High-intensity interval training blunts exercise-induced changes in markers of mitochondrial biogenesis in human skeletal muscle

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Running title: Blunted mitochondrial adaptations post training

15 **ABSTRACT**

16 Exercise-induced increases in peroxisome proliferator-activated receptor γ coactivator-1α (PGC-
17 1α) and p53 protein content in the nucleus mediate the initial phase of exercise-induced
18 mitochondrial biogenesis. Here we investigated, for the first time, if these exercise-induced
19 increases in these and other markers of mitochondrial biogenesis were altered after 40 sessions of
20 twice-daily high-volume high-intensity interval training (HVT) in human skeletal muscle. Vastus
21 lateralis muscle biopsies were collected from 10 healthy participants before, immediately post,
22 and 3h after a session of HIIE performed at the same absolute exercise intensity before and after
23 HVT (Pre-HVT and Post-HVT, respectively). Protein contents of common markers of exercise-
24 induced mitochondrial biogenesis were assessed in nuclear- and cytosolic-enriched fractions by
25 immunoblotting; mRNA contents of key transcription factors and mitochondrial genes were
26 assessed by qPCR. Despite exercise-induced increases in PGC-1α, p53, and plant homeodomain
27 finger-containing protein 20 (PHF20) protein content, the phosphorylation of p53 and acetyl-
28 CoA carboxylase (p-p53^{Ser15} and p-ACC^{Ser79}, respectively), and PGC-1α mRNA Pre-HVT, no
29 significant changes were observed Post-HVT. Forty sessions of twice-daily high-intensity
30 interval training blunted all of the measured exercise-induced molecular events associated with
31 mitochondrial biogenesis that were observed Pre-HVT. Future studies should determine if this
32 loss relates to the decrease in relative exercise intensity, habituation to the same exercise
33 stimulus, or a combination of both.

34 **Keywords**: endurance exercise, HIIT, mitochondrial adaptations, p53, PGC-1α

35 **Introduction**

36 Mitochondria are responsible for the production of the majority of the energy required to sustain
37 daily activities and are a key regulator of energy homeostasis (26). The importance of
38 mitochondria is underlined by the links between a healthy mitochondrial pool and enhanced
39 endurance performance (32), improved health (45), and a reduced risk of several lifestyle-related
40 chronic diseases (6, 40). Exercise has long been known to induce mitochondrial biogenesis (29) -
41 the making of new components of the mitochondrial reticulum (19). However, while much is
42 known about changes in molecular signals mediating mitochondrial adaptations in response to a
43 single session of exercise (16, 18, 30), no studies have investigated if the nuclear abundance of
44 important transcription factors and cofactors post-exercise, is altered by training. This is
45 important information that may help explain the common observation of smaller fitness gains as
46 training progresses (36, 39). Furthermore, a better understanding of how molecular signals are
47 altered when an exercise stimulus is repeated may inform strategies to maintain the effectiveness
48 of exercise to stimulate mitochondrial biogenesis.

49 A key regulator of exercise-induced mitochondrial biogenesis is peroxisome proliferator-
50 activated receptor γ coactivator-1α (PGC-1α) (65). In both rat (64) and human (21, 28, 37, 38)
51 skeletal muscle it has been observed that there is a post-exercise increase of PGC-1α protein
52 content in the nucleus, where PGC-1α performs its transcriptional activity (54). In addition, the
53 PGC-1α protein itself (25), as well as other proteins (e.g., p53 (31)), contribute to the exercise-
54 induced upregulation of PGC-1α mRNA (50), the upregulation of which has been shown to be
55 reduced as a training intervention progresses (42, 48, 61). Despite this, no study has investigated
56 exercise-induced changes in the content of nuclear proteins before and after a training
57 intervention. Given that increased PGC-1α protein content in the nucleus represents an important

58 process that contributes to the initial phase of exercise-induced mitochondrial biogenesis (64), it
59 is important to better understand how the molecular response to exercise changes with training.

60 p53 is another important regulator of exercise-induced mitochondrial biogenesis in human
61 skeletal muscle (56). Nuclear accumulation of p53 protein has been reported immediately (21),
62 or 3 hours (62), after a single session of exercise. While the mechanisms underlying the nuclear
63 accumulation of p53 are complex (41, 46), they have partly been attributed to phosphorylation of
64 p53 at serine 15 (p-p53^{Ser15}) (46) - a posttranslational modification that enhances p53 protein
65 stability (60) and prevents its nuclear export and cytosolic degradation (27, 46). However, once
66 again, these molecular events have only been investigated following a single exercise session,
67 and it is not known if they are altered by training. Given that the majority of the p53 activity
68 takes place in the nucleus (46), it is important to determine if the early events of the p53-
69 mediated exercise-induced mitochondrial biogenesis are differentially regulated in this
70 subcellular compartment as the training intervention progresses.

71 Therefore, the aim of our study was to investigate if a session of high-intensity interval exercise
72 (HIIE), performed at the same absolute workload before and after a period of high-volume
73 training (HVT; 40 sessions of high-intensity interval training [HIIT] performed twice-daily for
74 20 consecutive days), induces similar increases in the protein content of PGC-1α, p53, and p-
75 p53^{Ser15} in the nucleus. Upstream signaling, as well as mRNA content of several genes involved
76 in exercise-induced mitochondrial biogenesis, were also investigated before and after HVT. The
77 same absolute workload was chosen as this approach is often used in training studies (19), as
78 well as in practice, where individuals regularly repeat the same exercise session. We
79 hypothesized that 40 sessions of HIIT would result in significantly reduced exercise-induced
80 increases in these events mediating exercise-induced mitochondrial biogenesis.

81 **Materials and methods**

82 *Participants*

83 Ten healthy men (20 ± 2 y; 180 ± 12 cm; 80 ± 15 kg; 46.2 ± 7.6 mL · min^{-1} · kg^{-1}), who were not
84 regularly engaged in cycling-based sports, were moderately-trained (i.e., undertaking moderate,
85 unstructured aerobic activity for less than 3 to 4 hours per week for at least 6 months prior to the
86 study), and were non-smokers and free of medications, volunteered to participate in this study.
87 Upon passing an initial medical screening participants were informed of the study requirements,
88 risks, and benefits, before giving written informed consent. All experimental protocols and
89 study's procedures were approved by the Victoria University Human Research Ethics Committee
90 and conformed to the standards set by the latest revision of the Declaration of Helsinki. All
91 participants completed the study; however, due to the limited amount of muscle tissue harvested
92 during the second biopsy trial, data from one participant were excluded (including physiological
93 and performance data).

94 *Study design and testing*

95 This research was part of a larger, previously-published study investigating the effect of different
96 training volumes on mitochondrial adaptations (20). The experimental protocol specific to the
97 portion of the study described in this manuscript consisted of three tests, each separated by 48 to
98 72 hours, repeated before and after the HVT: a 20-km cycling time trial (20k-TT), a graded
99 exercise test (GXT) and a HIIE biopsy trial (Pre-HVT and Post-HVT). During the 20 days of
100 HVT participants performed HIIT twice a day (Figure 1). Prior to beginning this phase of the
101 larger study, participants were familiarized with the 20k-TT, the GXT and the HIIE, and
102 completed the normal volume training (NVT) phase, (12 HIIT sessions in 4 weeks; Figure 1). It

103 has been reported that the transcriptional response to the first session of exercise is reflective of
104 muscle damage (43) and can differ significantly from the response to subsequent exercise
105 sessions (48). Thus, the NVT phase served to habituate participants to the exercise stimulus.
106 Participants were required to refrain from vigorous exercise for the 72 h preceding each test,
107 from alcohol for 24 h before testing, and from food and caffeine consumption for 3 h before each
108 test.

109 *20k-TT.* Cycling time trials were performed on an electronically-braked cycle ergometer
110 (Velotron, RacerMate, USA) after a 6-min warm-up were participants cycled for 4 min at 66% of
111 the power attained at the lactate threshold ($\dot{W}_{LT}$), followed by 2 min at $\dot{W}_{LT}$, and 2 min of rest.
112 During these tests, participants were only allowed access to cadence and completed distance.
113 Heart rate was monitored (Polar-Electro, Finland) during all exercise trials and training sessions.

