES: 0.67, ± 0.70) for total p70S6K protein
440 (Figure 3B), which was unchanged at POST for both groups combined (time main effect: $P =$
441 0.888, ES: 0.03; ± 0.74).

442

443 *Total rps6 protein*

444 There was no group \times time interaction ($P = 0.577$, ES: 0.51, ± 1.33) for total rps6 protein
445 (Figure 3D), which increased at POST for both groups combined (time main effect: $P =$
446 0.009, Table 3).

447

448 *Total 4E-BP1 protein*

449 There was no group \times time interaction ($P = 0.128$, ES: 0.33, ± 0.43) nor main effect of time ($P =$
450 0.061, ES: 0.26; ± 0.35) for total 4E-BP1 protein (Figure 3F).

451

452 *Total FOX-O1 protein*

453 There was no group \times time interaction ($P = 0.108$) for total FOX-O1 protein (Figure 4B),
454 which increased at POST for both groups combined (time main effect: $P = 0.007$, Table 3).

455 There was, however, a greater PRE-POST change in total FOX-O1 protein for COLD vs.
456 CON (Table 4).

457 *Total FOX-O3a protein*

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

461

462 *Total MuRF-1 protein*

463 There was no group \times time interaction ($P = 0.596$, ES: 0.10, ± 0.36) for total MuRF-1 protein
464 (Figure 4E), which was unchanged at POST for both groups combined (time main effect: $P =$
465 0.313, ES: 0.10, ± 0.25).

466

467 *Total HSP27 protein*

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

471

472 *Total HSP72 protein*

473 There was no group \times time interaction ($P = 0.465$) for total HSP72 protein (Figure 5D),
474 which decreased at POST for both groups combined (time main effect: $P < 0.013$, Table 3),
475 due to a reduction for COLD (Table 3) and not for CON (-0.8-fold \times / \div 1.4, ES: -0.33,
476 ± 0.65).

477

478 *Total $\alpha\beta$ crystallin protein*

479 There was no group \times time interaction ($P = 0.488$, ES: 0.29, ± 0.88) for total $\alpha\beta$ crystallin
480 protein (Figure 5F), which increased at POST for both groups combined (time main effect: P
481 = 0.004, Table 3).

482

483 **Responses to single exercise sessions before and after training**

484 **mTORC1 signalling responses**

485 *p-p70S6K^{Thr389}*

486 There was no group × time interaction ($P = 0.411$), nor influence of training status ($P =$
487 0.369), for p70S6K^{Thr389} phosphorylation (Figure 3A). p70S6K^{Thr389} phosphorylation was,
488 however, increased for both groups combined at PRE +1 h, PRE +48 h, and POST +48 h
489 (time main effect: $P = 0.001$, Table 3).

490

491 *p-rps6^{Ser235/236}*

492 There was no group × time interaction ($P = 0.154$), nor influence of training status ($P =$
493 0.707), for rps6^{Ser235/236} phosphorylation (Figure 3C), which was increased for both groups
494 combined at PRE +1 h, POST +1 h, and POST +48 h (time main effect: $P < 0.001$, Table 3).
495 There were also greater increases in rps6^{Ser235/236} phosphorylation for CON vs. COLD at both
496 POST +1 h and POST +48 h (Table 4).

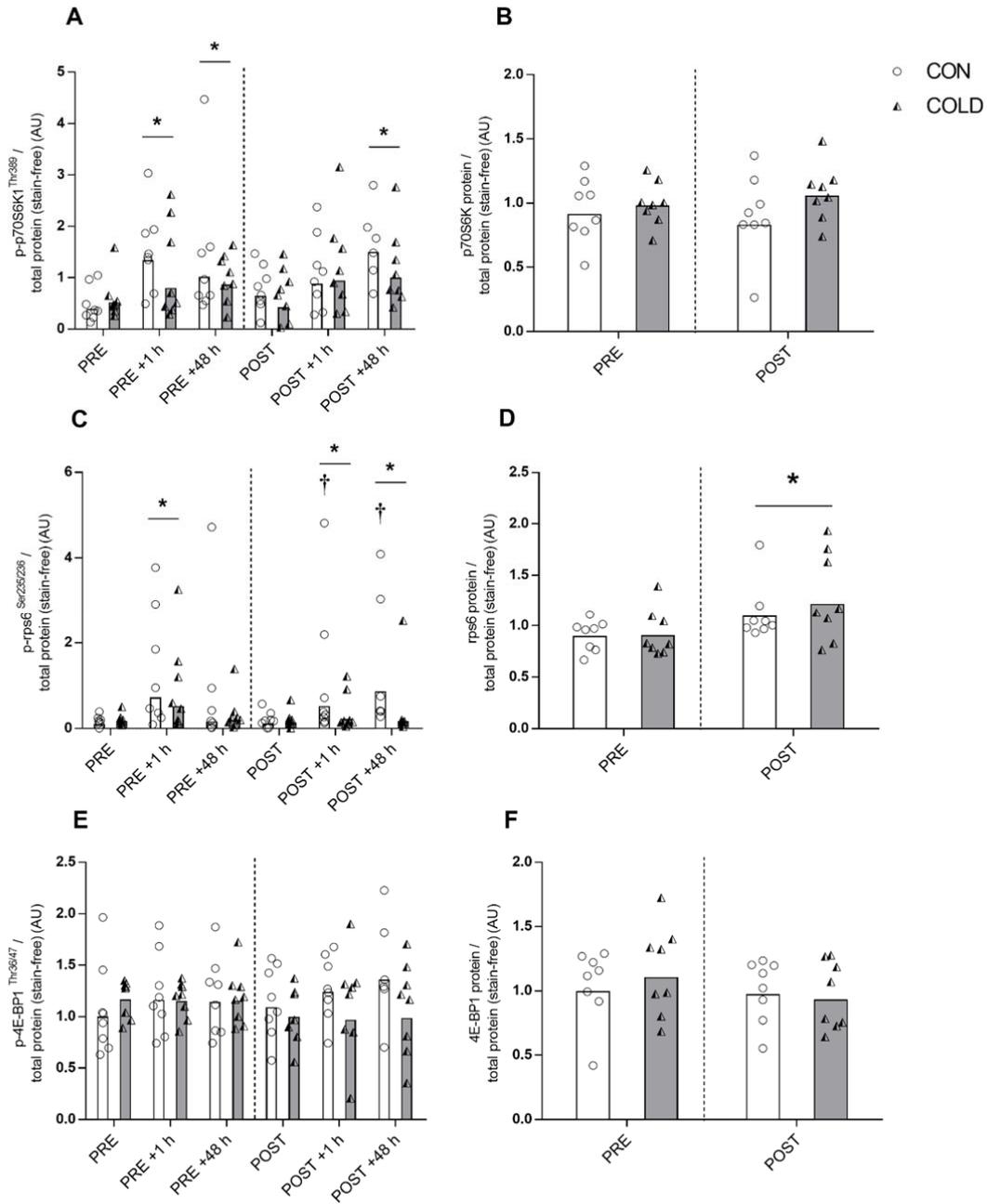
497

498 *p-4E-BP1^{Thr36/47}*

499 There was no group × time interaction ($P = 0.440$) nor main effects of training status ($P =$
500 0.94) or time ($P = 0.395$) for 4E-BP1^{Thr36/47} phosphorylation (Figure 3E). There was,
501 however, a greater increase in 4E-BP1^{Thr36/47} phosphorylation for CON vs. COLD from PRE-
502 PRE +1 h (Table 4).

503

504



505

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

513 * = $P < 0.05$ vs. PRE, † = substantially greater change vs. COLD.

514

515

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INSERT FIGURE 3 ABOUT HERE

517

518

519 **Protein degradation responses**

520 *p-FOX-O1^{Ser256}*

521 There was no group \times time interaction ($P = 0.311$) nor influence of training status ($P = 0.202$)

522 for FOX-O1^{Ser256} phosphorylation (Figure 4A), which was unchanged over time for both

523 groups combined ($P = 0.302$). There was, however, a greater increase for CON vs. COLD at

524 both POST +1 h and POST +48 h (Table 4).

525

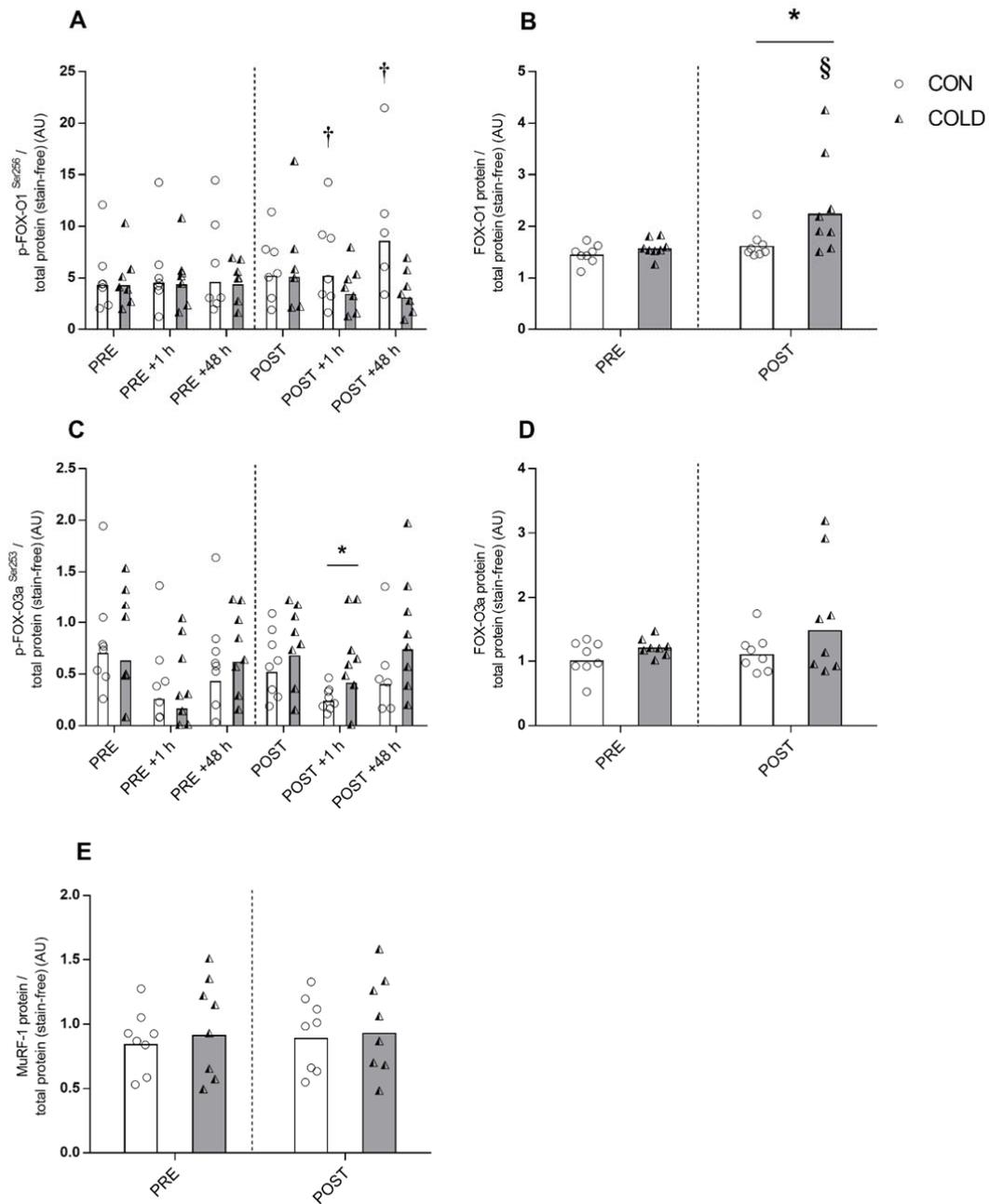
526 *p-FOX-O3a^{Ser253}*

527 There was no group \times time interaction ($P = 0.414$) nor influence of training status ($P = 0.688$)

528 for FOX-O3a^{Ser253} phosphorylation (Figure 4C), which decreased at POST +1 h for both

529 groups combined (time main effect: $P = 0.010$, Table 3).