114 *GXT.* A discontinuous graded exercise test was performed on an electronically-braked cycle
115 ergometer (Lode Excalibur, v2.0, The Netherlands) to determine peak oxygen uptake ($\dot{V}O_{2peak}$),
116 peak power ($\dot{W}_{peak}$), and $\dot{W}_{LT}$ (using the modified D_{max} method (5)), and the exercise intensity for
117 both the biopsy trial and the HVT training sessions, as previously described (22). Briefly, the test
118 began at 60, 90, or 120 W, depending on participants' fitness levels, and was increased by 30 W
119 every 4 min. Stages were interspersed with 30-s breaks for measurement of fingertip capillary
120 blood lactate concentration using a pre-calibrated blood-lactate analyzer (YSI 2300 STAT Plus,
121 YSI, USA). Participants were instructed to keep a cadence above 60 rpm and were only allowed
122 access to cadence and elapsed time; the GXT was terminated when participants reached
123 volitional exhaustion or cadence dropped below 60 rpm. The $\dot{W}_{peak}$ was determined as the power
124 of the last completed stage plus 7.5 W for every additional minute completed. O_2 and CO_2
125 concentrations were analyzed from expired air using a pre-calibrated gas analyzer (Moxus 2010,

126 AEI technologies, USA), and $\dot{V}O_2$ values were recorded every 15 s. The average of the two
127 highest consecutive 15-s values was recorded as a participant's $\dot{V}O_{2peak}$. The same GXT was
128 performed after 20 days of training to determine the relative exercise intensity of the Post-HVT
129 biopsy trial.

130 *Pre- and Post-HVT HIIE biopsy trials.* Each participant performed the two biopsy trials in the
131 morning and at the same time, to avoid variations caused by circadian rhythms. Participants were
132 provided with a standardized dinner (55 $kJ \cdot kg^{-1}$ body mass (BM), providing 2.1 g
133 carbohydrate$\cdot kg^{-1}$ BM, 0.3 g fat$\cdot kg^{-1}$ BM, and 0.6 g protein$\cdot kg^{-1}$ BM) and breakfast (41 $kJ \cdot kg^{-1}$
134 BM, providing 1.8 g carbohydrate$\cdot kg^{-1}$ BM, 0.2 g fat$\cdot kg^{-1}$ BM, and 0.3 g protein$\cdot kg^{-1}$ BM) to
135 minimize variability in muscle gene and protein expression attributable to diet, as previously
136 described (20). While participants rested in the supine position, and after injection of local
137 anesthetic (1% xylocaine) into the skin and fascia of the vastus lateralis muscle, three small
138 incisions were made about 2-3 cm apart. A resting muscle biopsy was taken (Rest) using a
139 biopsy needle with suction. Approximately ten minutes after participants were helped to an
140 electronically-braked cycle ergometer (Velotron, RacerMate, USA) and began a warm up
141 consisting of cycling for four minutes at 66% of $\dot{W}_{LT}$, followed by 2 min at $\dot{W}_{LT}$, and 2 min of
142 rest, after which the Pre-HVT HIIE session began. HIIE consisted of five 4-min intervals at an
143 exercise intensity equal to $\dot{W}_{LT} + 0.2\ (\dot{W}_{peak} - \dot{W}_{LT})$, interspersed with two minutes of recovery at
144 60 W. Immediately after termination of HIIE (~5 to 10 s) a second skeletal muscle biopsy was
145 taken (+0 h), while a third one was obtained after three hours of recovery (+3 h), during which
146 time participants were allowed access to water *ab libitum* and had no access to food. Skeletal
147 muscle samples were rapidly cleaned of excess blood, fat, and connective tissue, were snap
148 frozen in liquid nitrogen, and later stored at -80°C for subsequent analyses. By design, the Post-

149 HVT HIIE biopsy trial was performed at the same absolute exercise intensity used during the
150 Pre-HVT trial, and followed an identical format.

151 *HVT.* The day following the Pre-HVT HIIE biopsy trial participants began HIIT twice a day for
152 20 consecutive days. Training sessions were performed in the morning and afternoon and
153 consisted of either five to twelve 4-min intervals at relative exercise intensities ranging from $\dot{W}_{LT}$
154 + 0.3 ($\dot{W}_{peak}$ - $\dot{W}_{LT}$) to $\dot{W}_{LT}$ + 0.8 ($\dot{W}_{peak}$ - $\dot{W}_{LT}$), interspersed with a 2-min recovery at 60 W, or
155 eight to twenty-two 2-min intervals at relative exercise intensities ranging from $\dot{W}_{LT}$ + 0.5 ($\dot{W}_{peak}$
156 - $\dot{W}_{LT}$) to $\dot{W}_{LT}$ + 0.8 ($\dot{W}_{peak}$ - $\dot{W}_{LT}$), interspersed with a 1-min recovery at 60 W (20). Single-
157 session duration increased from 30–35 min to 70–80 min. A 10 km cycling time trial was
158 performed before, and at regular weekly intervals during, the HVT to monitor participants for
159 signs of overreaching, as previously described (20). If performance would have decreased by
160 more than 10%, the training load would have been reduced to prevent overreaching (23). No
161 participants experienced a performance loss throughout the entire study, and the training protocol
162 was completed as planned. All participants completed a minimum of 36 (equivalent to 90%)
163 training sessions; average compliance was 96.5% of the prescribed number of sessions.

164 *Skeletal muscle analyses*

165 *Subcellular fractionation.* Nuclear and cytosolic fractions were prepared from 35 to 50 mg of
166 skeletal muscle using a commercially-available nuclear extraction kit (NE-PER, Pierce, USA).
167 Briefly, muscle samples were washed in phosphate-buffered saline (PBS), homogenized in CER-
168 I buffer containing a protease/phosphatase inhibitor cocktail (Cell Signaling Technology [CST],
169 5872) and centrifuged at ~16,000 g. The supernatant was taken as the crude cytosolic fraction.
170 The pellets containing nuclei was washed six times in PBS to minimize cytosolic contamination

171 and nuclear protein were extracted by centrifugation (~16,000 g) in a high-salt NER buffer
172 supplemented with the same inhibitors cocktail and following the manufacturers' instructions.
173 Protein concentration was determined in triplicate using a commercial colorimetric assay (Bio-
174 Rad Protein Assay kit-II; Bio-Rad, Gladesville, NSW, Australia). Nuclear and cytosolic fraction
175 enrichment was confirmed by blotting the separated fractions against a nuclear (histone H3) and
176 a cytosolic (lactate dehydrogenase A [LDHA]) protein; histone H3 was mainly detected in
177 nuclear fractions, whereas LDHA was mainly detected in cytosolic fractions (Figure 2A),
178 indicating the subcellular fractionation enrichment was successful.

179 *Immunoblotting.* Muscle lysates (10 to 50 μg) were separated by electrophoresis using SDS-
180 PAGE gels (8-15%) as previously described (21). An internal standard was loaded in each gel,
181 and each lane was normalized to this value to reduce gel-to-gel variability. Whole-lane
182 Coomassie blue staining (63) was performed to verify correct loading and equal transfer between
183 lanes (Figure 2B). The following primary antibodies were used (supplier, catalogue number):
184 histone H3 (CST, 9715), LDHA (CST, 2012), p53 (CST, 2527), p-acetyl-CoA carboxylase (p-
185 ACC^{Ser79}; CST, 3661), PGC-1α (Calbiochem, st-1202), plant homeodomain finger-containing
186 protein 20 (PHF20; CST, 3934), and $p\text{-}p53^{Ser15}$ (CST, 9284). Representative images for all target
187 proteins are presented in Figure 2C.

188 *Total RNA isolation.* Total RNA was isolated from ~15 mg of muscle tissue as previously
189 described (13). Briefly, samples were homogenized (FastPrep FP120 Homogenizer; Thermo
190 Savant) in the presence of 1 g of zirconia/silica beads (1.0 mm; Daintree Scientific, St. Helens,
191 TAS, Australia) and 800 μL of TRIzol® Reagent (Invitrogen, Melbourne, Australia). Lysates
192 were centrifuged at 13,000 rpm for 15 min at 4°C; the supernatant was collected, combined with
193 chloroform (Sigma-Aldrich, St Louis, USA), and total RNA was extracted using the TRIzol®

194 protocol as per manufacturer's instructions. RNA precipitation was performed for at least 2 h at -
195 20°C in the presence of 400 µL of isopropanol and 10 µL of 5 M NaCl (both Sigma-Aldrich, St
196 Louis, USA). RNA concentration was determined spectrophotometrically (Nanodrop ND1000,
197 Thermo Fisher Scientific, USA) by measuring the absorbance at 260 (A260) and 280 (A280) nm,
198 with A260/A280 ratios > 1.8 indicating high-quality RNA (35). To ensure RNA was free of
199 DNA contamination samples were DNase treated using an RQ1 RNase-free DNase kit (Promega
200 Corporations, Madison, USA).