530



531

532

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

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

542

543

544

545

INSERT FIGURE 4 ABOUT HERE

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547

548 **Heat shock protein responses**

549 *p-HSP27^{Ser15}*

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

554

555 *p-HSP27^{Ser82}*

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

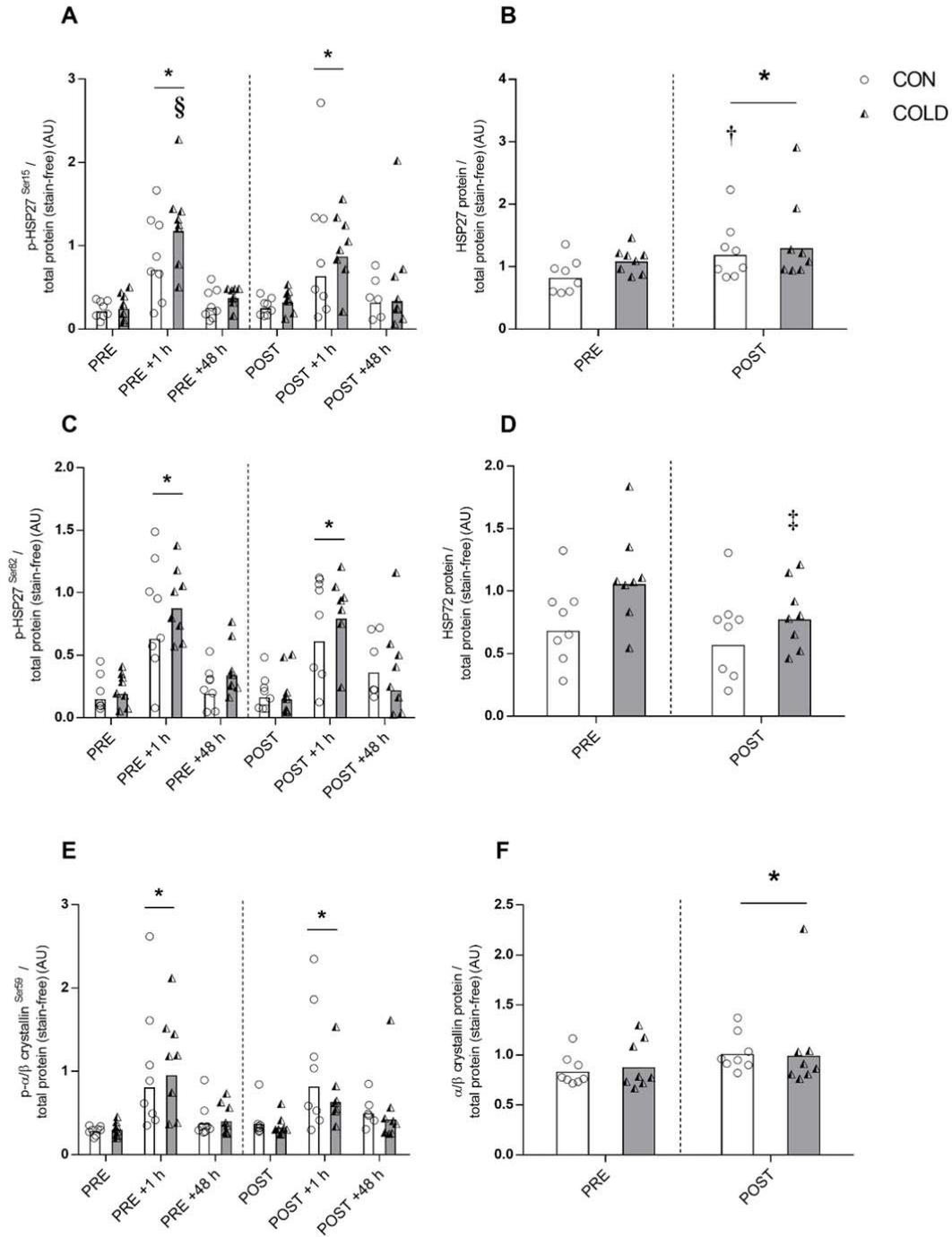
559

560 *p- $\alpha\beta$ crystallin^{Ser59}*

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

564

565



566

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

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

576

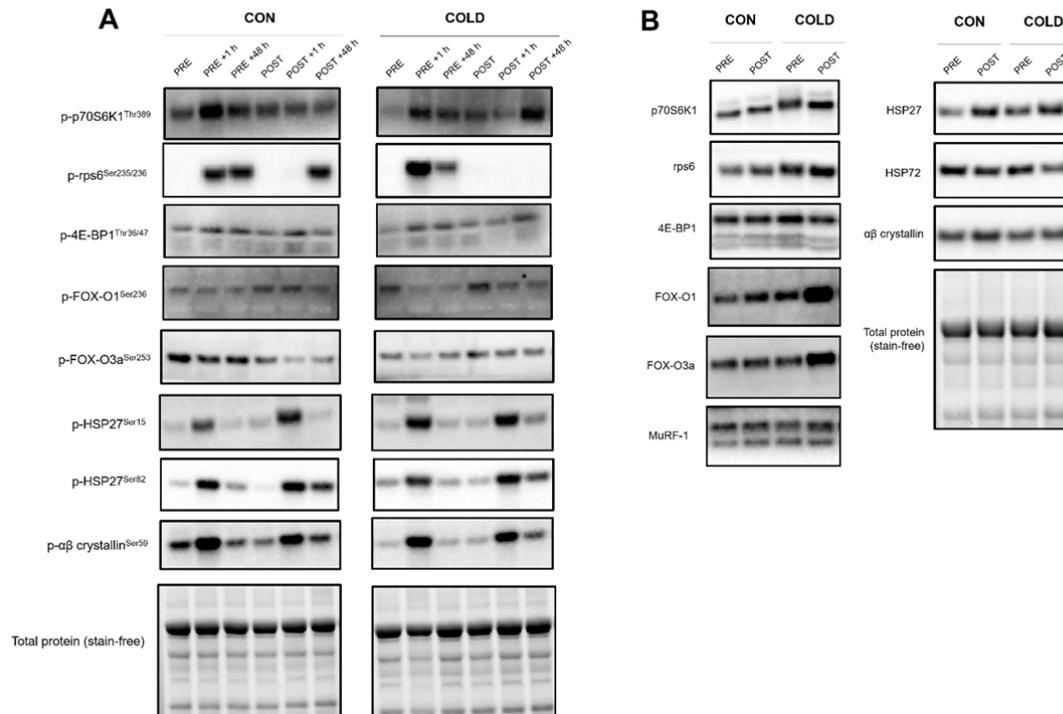
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581

582

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

588

589

590

INSERT FIGURE 6 ABOUT HERE

591 **6. Discussion**

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

605

606 The findings that CWI attenuated post-exercise anabolic signalling responses to single
607 resistance training sessions, together with blunted type II muscle hypertrophy, are in
608 agreement with previous work (56). Roberts et al. (56) also reported an attenuated increase in
609 *vastus lateralis* type II fiber size following resistance training coupled with post-exercise
610 CWI compared with an active recovery. In a separate sub-study (56), these responses
611 occurred alongside a blunted increase in p70S6K phosphorylation after the first training
612 session (at both 2 and 24 hours post-exercise) and attenuated myonuclei accretion after the
613 training period. This blunting of p70S6K phosphorylation did not, however, influence the
614 phosphorylation response of rps6, a key downstream target of p70S6K (54), nor other key
615 proteins that regulate MPS, such as 4E-BP1 (eIF4E binding protein 1) (56). In contrast to

616 these findings (56), we noted similar post-exercise p70S6K phosphorylation with CWI
617 application compared with passive recovery, which was elevated for both conditions before
618 (at +1 and +48 h) and after the training period (at +48 h), and instead saw blunted post-
619 exercise phosphorylation of rps6, a key downstream target of p70S6K, after the training
620 period.

621

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

641 have resulted from a lower net energy balance (stimulated by shivering and non-shivering
642 thermogenesis) (63) following each CWI session.

643

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

666

667 Unlike previous work (56), attenuated muscle fiber hypertrophy with lower-body CWI did
668 not occur alongside blunted maximal lower-body strength gain. Although muscle
669 hypertrophy has traditionally been associated with muscle strength gain (40), recent work has
670 questioned the role of training-induced muscle hypertrophy in improved maximal strength
671 (11). From this perspective, any influence of CWI on muscle hypertrophy may have little
672 influence on strength, particularly when assessed during complex, dynamic tasks. Since
673 strength is a highly task-specific phenomenon (41), it is also possible our findings were
674 influenced by the particular measure of strength chosen. Since the contribution of neural
675 factors (i.e., learning and coordination) to strength gain is larger during higher-complexity
676 tasks (58), any attenuation of muscle hypertrophy may have less influence on strength gain
677 when assessed during higher- versus lower-complexity tasks. It is interesting to note the
678 magnitude of attenuated strength gain with CWI application in a previous study (56)
679 appeared greater when assessed during lower- versus higher-complexity strength tasks (i.e.,
680 1-RM leg extension vs. leg press). As we employed a relatively high-complexity task (1-RM
681 leg press) as the only strength outcome measure, this may explain why we did not observe
682 any influence of blunted hypertrophy on maximal strength gain. Nevertheless, our results are
683 in agreement with others showing relatively weak relationships between training-induced
684 muscle hypertrophy and strength (2, 12, 18), and suggest blunted muscle hypertrophy with
685 application of CWI can occur without any influence on dynamic strength development.
686 However, although we did not observe impaired 1-RM strength gains with CWI application,
687 we did observe a blunting of peak force during the CMJ. While not directly assessed in this
688 study, this finding aligns with previous observations of blunted improvement in rate of force
689 development after resistance training with CWI application (56) and suggests improvement in
690 force-generating capacity during rapid, dynamic movements may be compromised with CWI.

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

693

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

716

717 The heat-shock family of proteins are important for cellular homeostasis, protein preservation
718 and degradation (46), and play key roles in several processes involved in exercise
719 adaptations. For example, HSP72 regulates mRNA elongation rate (35) and inhibits several
720 steps involved in protein degradation (5, 16, 61, 79). HSP27 and $\alpha\beta$ -crystallin also inhibit
721 protein degradation pathways (1, 15, 71) and bind to cytoskeletal and myofibrillar proteins
722 following muscle damaging exercise, where they are thought to stabilise disrupted elements
723 and assist in regeneration and remodelling (34, 49). Our data suggested a single session of
724 resistance exercise, performed before the training period, induced similar increases in
725 HSP27^{Ser15} phosphorylation at PRE +1 h for both conditions, although this change was
726 further enhanced for COLD (ES: 0.82; \pm 1.01). Similar post-exercise changes in HSP27^{Ser15}
727 phosphorylation were however noted between groups after the training period. A similar
728 pattern of response was also observed for HSP27^{Ser82} phosphorylation, with robust increases
729 during the early post-exercise period both before and after the intervention (i.e., at PRE +1 h
730 and POST +1 h), which was also not different between groups. Basal levels of HSP27 protein
731 were elevated after the training intervention for both groups, although this effect was greater
732 for CON (ES: 0.94; \pm 0.82). Total protein levels of $\alpha\beta$ -crystallin were similarly increased at
733 POST for both groups, while similar effects of a single exercise session on p- $\alpha\beta$ -crystallin^{Ser59}
734 were observed for both groups both before and after training, although there was a more
735 prolonged increase in p- $\alpha\beta$ -crystallin^{Ser59} before training for both CON and COLD. Taken
736 together, these data suggest repeated CWI blunts the chronic, but not acute, HSP27 response
737 to resistance exercise. These responses may have contributed to the blunted fiber hypertrophy
738 for COLD, given these small HSPs appear to be important for muscle remodelling (34, 49).
739 Moreover, while basal HSP72 protein levels were unchanged for CON, they were reduced
740 (0.7-fold) for COLD (ES: 0.79; \pm 0.57). Since HSP72 inhibits protein degradation (5, 16, 61,

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

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

762

763 **Conclusions**

764 The present study provides novel insights into the modulation of key adaptations to whole-
765 body resistance training combined with lower-body CWI. We provide additional evidence of

766 blunted muscle fiber hypertrophy following resistance training coupled with post-exercise
767 CWI. We provide evidence that CWI attenuates post-exercise anabolic responses both before
768 and after seven weeks of resistance training, and increases basal levels of protein degradation
769 markers post-training. The observation that the CWI-mediated blunting of anabolic responses
770 to single resistance exercise bouts persists after a period of training has implications for
771 muscle growth following longer-term training periods when coupled with CWI. Importantly,
772 the attenuation of muscle fiber hypertrophy with CWI did not impair maximal strength,
773 which potentially reflects the discordance between training-induced changes in muscle mass
774 and strength. Together, these data further highlight the ability of CWI to blunt resistance
775 training-induced muscle growth, but not strength, and suggest avoidance of post-exercise
776 CWI when muscle hypertrophy is a desired resistance training outcome.

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987 **7. Author contributions**

988 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
989 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.,
990 D.J.B. and A.C.P interpreted results; J.J.F prepared figures; J.J.F. and A.C.P. drafted
991 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
992 revised manuscript; all authors approved final version of manuscript.