201 *Real-time PCR (qPCR)*. First-strand cDNA synthesis was performed on 300 ng of total RNA
202 using a thermal cycler (S1000 Thermal Cycler; Bio-Rad; Bio-Rad, Gladesville, NSW, Australia)
203 and the commercially available iScript™ cDNA synthesis kit (Bio-Rad, Gladesville, NSW,
204 Australia) in the presence of random hexamers and oligo(dT)s, according to the manufacturer's
205 directions. Forward and reverse primers for all genes investigated (Table 1) were designed based
206 on NCBI RefSeq using NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/BLAST/), and specificity
207 of the amplified product was confirmed by melting point dissociation curves. The mRNA
208 expression of cytochrome *c* (cyt *c*), nuclear respiratory factor 1 (NRF-1) and 2 (NRF-2), p53,
209 PGC-1α, PHF20, and mitochondrial transcription factor A (TFAM) were quantified by
210 quantitative real-time PCR (Mastercycler® RealPlex2, Eppendorf, Germany), using SYBR
211 Green chemistry (iTaqTM Universal SYBR® Green Supermix; Bio-Rad, Gladesville, NSW,
212 Australia) (10 µL PCR reaction volume). All samples were run in duplicate simultaneously with
213 template free controls, using an automated pipetting system (epMotion 5070, Eppendorf,
214 Germany) to reduce technical variation (35). The following PCR cycling patterns were used:
215 initial denaturation at 95°C (3 min), 40 cycles of 95°C (15 s) and 60°C (60 s). Relative changes
216 in mRNA content were calculated using the $2^{-\Delta\Delta Ct}$ method. To account for the efficiency of RT

217 and initial RNA concentration, the mRNA expression of four housekeeping genes was
218 quantified, and their stability was determined using the BestKeeper software (49). Cyclophilin,
219 glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and beta-2-microglobulin (B2M) were
220 classified as stable, whereas TATA-binging protein (TBP) was reported as unstable and was
221 therefore excluded. These results were confirmed by the Normfinder algorithm (2).

222 *Statistical analysis*

223 All values are reported as mean ± SD unless otherwise specified. To investigate the influence of
224 exercise (Rest, +0 h, and +3 h) and training (Pre-HVT and Post-HVT), and the interaction
225 between these two variables, two-way ANOVA with repeated measures were performed on
226 absolute values, followed by Tukey's honestly significant difference post-hoc tests to assess
227 differences between time points. Specifically, significant interactions and main effects were
228 further analyzed with pre-planned contrasts comparing the Pre- and Post-HVT trials separately.
229 Resting protein and mRNA content values in the Pre- and Post-HVT trials were also compared
230 with a pre-planned paired t-test. Sigma Stat software (Jandel Scientific, USA) was used for all
231 statistical analyses. The level of statistical significance was set a priori at $P < 0.05$.

232 **Results**

233 *Total work during the biopsy trials*

234 By design, the Pre- and Post-HVT HIIE sessions were performed at the same absolute exercise
235 intensity (231.1 ± 33.1 W, Figure 3) and resulted in the same total work (277.3 ± 39.8 kJ). There
236 was an increase (9.0 ± 6.1%, $P = 0.002$) in the power attained at the lactate threshold ($\dot{W}_{LT}$)
237 following training (215.5 ± 32.2 *vs.* 234.7 ± 36.8 W, Pre- and Post-HVT, respectively; Figure 3),
238 which resulted in the relative exercise intensity of the Pre-HVT biopsy trial (107.4 ± 1.2% of

239 $\dot{W}_{LT}$) being greater than the Post-HVT biopsy trial (98.8 ± 5.2% of $\dot{W}_{LT}$). Following training,
240 there was also an increase in peak power ($\dot{W}_{peak}$) (7.8 ± 4.4%, P = 0.001; 292.5 ± 37.9 *vs.* 315.2
241 ± 42.3 W, Pre- and Post-HVT, respectively; Figure 3); consequently, the exercise intensity
242 expressed relative to $\dot{W}_{peak}$ was also greater in the Pre-HVT biopsy trial (78.9 ± 2.4% of $\dot{W}_{peak}$)
243 than the Post-HVT biopsy trial (73.3 ± 3.7% of $\dot{W}_{peak}$). Post-HVT, there was an increase in peak
244 oxygen uptake ($\dot{V}O_{2peak}$) (11.7 ± 7.6%, P = 0.001; 46.2 ± 7.6 *vs.* 51.4 ± 7.8 mL · min^{-1} · kg^{-1}, Pre-
245 and Post-HVT, respectively), whereas 20-km cycling time trial (20k-TT) time was decreased
246 (5.2 ± 2.3%, P < 0.001; 2140.8 ± 99.9 *vs.* 2028.1 ± 87.5 s, Pre- and Post-HVT, respectively). The
247 participants' BM did not change post training (0.2 ± 1.6 %, P = 0.720; 80.4 ± 14.8 *vs.* 80.6 ±
248 14.5 kg, Pre- and Post-HVT, respectively).

249 *Muscle analyses (representative immunoblots are presented in Figure 2C)*

250 *PGC-1α protein content.* There was an interaction effect in both subcellular compartments
251 (nucleus: P = 0.044, cytosol: P = 0.004). In the nucleus (Figure 4A), PGC-1α was increased at
252 +3 h compared with Rest during the Pre-HVT (3.1-fold, P = 0.002), but not during the Post-HVT
253 (1.0-fold, P = 0.869) biopsy trial. During the Pre-HVT, nuclear PGC-1α was also greater at +3 h
254 compared with Post-HVT (3.1-fold, P = 0.015). There was no significant difference in nuclear
255 PGC-1α protein content at Rest between the two biopsy trials (1.8-fold, P = 0.178).

256 In the cytosol (Figure 4B), PGC-1α increased compared with Rest both at +0 h (1.8-fold, P =
257 0.036) and +3 h (2.2-fold, P < 0.001) during the Pre-HVT, but not during the Post-HVT (1.1-
258 fold, P = 1.000 at +0 h; 0.8-fold, P = 0.070 at +3 h) biopsy trial. During the Pre-HVT biopsy
259 trial, cytosolic PGC-1α was also greater at +3 h (1.5-fold, P = 0.017) compared with the same

260 time point of the Post-HVT biopsy trial. At Rest, cytosolic PGC-1α was greater Post-HVT
261 compared with Pre-HVT (2.0-fold, $P = 0.002$).

262 *Gene expression*. There was an interaction effect for PGC-1α mRNA content ($P = 0.020$; Figure
263 5A), which was increased at +3 h compared with Rest during the Pre-HVT (3.6-fold, $P < 0.001$),
264 but not during the Post-HVT (2.0-fold, $P = 0.129$) biopsy trial. During the Pre-HVT biopsy trial,
265 the mRNA content of PGC-1α at +3 h was also greater (1.9-fold, $P < 0.001$) compared with that
266 recorded at the same time point during the Post-HVT biopsy trial. There was no change in p53
267 mRNA content throughout (interaction: $P = 0.425$; main effect of exercise: $P = 0.379$; Figure
268 5B). Results for the mRNA content of cyt *c*, NRF-1 and NRF-2, PHF20, and TFAM are reported
269 in Table 2.

270 *Phosphorylation of acetyl-CoA carboxylase (ACC) at serine 79 (p-ACC^{Ser79}) protein content.* p-
271 ACC^{Ser79} was not detected in nuclear fractions (Figure 2C). In the cytosol (Figure 6), no
272 interaction effect was reported ($P = 0.774$); however, there was a main effect of exercise ($P <$
273 0.001), whereby p-ACC^{Ser79} was greater compared with Rest at +0 h (1.7-fold, $P < 0.001$). Pre-
274 planned comparisons within biopsy trials indicated that at +0 h cytosolic p-ACC^{Ser79} was greater
275 compared with Rest during the Pre-HVT (2.0-fold, $P = 0.013$), but not during the Post-HVT (1.4-
276 fold, $P = 0.114$) biopsy trial.

277 *p53 protein content.* In the nucleus (Figure 7A), there was an interaction effect ($P = 0.016$);
278 nuclear p53 was increased at +3 h compared with Rest during the Pre-HVT (2.8-fold; $P = 0.004$),
279 but not during the Post-HVT (1.2-fold, $P = 0.328$) biopsy trial. At Rest, nuclear p53 was greater
280 Post-HVT compared with Pre-HVT (1.6-fold, $P = 0.042$)

281 In the cytosol (Figure 7B), the interaction effect was not statistically significant ($P = 0.051$);
282 however, there was a main effect of exercise ($P = 0.003$). Cytosolic p53 increased compared with
283 Rest both at +0 h (1.9-fold, $P = 0.019$) and +3 h (2.2-fold, $P = 0.004$). Pre-planned comparisons
284 within trials revealed that during the Pre-HVT biopsy trial cytosolic p53 was greater compared
285 with Rest at both +0 h (2.6-fold, $P = 0.020$) and +3 h (3.2-fold, $P < 0.001$); however, during the
286 Post-HVT biopsy trial no differences compared with Rest were reported at +0h (1.3-fold, $P =$
287 0.440) and +3 h (1.2-fold, $P = 0.835$). There was no difference between the two biopsy trials at
288 Rest (1.9-fold, $P = 0.108$).

289 *PHF20 protein content.* No interaction effects were observed in the nuclear ($P = 0.151$) and
290 cytosolic ($P = 0.132$) fractions. In the nucleus (Figure 7C), there was a main effect of exercise (P
291 $= 0.049$), and PHF20 was greater compared with Rest at +3 h (2.0-fold, $P = 0.040$). Pre-planned
292 comparisons within biopsy trials indicated that at +3 h nuclear PHF20 was greater compared
293 with Rest during the Pre-HVT (2.8-fold, $P = 0.025$), but not during the Post-HVT (1.3-fold, $P =$
294 0.991) biopsy trial. There was no difference between the two biopsy trials at Rest (1.9-fold, $P =$
295 0.339).

296 In the cytosol (Figure 7D), a main effect of exercise ($P = 0.014$) was also reported; cytosolic
297 PHF20 was increased compared with Rest at +3 h (1.6-fold, $P = 0.012$). Pre-planned
298 comparisons within biopsy trials indicated that at +3 h cytosolic PHF20 was greater compared
299 with Rest during the Pre-HVT (2.2-fold, $P = 0.016$), but not the Post-HVT (1.1-fold, $P = 0.988$)
300 biopsy trial. There was no difference between the two biopsy trials at Rest (1.5-fold, $P = 0.618$).

301 *p-p53^{Ser15} protein content.* In the nucleus (Figure 7E), there was an interaction effect (P = 0.021);
302 nuclear p-p53^{Ser15} was increased compared with Rest at +3 h during the Pre-HVT (2.2-fold; P =
303 0.001), but not during the Post-HVT (1.3-fold, P = 0.970) biopsy trial.

304 In the cytosol (Figure 7F), there was no interaction effect (P = 0.136); however, there was a main
305 effect of exercise (P = 0.017). Cytosolic p-p53^{Ser15} was increased compared with Rest at +3 h
306 (2.1-fold, P = 0.014). Pre-planned comparisons within biopsy trials indicated that cytosolic p-
307 p53^{Ser15} was greater compared with Rest at +3 h during the Pre-HVT (3.2-fold, P = 0.003), but
308 not during the Post-HVT (1.1-fold, P = 0.963) biopsy trial.

309 **Discussion**

310 We report for the first time that 40 sessions of HIIT resulted in the loss of all measured exercise-
311 induced molecular changes recorded Pre-HVT. Despite exercise-induced increases in the protein
312 content of PGC-1α, p-ACC^{Ser79}, p53, PHF20, and p-p53^{Ser15}, and the mRNA content of PGC-1α,
313 prior to the HVT, there were no significant changes in any of these parameters when a session of
314 HIIE was repeated at the same absolute exercise intensity post training. In most instances, this
315 did not seem to be explained by increases in resting protein values post HVT. It remains to be
316 determined if this loss stems from the decrease in the relative exercise intensity post-training
317 (98.8 *vs.* 107.4% of $\dot{W}_{LT}$ for Post- and Pre-HVT, respectively), habituation to the HIIE stimulus,
318 or a combination of both.

319 We observed a significant exercise-induced increase in PGC-1α protein content in both the
320 nuclear and cytosolic fractions Pre-HVT, consistent with most previous research (21, 28, 37, 38).
321 However, for the first time we report that these exercise-induced increases were absent post
322 training in both subcellular fractions. A possible explanation for our findings is that Post-HVT

323 the relative exercise intensity elicited during the session was lower compared with Pre-HVT
324 (98.8 *vs.* 107.4% of $\dot{W}_{LT}$ for Post- and Pre-HVT, respectively), suggesting that metabolic
325 perturbations may also have been reduced post-training. It has been proposed that metabolic
326 perturbations (e.g., increases in intracellular calcium [Ca^{2+}], adenosine monophosphate [AMP] to
327 adenosine triphosphate [ATP] ratio, oxidized nicotinamide adenine dinucleotide [NAD^{+}] to
328 NADH ratio, reactive oxygen species [ROS] production) provide an important stimulus for
329 exercise-induced mitochondrial biogenesis (16), and promote an increase in the nuclear content
330 of PGC-1α protein (64). Indeed, as described more in depth later in the discussion, we also
331 observed no significant changes in cytosolic p-ACC^{Ser79} Post-HVT, which supports this
332 hypothesis.

333 A strength of our study design is that participants were habituated to HIIE (12 sessions) during
334 the NVT phase (20). This reduces the possibility that the different molecular responses to the
335 Pre- and Post-HVT trials were due to muscle damage (43) or being unfamiliar with this type of
336 exercise during the Pre-HVT trial (4). Future studies should investigate the effects of a single
337 session of exercise performed at the same relative exercise intensity before, at different time
338 points during, and after, a high volume of training (e.g., >25-30 sessions), to determine if the
339 complete loss of the exercise-induced increases in nuclear and cytosolic PGC-1α protein content
340 also occurs if the relative exercise intensity is maintained pre- and post-training.

341 The reported increase in PGC-1α protein content in both the nucleus and the cytosol may be
342 attributable, at least in part, to increased protein stability (55). Both p38 mitogen-activated
343 protein kinase (MAPK) (53) and AMP-activated protein kinase (AMPK) (7) act as signaling
344 proteins that increase PGC-1α stability via phosphorylation (and they act in similar fashion to
345 also increase the stability of the p53 protein (34, 59)). Due to the limited amount of enriched

346 lysates obtained during subcellular fractionation, we could not measure phosphorylation of p38
347 MAPK and/or AMPK directly. However, due to its molecular weight (~280 kDa), when blotting
348 for lower molecular weight proteins we were also able to measure p-ACC^{Ser79}, a downstream
349 target and commonly-used marker of AMPK activation (9, 10, 33). As previously reported, p-
350 ACC^{Ser79} was not detected in nuclear fractions as (21, 38). However, we were able to measure
351 cytosolic p-ACC^{Ser79} and make the novel observation that despite a robust post-exercise increase
352 Pre-HVT, there was no significant change Post-HVT. This suggests that abrogation of AMPK
353 signaling may have contributed to the abrogation of exercise-induced increases in PGC-1α (and
354 p53) protein content post-training. Subcellular translocation is another factor that has been
355 associated with increased PGC-1α protein content in the nucleus (64). While our data do not
356 seem to indicate cytosolic/nuclear shuttling of PGC-1α, protein translocation is a complex series
357 of cellular processes that cannot be assessed by subcellular fractionation coupled with the
358 immunoblotting technique (1).