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

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1000 **8. Disclosures**

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1003 declared by the authors.

1004

1005 **9. Figure legends**

1006 **Figure 1.** Study overview. DXA, dual x-ray absorptiometry scan; BEP, ballistic exercise
1007 performance (countermovement jump, squat jump, ballistic push-up) testing; 1-RM, one-
1008 repetition maximum (leg press and bench press) testing.

1009
1010 **Figure 2.** Type I (A) and type II (B) muscle fiber cross-sectional area (CSA) before (PRE),
1011 and after (POST) seven weeks of resistance training with either cold-water immersion
1012 (COLD) or passive control (CON) applied after each training session. Data are mean values \pm
1013 SD. Representative confocal microscope immunofluorescence images of muscle cross-
1014 sections obtained before (PRE) and after (POST) seven weeks of resistance training with
1015 application of either control (CON; images C and D, respectively) or cold-water immersion
1016 (COLD; images E and F, respectively) or after each training session. Muscle fiber
1017 membranes are visualized green, type I muscle fibers are visualized red, and type II muscle
1018 fibers are unstained. Scale bar = 200 μm . † = Substantially greater change for CON vs.
1019 COLD.

1020
1021 **Figure 3. mTORC1 signalling responses.** Phosphorylation and total proteins levels of
1022 p70S6K^{Thr389} (A, B respectively), rps6^{Ser235/236} (C, D respectively), and 4E-BP1^{Thr36/47} (E, F
1023 respectively) before (PRE) and after (POST) seven weeks of resistance training with either
1024 cold-water immersion (COLD) or passive control (CON) applied after each training session,
1025 as well as 1 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h)
1026 and after (POST +1 h, POST +48 h) the training period (phosphorylated proteins only). Data
1027 shown are back-transformed individual participant values and geometric means. * = $P < 0.05$
1028 vs. PRE, † = substantially greater change vs. COLD.

1029
1030 **Figure 4. Protein degradation-related responses.** Phosphorylation and total proteins levels
1031 of FOX-O1^{Ser256} (A, B respectively), FOX-O3a^{Ser253} (C, D respectively) and MuRF-1 (E)
1032 before (PRE) and after (POST) seven weeks of resistance training with either cold-water
1033 immersion (COLD) or passive control (CON) applied after each training session, as well as 1
1034 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h) and after
1035 (POST +1 h, POST +48 h) the training period (phosphorylated proteins only). Data shown
1036 are back-transformed individual participant values and geometric means. * = $P < 0.05$ vs.
1037 PRE, † = substantially greater change vs. COLD, § = substantially greater change vs. CON.

1038
1039 **Figure 5. Heat shock protein responses.** Phosphorylation of HSP27^{Ser15} (A), HSP27^{Ser82} (C)
1040 and $\alpha\beta$ crystallin^{Ser59} (E), and total protein levels of HSP27 (B), HSP72 (D), and $\alpha\beta$ crystallin
1041 (F) before (PRE) and after (POST) seven weeks of resistance training with either cold-water
1042 immersion (COLD) or passive control (CON) applied after each training session, as well as 1
1043 h and 48 h after single exercise bouts performed before (PRE +1 h, PRE +48 h) and after
1044 (POST +1 h, POST +48 h) the training period (phosphorylated proteins only). Data shown
1045 are back-transformed individual participant values and geometric means. * = $P < 0.05$ vs.

1046 PRE, ‡ = substantial change vs. PRE. † = substantially greater change vs. COLD, § =
1047 substantially greater change vs. CON.

1048

1049 **Figure 6.** Representative Western blot images for analysed phosphorylated proteins (A) and
1050 total protein content (B) before (PRE) and after (POST) seven weeks of resistance training
1051 with either cold-water immersion (COLD) or passive control (CON) applied after each
1052 training session, as well as 1 h and 48 h after single exercise bouts performed before (PRE +1
1053 h, PRE +48 h) and after (POST +1 h, POST +48 h) the training period.

1054

1055 **10. Tables**

1056

1057 **Table 1.** Participant physical characteristics, exercise performance and body composition
1058 data for the control (CON) and cold water immersion (COLD) training groups. Data shown
1059 are group means \pm SD. * = $P < 0.05$ vs. PRE.

1060

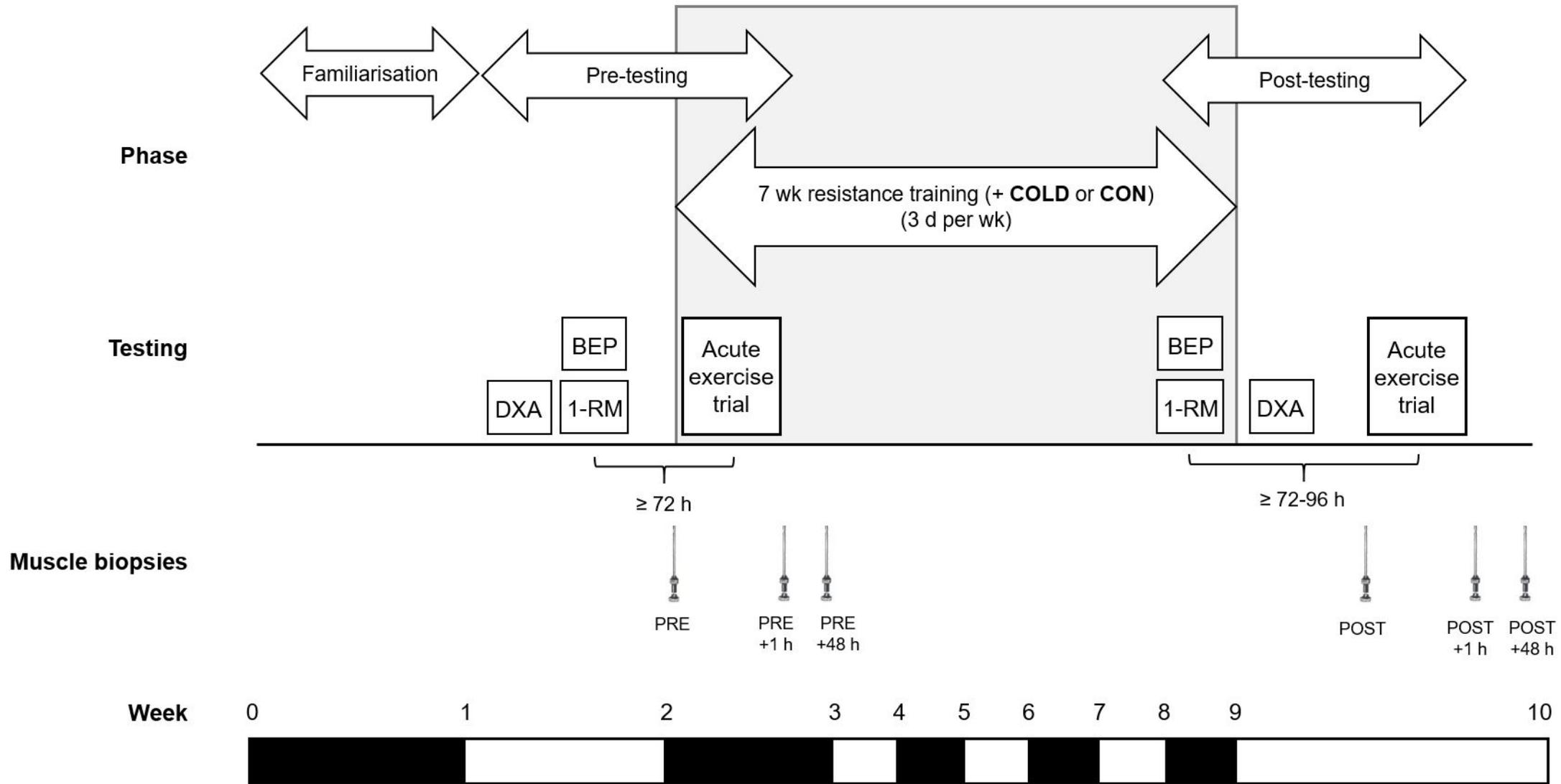
1061 **Table 2.** Details of the resistance training (RT) intervention performed by both the control
1062 (CON) and cold water immersion (COLD) groups.

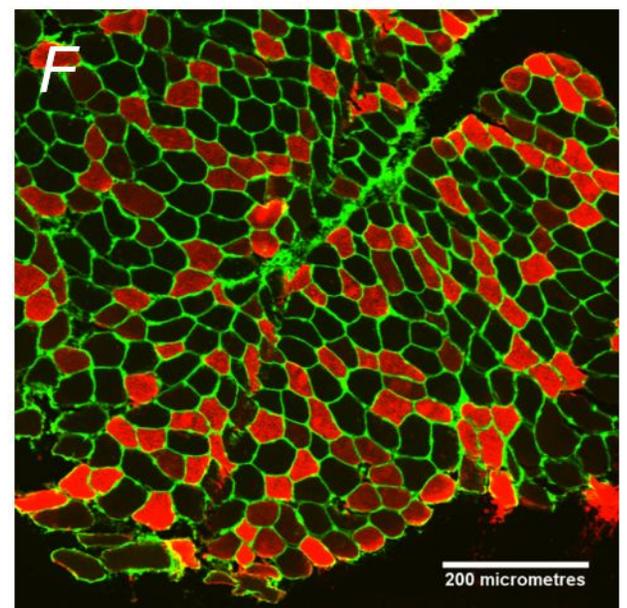
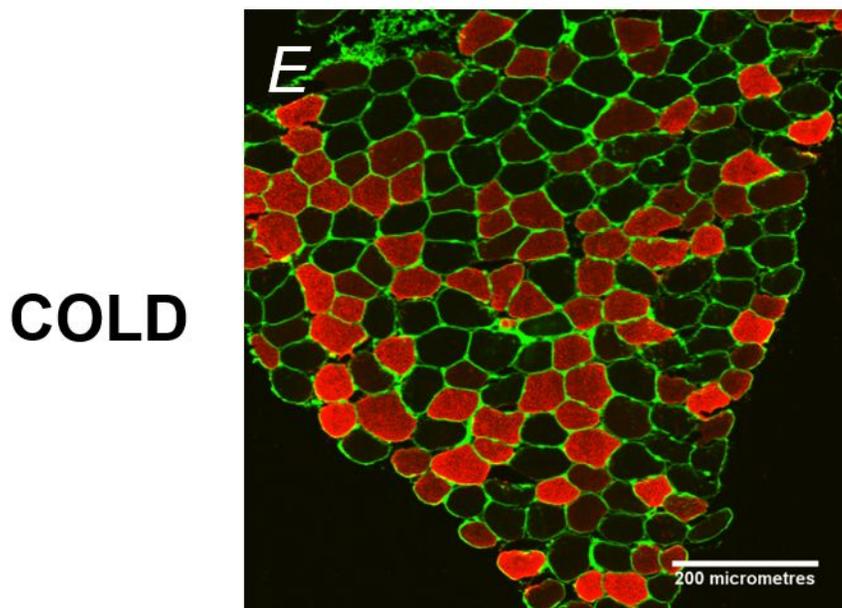
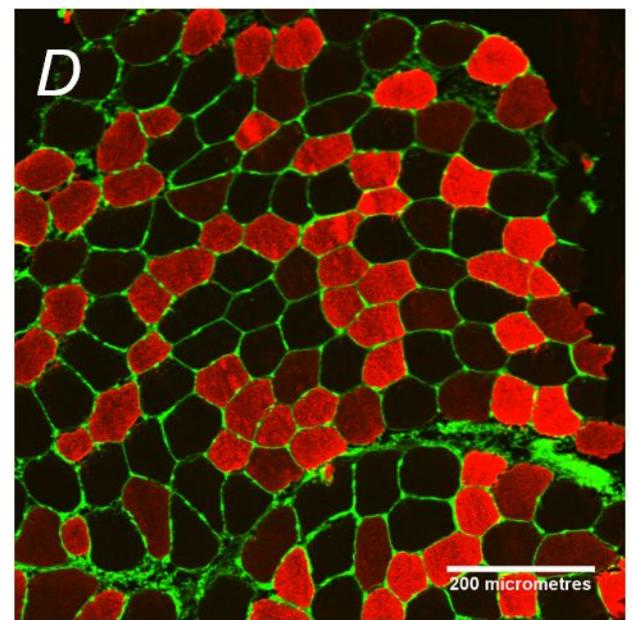
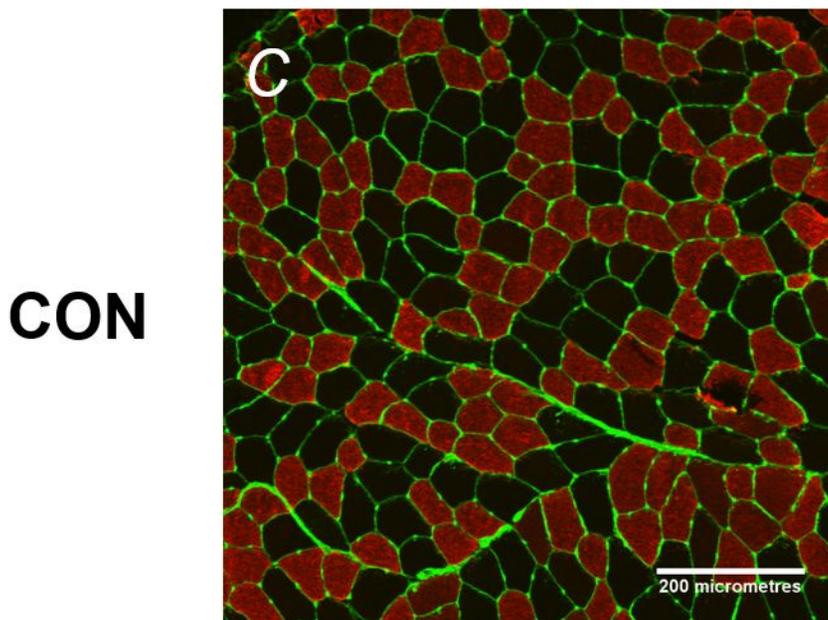
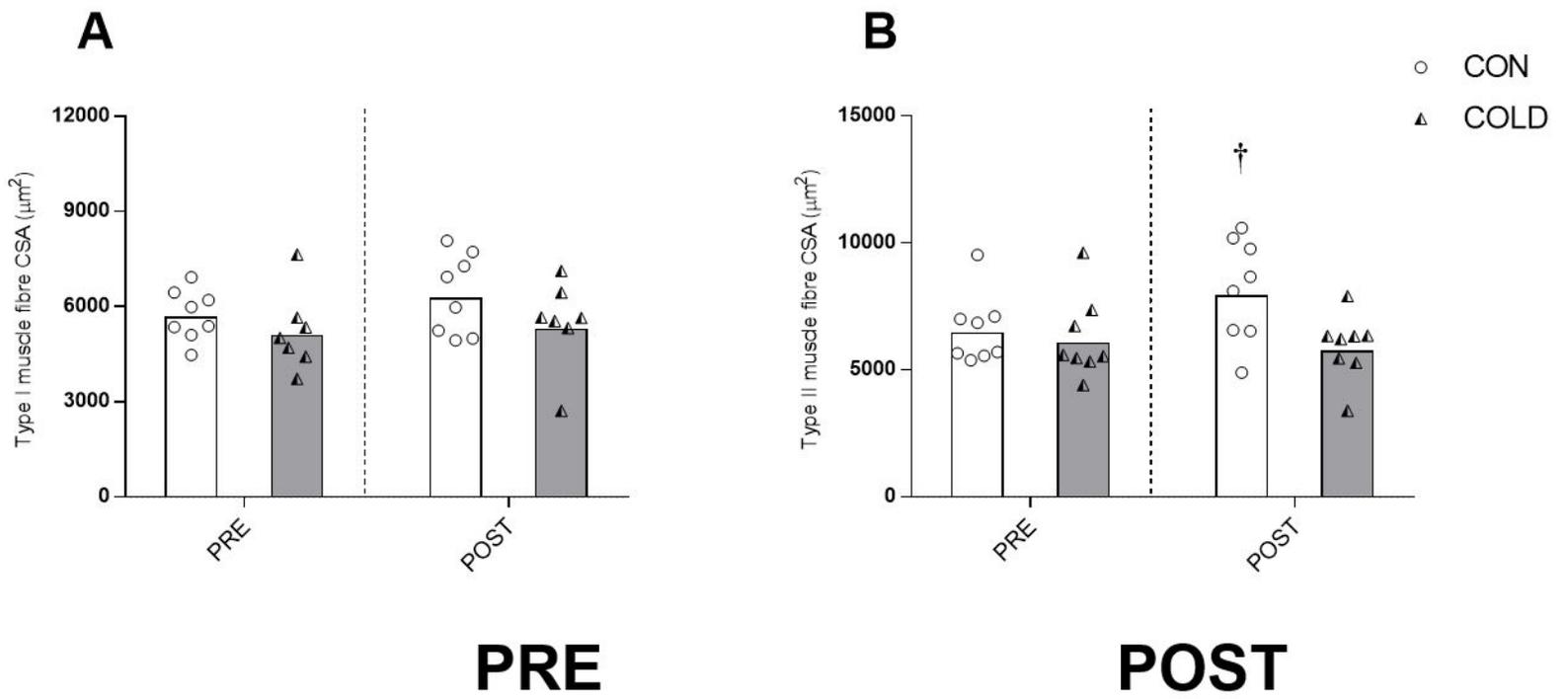
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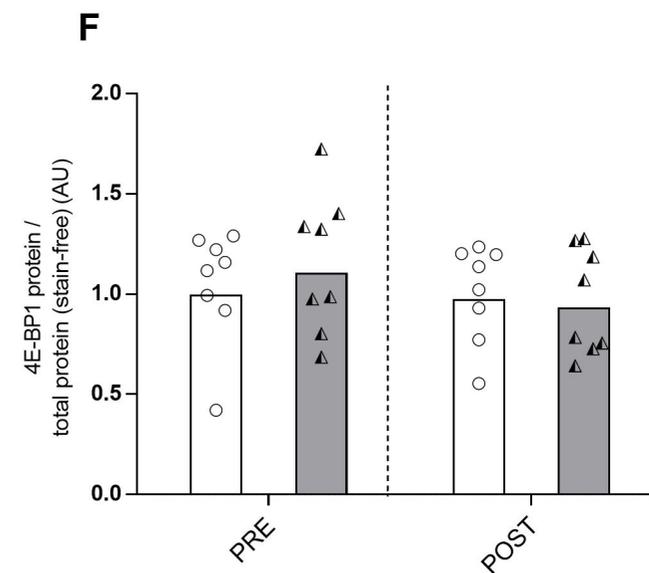
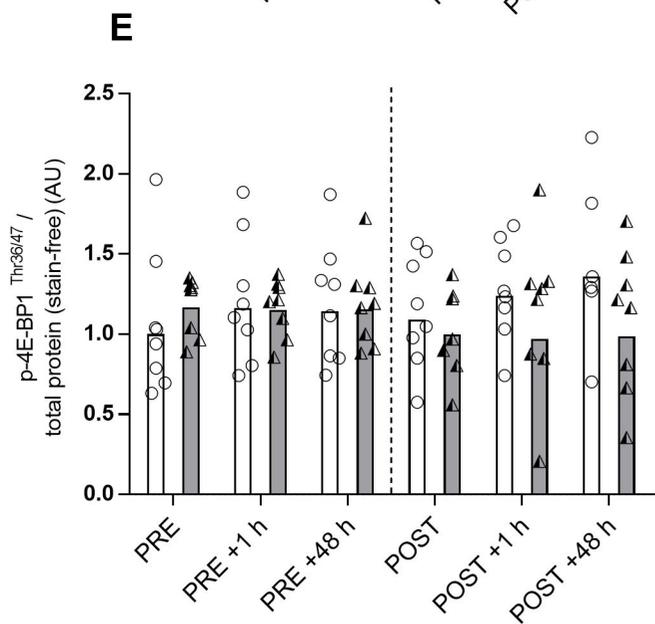
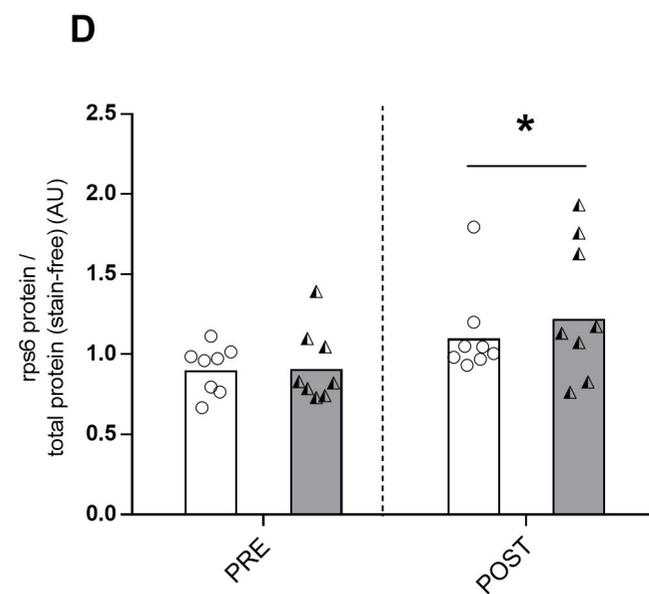
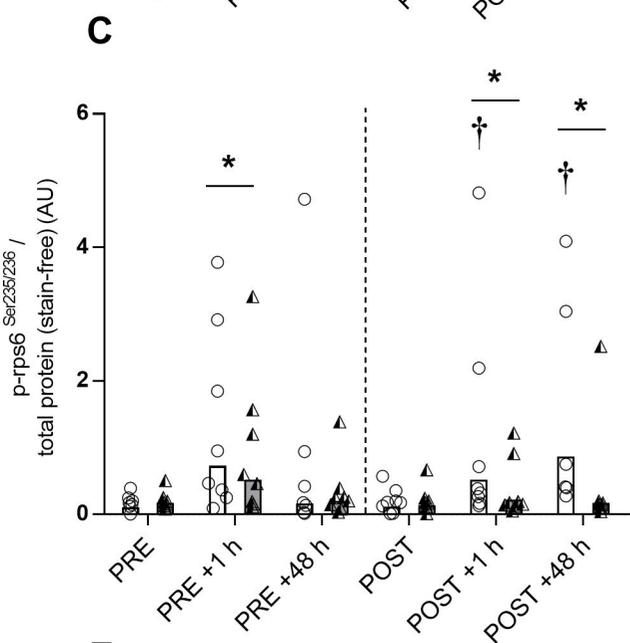
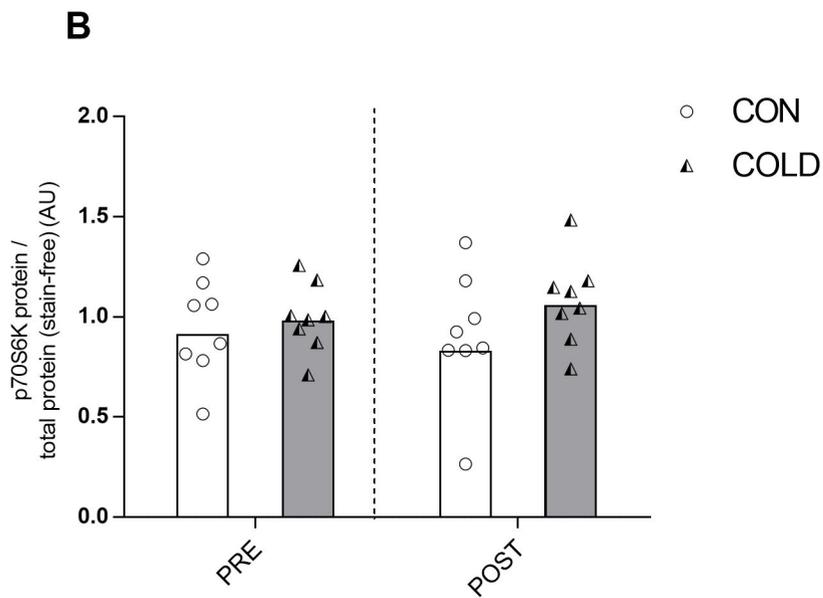
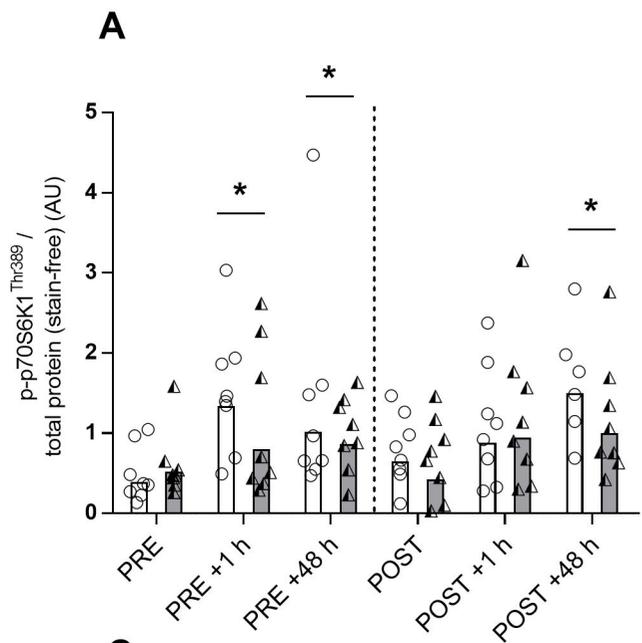
1064 **Table 3.** Summary of all within-group effects considered substantial in magnitude.