359 The PGC-1α protein itself has been reported to increase PGC-1α transcriptional activity
360 following exercise (31, 50) via an autoregulatory loop (25). The exercise-induced increase in
361 PGC-1α mRNA content observed Pre-HVT is consistent with previous findings investigating
362 HIIE (12, 14, 42, 44, 48, 51, 52) and with the notion that increased nuclear PGC-1α protein
363 content and stability is associated with greater PGC-1α transcriptional activity (3). No exercise-
364 induced increase in PGC-1α mRNA content was reported Post-HVT, suggesting that 20 days of
365 HVT also blunted the exercise-induced increase in PGC-1α transcription. However, previous
366 studies have reported a reduction (rather than complete loss) of the exercise-induced
367 upregulation of PGC-1α mRNA content post-training compared to pre-training when the exercise
368 session was repeated at the same relative (48) or absolute (42, 61) exercise intensity. This

369 discrepancy may relate to the much greater number of sessions performed between exercise
370 biopsy trials in our study compared with these three previous studies (40 *vs.* 7 to 12,
371 respectively), and a likely greater reduction in the relative exercise intensity between the Pre-
372 and Post-HVT trials. Moreover, in contrast to the three previous studies, our participants were
373 habituated to HIIE; this raises the possibility that the greater molecular response recorded pre-
374 training in the previous studies may be partly attributable to the "first bout" effect (4, 43).

375 To better characterize the effect of 40 sessions of HIIT on exercise-induced mitochondrial
376 adaptations to HIIE, we also measured the mRNA content of nuclear (NRF-1 and NRF-2 (57))
377 and mitochondrial (TFAM (58)) transcription factors regulating mitochondrial biogenesis that
378 are transcriptionally controlled by PGC-1α (65). The mRNA content of cyt *c* (a gene under the
379 regulation of PGC-1α and NRF1 (65)), p53 (a transcriptional regulator of PGC-1α gene
380 expression (31)) and PHF20 (a transcription factor that activates p53 gene expression (47)), were
381 also measured. Apart from a decrease in cyt *c* mRNA content at +0 h in both HIIE trials, we
382 observed no exercise-induced increases in any other gene either Pre- or Post-HVT. A possible
383 explanation for our findings may relate to biopsy timing, as there is some evidence that the
384 exercise-induced upregulation of some of these genes peaks more than 3 hours post-exercise (8,
385 12, 15, 18, 24, 47).

386 Similar to our results for PGC-1α protein we observed an exercise-induced increase in p53
387 protein content pre-training in the nuclear and cytosolic fractions, as previously demonstrated
388 (21, 62). However, this exercise-induced increase in both subcellular fractions was blunted
389 following 40 training sessions. No other study has investigated exercise-induced changes in p53
390 protein content pre- and post-training in subcellular fractions. Nonetheless, our results are
391 consistent with findings showing reduced/blunted exercise-induced mitochondrial adaptations

392 (e.g., PGC-1α mRNA, PGC-1α protein in whole-muscle lysates) when the same exercise session
393 is repeated post-training both at the same absolute (42, 61) or relative (48) exercise intensity.

394 A possible factor contributing to the lack of exercise-induced changes in nuclear p53 protein
395 content Post-HVT is that 40 sessions of HIIT increased the resting values of p53 in the nucleus.
396 This does not apply to the cytosolic fraction, however, given that resting values of p53 protein
397 remained unchanged. A second factor relates to a possible decrease in subcellular shuttling (17);
398 however, simply immunoblotting subcellular enriched fractions for p53 (or PGC-1α) protein is
399 not a valid technique to demonstrate p53 (similar to PGC-1α) nuclear/cytosolic shuttling - a
400 process requiring an intricate and tightly synchronized series of events (17, 41). Nonetheless, a
401 further novel observation is that there was a concomitant increase in p53 and PHF20 protein
402 content in the nucleus pre-HVT, but not Post-HVT. In this regard, PHF20 has been reported to
403 increase p53 protein stability (46) by disrupting the murine double minute-2 (MDM2)-p53
404 interaction (11) responsible for p53 protein degradation (27, 46). Although we were not able to
405 measure the interaction between these two proteins due to limited lysate availability, it is
406 plausible that our findings may indicate greater p53-PHF20, and reduced p53-MDM2,
407 interaction Pre- vs Post-HVT. In the cytosolic enriched fractions, findings are similar; however,
408 the increase in p53 protein content at +0 h was not matched by an increase in PHF20 protein
409 content. This could suggest that cellular events other than the p53 interaction with PHF20 may
410 also induce p53 protein stability.

411 A second important event disrupting the p53-MDM2 interaction and promoting p53 stability is
412 phosphorylation of p53 at serine 15 (60). Pre-HVT, and consistent with this notion, nuclear p-
413 $p53^{Ser15}$ increased in parallel with the increase in p53 protein content, as previously reported (21).
414 In the cytosol, however, p53 protein content increased earlier than p-$p53^{Ser15}$ (+0 h *vs.* +3 h,

415 respectively), indicating once again that other cellular mechanisms may be involved in the
416 regulation of the p53 protein stability. In contrast, we report for the first time that there were no
417 exercise-induced changes in p-p53^{Ser15} in either the nuclear or cytosolic fractions after a period of
418 training (i.e., post-HVT).

419 This research adds novel information regarding the early molecular events regulating the
420 exercise-induced mitochondrial adaptations and how these are altered by an exercise training
421 intervention. We provide evidence that 40 sessions of HIIT blunted the exercise-induced
422 increases recorded pre-training in all of the molecular events measured. Future studies should
423 investigate if the loss (or reduction) of the exercise-induced increases in markers of
424 mitochondrial adaptations post-training relates solely to the decrease in relative exercise
425 intensity, and/or if this is exacerbated by the continuous repetition of the same exercise stimulus
426 during the training intervention. Well-designed experiments comparing exercise sessions
427 repeated pre- and post-training at the same relative exercise intensity and at different time points
428 during the training intervention (even after only 1 or 2 training sessions to determine the role, if
429 any, of the "first bout effect") should provide valuable insight into the mechanisms driving this
430 phenomenon.

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Author contributions

D. J. Bishop and C. Granata designed the research; C. Granata and R. S. F. Oliveira conducted the research; C. Granata, R. S. F. Oliveira, J. P. Little, and D. J. Bishop analyzed and interpreted the data; C. Granata wrote the manuscript; C. Granata, R. S. F. Oliveira, J. P. Little, and D. J. Bishop critically revised and contributed to the manuscript; C. Granata and D. J. Bishop have primary responsibility for final content. Data collection took place at Victoria University. Muscle analysis took place at Victoria University and the University of British Columbia Okanagan. All persons designated as authors qualify for authorship, and all those qualifying for authorship are listed. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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632

633 Tables

634 Table 1. Primers used for real-time PCR analyses of mRNA expression.

Gene	**Primer efficiency**	**Forward primer (5'→3')**	**Reverse primer (5'→3')**
ACTB	107%	GAGCACAGAGCCTCGCCTTT	TCATCATCCATGGTGAGCTGGC
B2M	98%	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
cyt *c*	98.8%	GGGCCAAATCTCCATGGTCT	TCTCCCCAGATGATGCCTTT
GAPDH	106%	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA
NRF-1	80.7%	CTACTCGTGTGGGACAGCAA	AGCAGACTCCAGGTCTTCCA
NRF-2	92%	AAGTGACAAGATGGGCTGCT	TGGACCACTGTATGGGATCA
p53	101.8%	GTTCCGAGAGCTGAATGAGG	TTATGGCGGGAGGTAGACTG
PGC-1α	103.6%	GGCAGAAGGCAATTGAAGAG	TCAAAACGGTCCCTCAGTTC
PHF20	117.5%	GTGGGGCCGTGAGGAGAATA	AACTGGGCTCCCACTTCAAA
TBP	99%	CAGTGACCCAGCAGCATCACT	AGGCCAAGCCCTGAGCGTAA
TFAM	109.3%	CCGAGGTGGTTTTCATCTGT	GCATCTGGGTTCTGAGCTTT