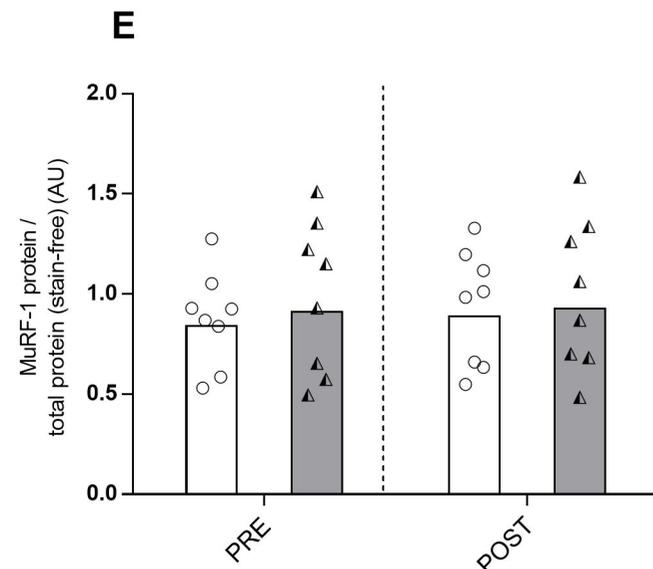
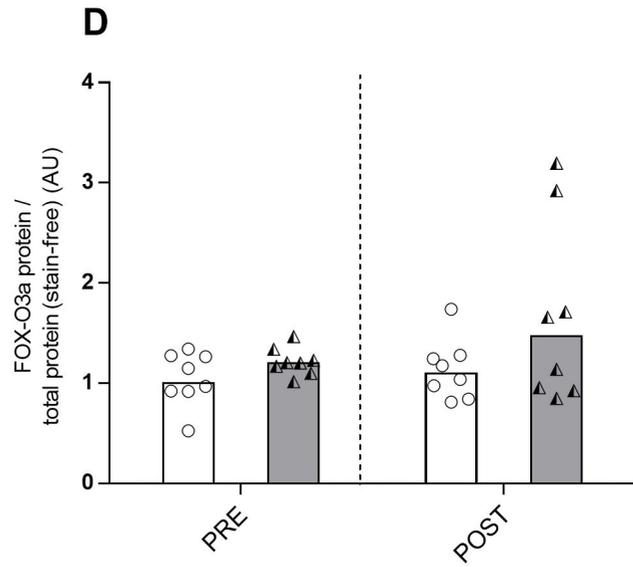
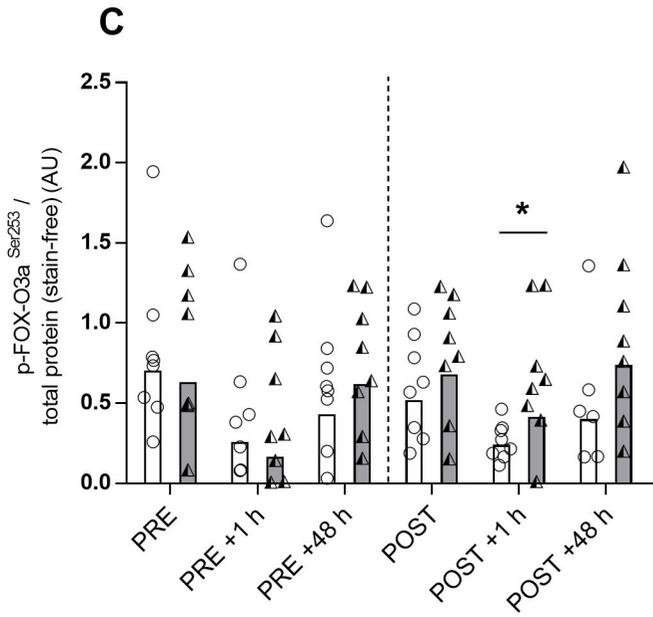
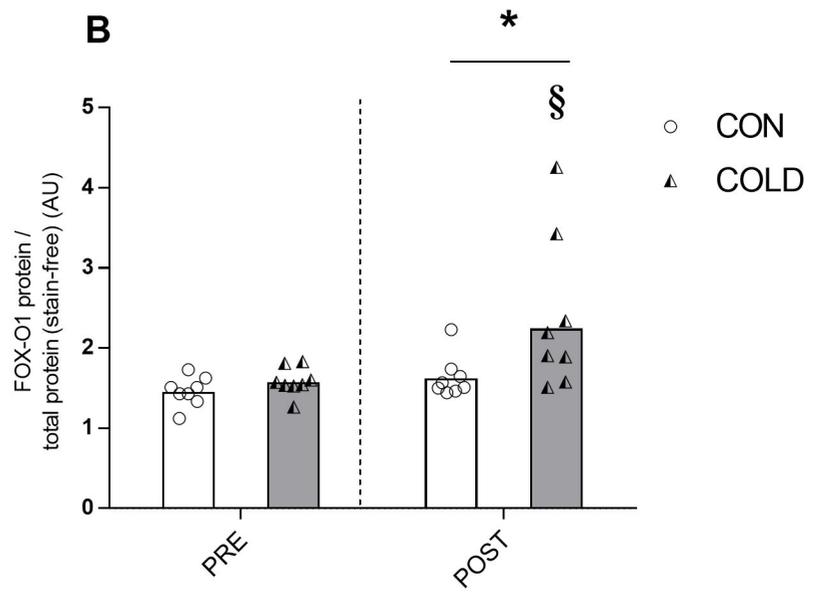
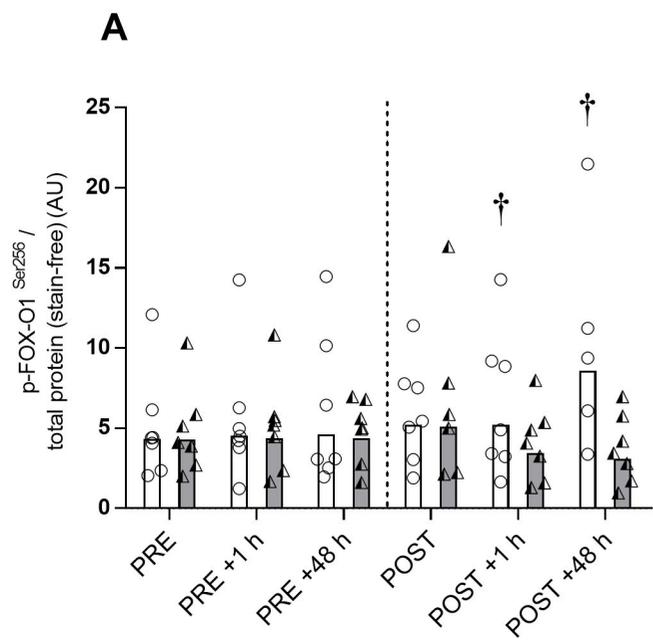
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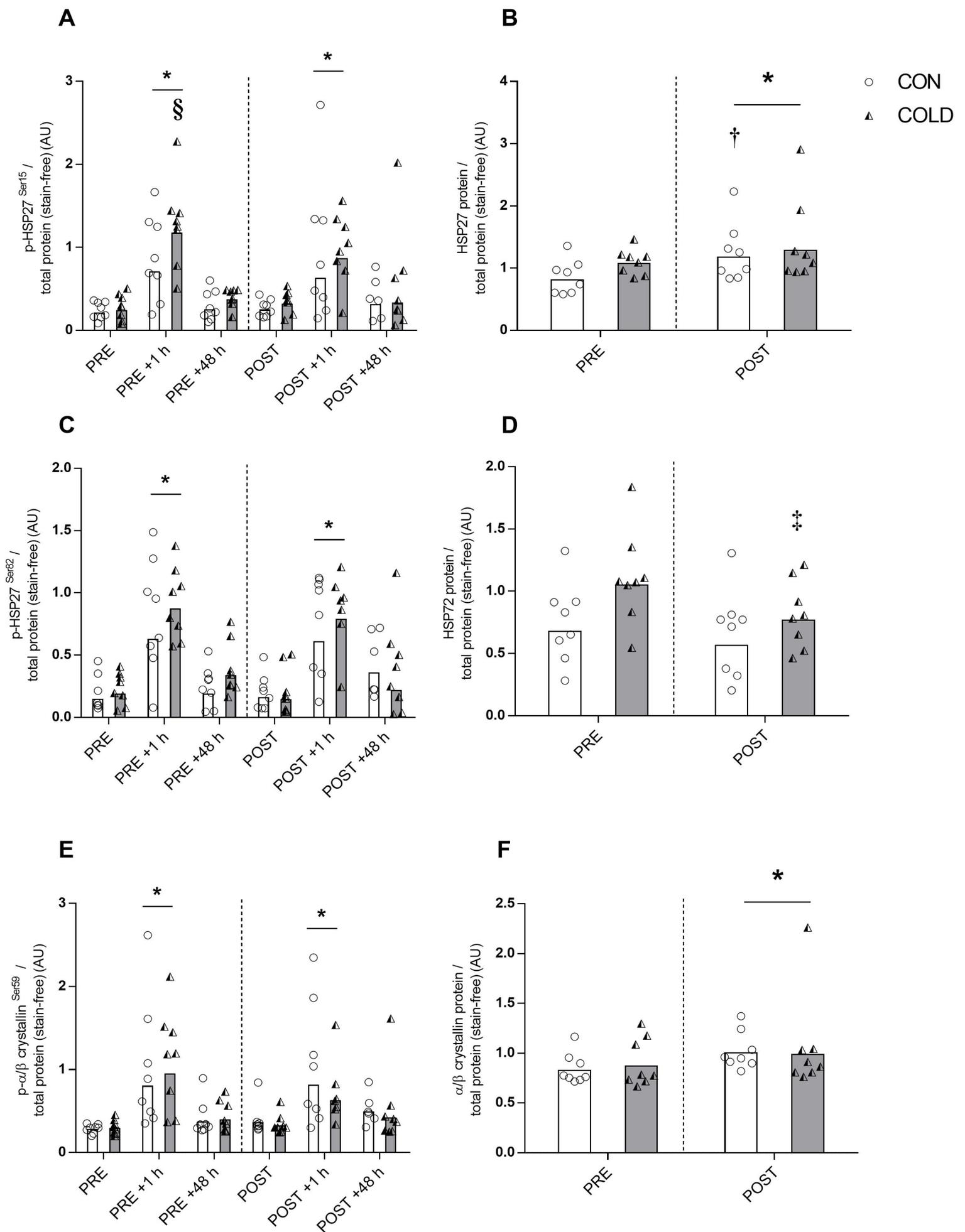
1066 **Table 4.** Summary of all between-group effects considered substantial in magnitude.











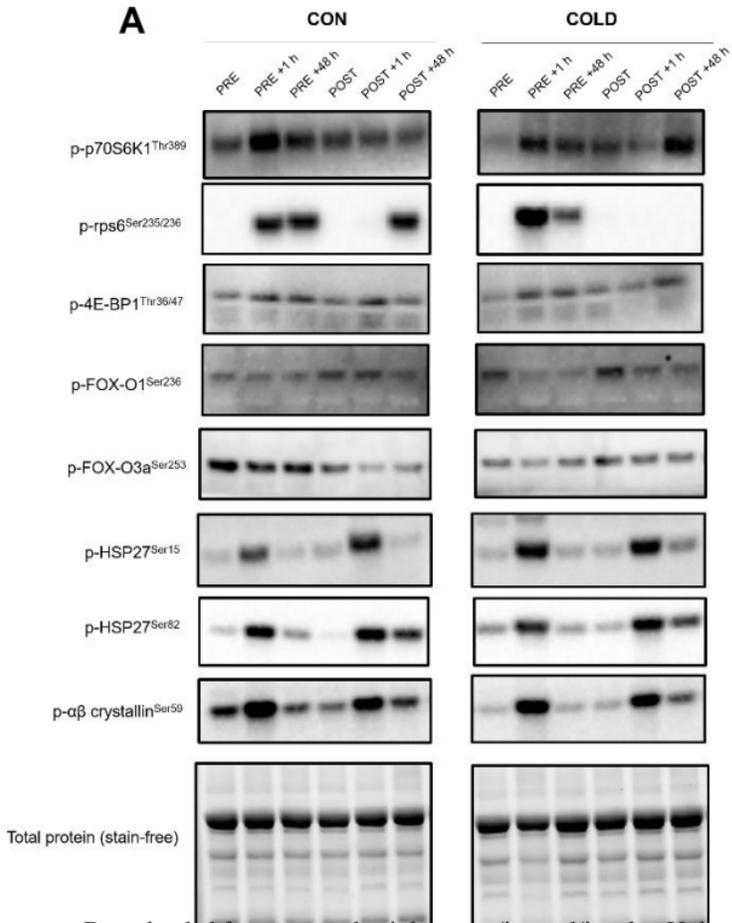
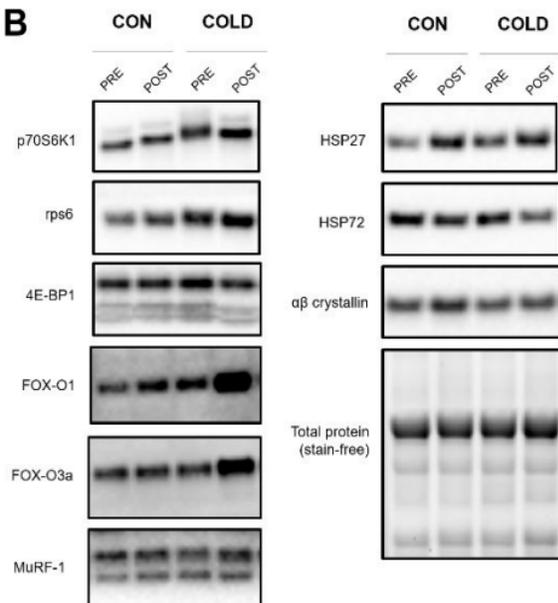
A**B**