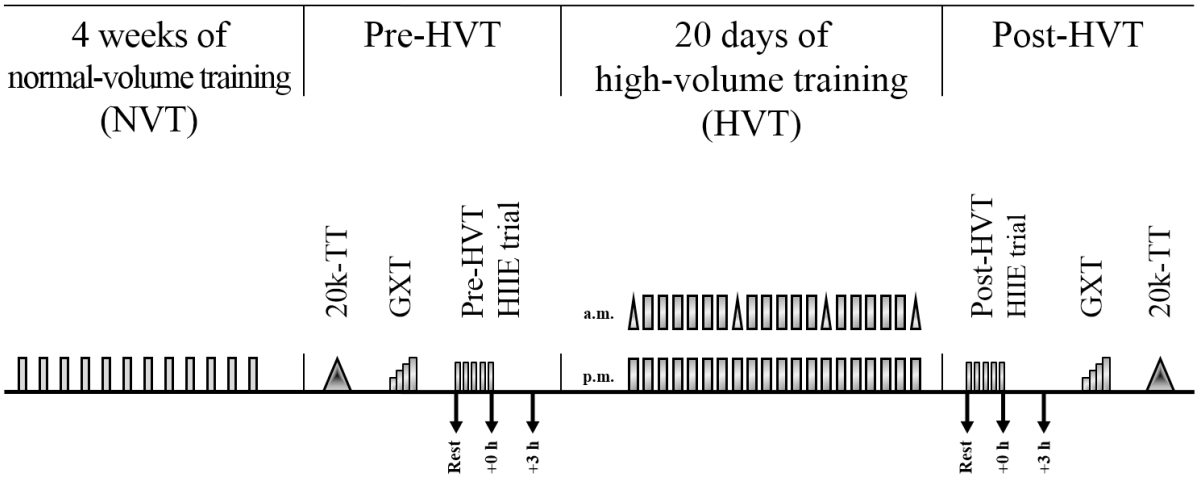
635 ACTB, β-actin; B2M, beta-2-microglobulin; cyt *c*, cytochrome *c*; GAPDH, glyceraldehyde 3-phosphate
636 dehydrogenase; NRF1, nuclear respiratory factor 1; NRF2, nuclear respiratory factor 2; PGC-1α,
637 peroxisome proliferator-activated receptor γ coactivator-1α; PHF20, plant homeodomain finger-
638 containing protein 20; TBP, TATA-binging protein; TFAM, mitochondrial transcription factor A.

639 Table 2. mRNA content of cytochrome *c* (cyt *c*), nuclear respiratory factor 1 (NRF-1) and 2
640 (NRF-2), plant homeodomain finger-containing protein 20 (PHF20), and mitochondrial
641 transcription factor A (TFAM) measured immediately post (+0 h) and 3 h (+3 h) after a single
642 session of high-intensity interval exercise (HIIE) performed at the same absolute intensity before
643 (Pre-HVT) and after (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval
644 training (HVT), in the vastus lateralis muscle of young healthy men. Values are expressed
645 relative to TATA-binging protein (TBP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH),
646 and β-actin (ACTB) housekeeping genes.

Gene	**Time Point**	**Pre-HVT**	**Post-HVT**
	Rest	2.06 ± 0.89	2.79 ± 1.55
cyt *c*	+0 h [#]	1.63 ± 0.44	1.46 ± 0.67
	+3 h	1.70 ± 0.64	2.48 ± 1.37
	Rest	0.14 ± 0.08	0.13 ± 0.06
NRF-1	+0 h	0.19 ± 0.09	0.14 ± 0.04
	+3 h	0.15 ± 0.05	0.16 ± 0.05
	Rest	0.23 ± 0.07	0.21 ± 0.06
NRF-2	+0 h	0.23 ± 0.09	0.19 ± 0.05
	+3 h	0.28 ± 0.13	0.26 ± 0.15
	Rest	0.33 ± 0.08	0.27 ± 0.04
PHF20	+0 h	0.31 ± 0.07	0.24 ± 0.05
	+3 h	0.31 ± 0.08	0.46 ± 0.50
	Rest	0.48 ± 0.14	0.44 ± 0.09
TFAM	+0 h	0.40 ± 0.11	0.37 ± 0.08
	+3 h	0.47 ± 0.13	0.44 ± 0.12

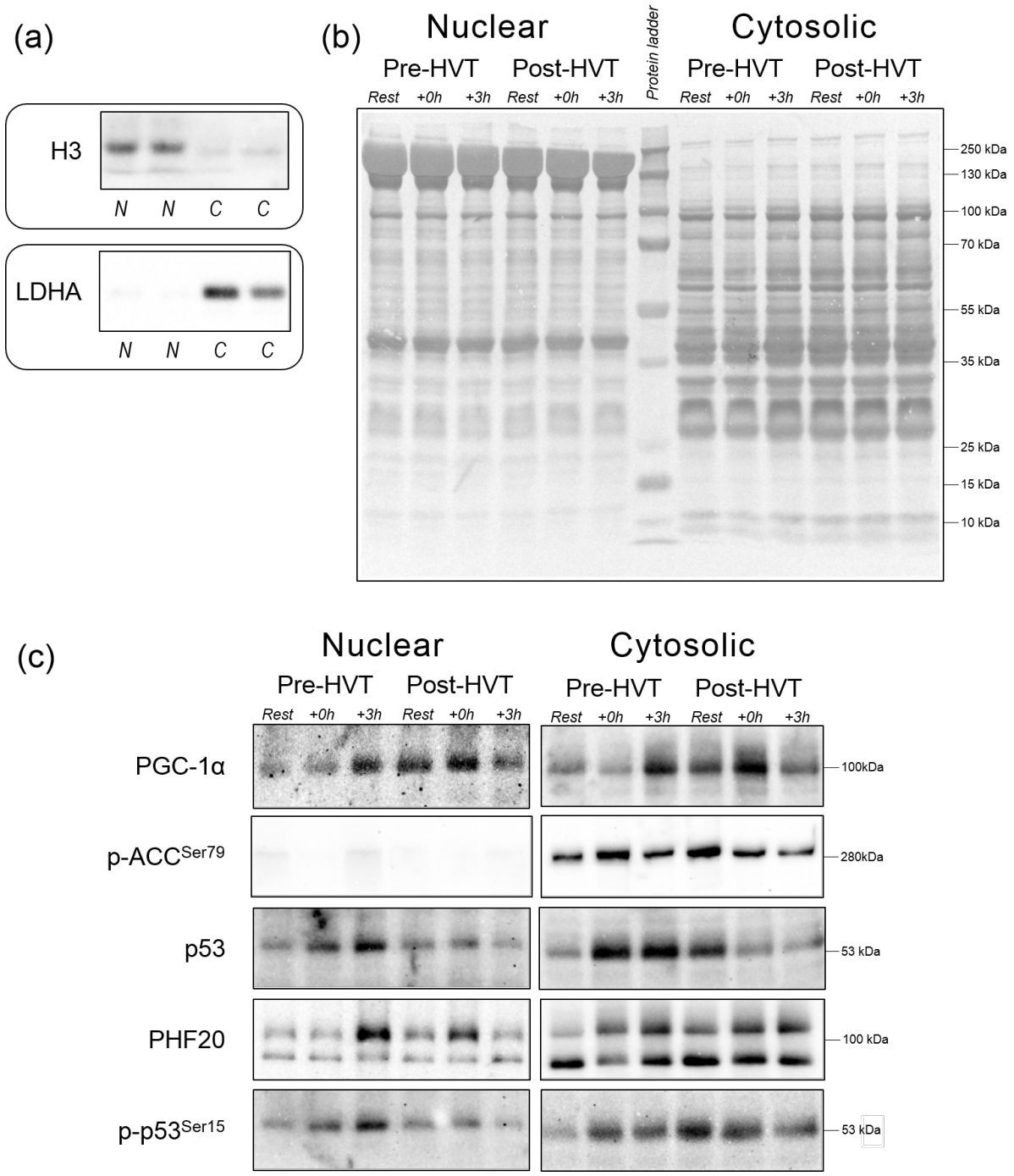
647 [#] Main effect of exercise ($P < 0.05$) *vs.* Rest by two-way ANOVA with repeated measures followed by
648 Tukey's honestly significant difference post-hoc test. All values are mean ± SD. n = 9 for NRF1; n = 8 for
649 all other genes.