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 \pm SD. * = $P < 0.05$ vs. PRE.

	CON		COLD	
	PRE	POST	PRE	POST
Physical characteristics				
Age (y)	25.0 \pm 4.9	-	20.9 \pm 3.4	-
Height (m)	1.84 \pm 0.06	-	1.80 \pm 0.08	-
Body mass (kg)	88.5 \pm 22.3	90.3 \pm 22.5*	80.4 \pm 10.7	81.2 \pm 11
Maximal strength				
1-RM leg press (kg)	338 \pm 78	464 \pm 111*	346 \pm 55	480 \pm 108*
1-RM bench press (kg)	79.5 \pm 17.2	86.4 \pm 20.6*	75.6 \pm 16	83.4 \pm 14.5*
Ballistic exercise performance				
CMJ peak force (N)	1850 \pm 380	1948 \pm 425*†	1908 \pm 324	1846 \pm 318
Squat jump peak force (N)	1997 \pm 451	2129 \pm 495	2008 \pm 372	1987 \pm 323
Ballistic push-up peak force (N)	881 \pm 188	884 \pm 176	855 \pm 102	856 \pm 74
Body composition				
Upper-body lean mass (kg)	38.9 \pm 7.0	40.8 \pm 7.1*	36.3 \pm 3.4	37.7 \pm 4.0*
Lower-body lean mass (kg)	21.6 \pm 2.0	22.5 \pm 3.3*	20.5 \pm 2.1	21.4 \pm 2.4*
Total lean mass (kg)	60.6 \pm 8.9	63.3 \pm 10.3*	55.7 \pm 5.3	59.1 \pm 6.2*
Body fat (%)	19.6 \pm 12.4	18.5 \pm 11.4*	15.6 \pm 6.8	13.9 \pm 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 (<i>d</i>)	±90% CI	
<i>Performance measures</i>							
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
<i>Body composition</i>							
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
<i>Total protein content</i>							
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 (<i>d</i>)	±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
<i>Protein phosphorylation</i>							
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 (<i>d</i>)	±90% CI	
<i>Performance measures</i>							
Peak CMJ force	CON vs. COLD	PRE-POST	160 N	± 73	0.44	0.27	small
<i>Muscle fiber CSA</i>							
Type II muscle fiber CSA	CON vs. COLD	PRE-POST	1915 μM ²	± 1675	1.37	0.99	large
<i>Total protein content</i>							
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
<i>Protein phosphorylation</i>							
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