650 **Figures**



651

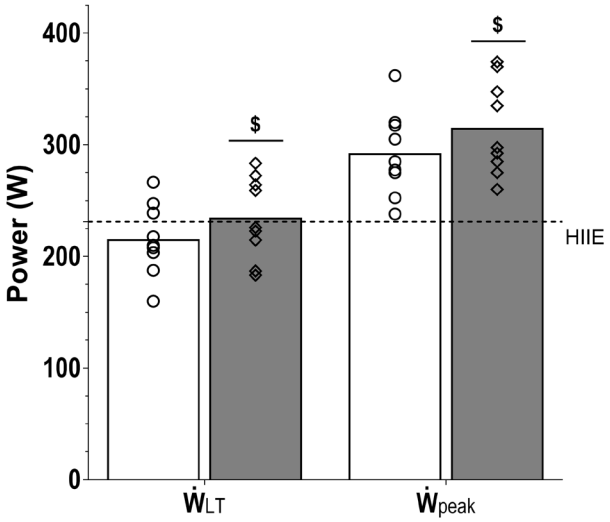
652 Figure 1. **Study design.** Grey rectangles indicate a HIIE session; grey triangles within HVT indicate a 10-
653 km cycling time trial; each rectangle and/or vertical pair of rectangles and/or vertical pair of rectangles
654 and triangles represents a training day; arrows indicate a skeletal muscle biopsy. 20k-TT: 20-km cycling
655 time trial; GXT: graded exercise test; HIIE: high-intensity interval exercise; Rest: skeletal muscle biopsy
656 at rest; +0 h: skeletal muscle biopsy taken at the end of the HIIE session; +3 h: skeletal muscle biopsy
657 taken three hours after the completion of the HIIE session.



658

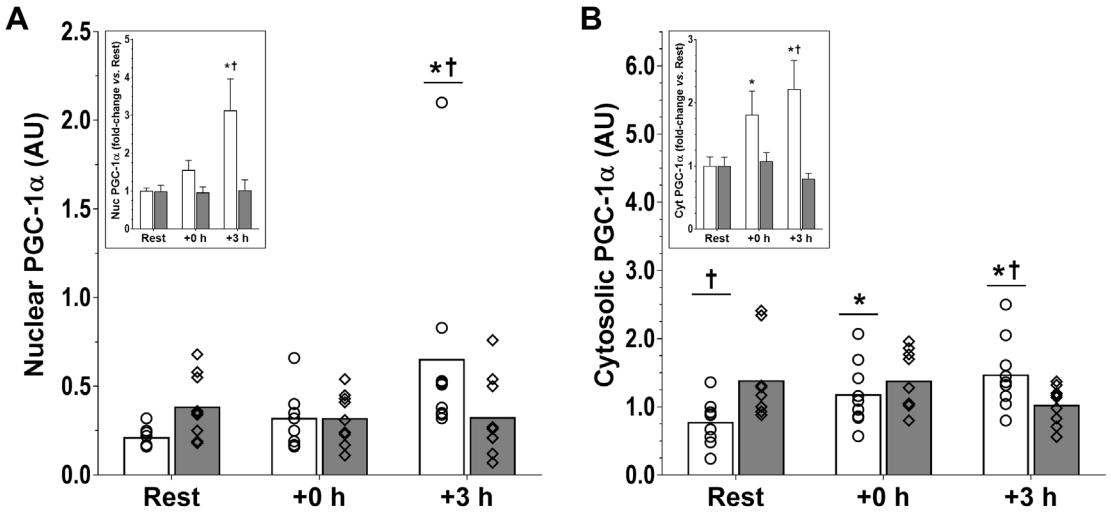
659 Figure 2. **Representative immunoblots, subcellular enrichment and protein loading controls.** (a)
660 Representative immunoblots of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α),
661 acetyl-CoA carboxylase phosphorylated at serine 79 (p-ACC^{Ser79}), p53, plant homeodomain finger-
662 containing protein 20 (PHF20), and p53 phosphorylated at serine 15 (p-p53^{Ser15}) measured in the nuclear

663 and cytosolic fractions obtained from human vastus lateralis muscle biopsies, before (Rest), immediately
664 post (+0 h), and 3 h (+3 h) after a single session of high-intensity interval exercise (HIIE) performed at
665 the same absolute intensity before (Pre-HVT) and after (Post-HVT) 40 sessions of twice-daily high-
666 volume high-intensity interval training (HVT). PHF20: top band at ~105 kDa. No band was detected in
667 the nuclear fractions for p-ACC^{Ser79}. (b) Histone H3 and lactate dehydrogenase A (LDHA) were used as
668 indicators of cytosolic and nuclear enrichment, respectively. N: nuclear fractions; C: cytosolic fractions.
669 (c) Whole-lane Coomassie blue staining for both nuclear and cytosolic fractions was used to verify equal
670 loading between lanes. The immunoblot and whole-lane Coomassie images in this figure were cropped to
671 improve the conciseness and clarity of the presentation.



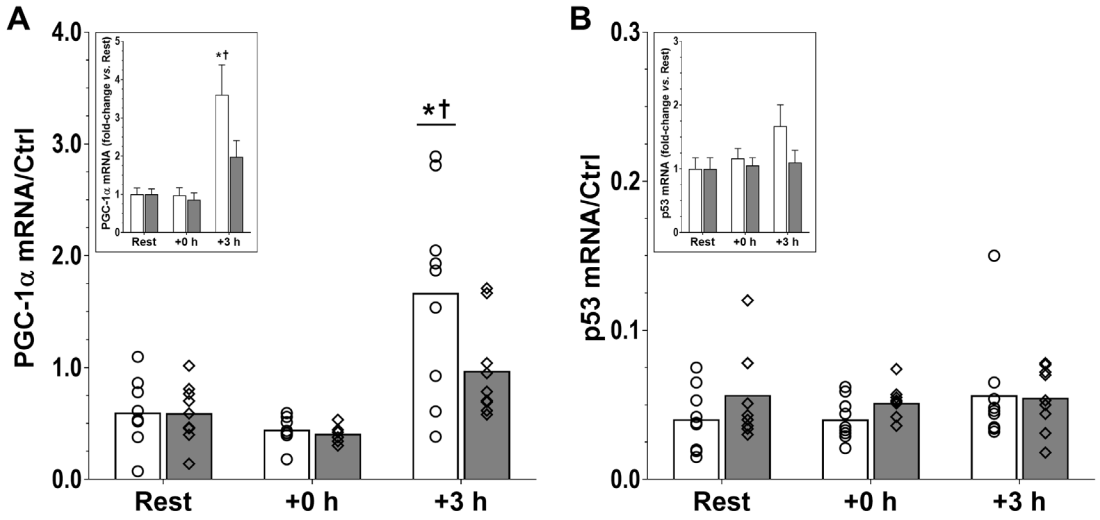
672

673 Figure 3. **Power attained at the lactate threshold ($\dot{W}_{LT}$), peak power achieved during the graded**
674 **exercise test ($\dot{W}_{peak}$), and mean power of the Pre- and Post-HVT high-intensity interval exercise**
675 **(HIIE) biopsy trials.** $\dot{W}_{LT}$ and $\dot{W}_{peak}$ were assessed before (Pre-HVT) and after (Post-HVT) 40 sessions
676 of twice-daily high-volume high-intensity interval training (HVT). Open circles (Pre-HVT) and open
677 diamonds (Post-HVT) represent individual values; white (Pre-HVT) and grey (Post-HVT) bars represent
678 mean values; the dotted line represents the mean power during the Pre- and Post-HVT HIIE biopsy trials.
679 n = 9. $^{\$}$ $P < 0.05$ *vs.* Pre-HVT Rest by two-way ANOVA with repeated measures followed by Tukey's
680 honestly significant difference post-hoc test.



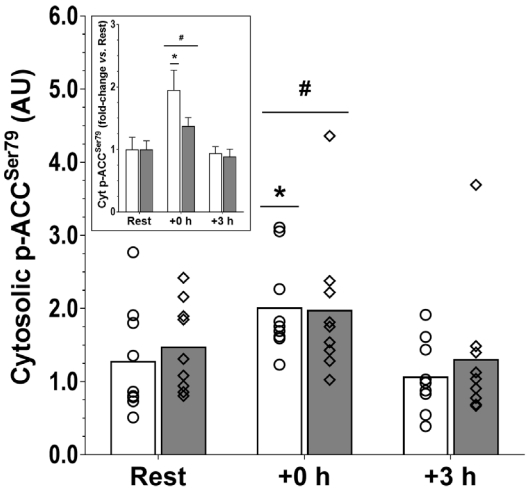
681

682 Figure 4. **Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) protein.** Protein
683 content of PGC-1α in nuclear (a), and cytosolic (b) sub fractions before (Rest), immediately post (+0 h),
684 and 3 h (+3 h) after a single session of high-intensity interval exercise (HIIE) performed at the same
685 absolute intensity before (Pre-HVT) and (Post-HVT) 40 sessions of twice-daily high-volume high-
686 intensity interval training (HVT), in the vastus lateralis muscle of young healthy men (n = 9). Open
687 circles (Pre-HVT) and open diamonds (Post-HVT) represent individual values; white (Pre-HVT) and grey
688 (Post-HVT) bars represent mean values. * $P < 0.05$ *vs.* Rest of the same group; † $P < 0.05$ *vs.* same time
689 point of Post-HVT trial by two-way ANOVA with repeated measures followed by Tukey's honestly
690 significant difference post-hoc test. To more clearly depict fold-changes in post-exercise values from
691 potentially different Rest values in the untrained and trained state, an inset has been added to each main
692 figure (note, significant differences between trained and untrained values at Rest are not reported in insets
693 as these values are both normalized to 1).



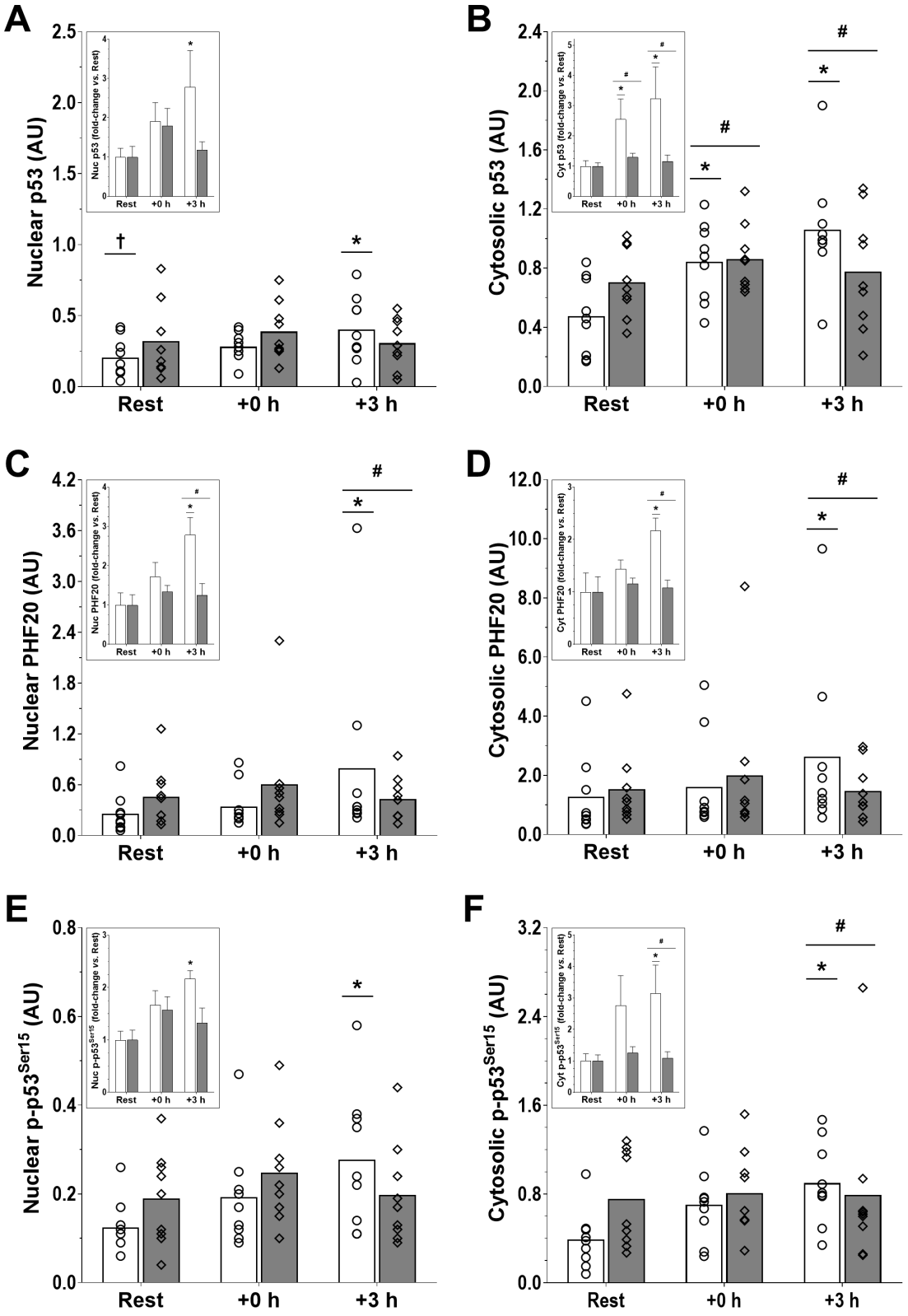
694

695 Figure 5. **Gene expression.** mRNA content of peroxisome proliferator-activated receptor γ coactivator-1α
696 (PGC-1α) (a), and p53 (b) before (Rest), immediately post (+0 h), and 3 h (+3 h) after a single session of
697 high-intensity interval exercise (HIIE) performed at the same absolute intensity before (Pre-HVT) and
698 after (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval training (HVT), in the
699 vastus lateralis muscle of young healthy men (n = 9). Values are expressed relative to TATA-binging
700 protein (TBP), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB) housekeeping
701 genes (Ctrl in the figure). Open circles (Pre-HVT) and open diamonds (Post-HVT) represent individual
702 values; white (Pre-HVT) and grey (Post-HVT) bars represent mean values. * $P < 0.05$ *vs.* Rest of the
703 same group; † $P < 0.05$ *vs.* same time point of Post-HVT trial by two-way ANOVA with repeated
704 measures followed by Tukey's honestly significant difference post-hoc test. To more clearly depict fold-
705 changes in post-exercise values from potentially different Rest values in the untrained and trained state,
706 an inset has been added to each main figure.



707

708 Figure 6. **Phosphorylation of acetyl-CoA carboxylase (ACC) at serine 79 (p-ACC^{Ser79}).** Protein
709 content of cytosolic p-ACC^{Ser79} before (Rest), immediately post (+0 h), and 3 h (+3 h) after a single
710 session of high-intensity interval exercise (HIIE) performed at the same absolute intensity before (Pre-
711 HVT) and after (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval training
712 (HVT), in the vastus lateralis muscle of young healthy men (n = 9). Open circles (Pre-HVT) and open
713 diamonds (Post-HVT) represent individual values; white (Pre-HVT) and grey (Post-HVT) bars represent
714 mean values. # main effect of exercise ($P < 0.05$) *vs.* Rest; * $P < 0.05$ vs. Rest of the same group by two-
715 way ANOVA with repeated measures followed by Tukey's honestly significant difference post-hoc test.
716 To more clearly depict fold-changes in post-exercise values from potentially different Rest values in the
717 untrained and trained state, an inset has been added to each main figure.


A
Nuclear p53 (AU)
Nuc p53 (fold-change vs. Rest)
Rest
+0 h
+3 h
B
Cytosolic p53 (AU)
Cyt p53 (fold-change vs. Rest)
C
Nuclear PHF20 (AU)
Nuc PHF20 (fold-change vs. Rest)
D
Cytosolic PHF20 (AU)
Cyt PHF20 (fold-change vs. Rest)
E
Nuclear p-p53Ser15 (AU)
Nuc p-p53Ser15 (fold-change vs. Rest)
F
Cytosolic p-p53Ser15 (AU)
Cyt p-p53Ser15 (fold-change vs. Rest)

718

719 Figure 7. **p53 and plant homeodomain finger-containing protein 20 (PHF20) protein.** Protein content
720 of nuclear (a) and cytosolic (b) p53, of nuclear (c) and cytosolic (d) PHF20, and of nuclear (e) and
721 cytosolic (f) p-p53^{Ser15} assessed before (Rest), immediately post (+0 h), and 3 h (+3 h) after a single
722 session of high-intensity interval exercise (HIIE) performed at the same absolute intensity before (Pre-
723 HVT) and after (Post-HVT) 40 sessions of twice-daily high-volume high-intensity interval training
724 (HVT), in the vastus lateralis muscle of young healthy men (n = 9). Open circles (Pre-HVT) and open
725 diamonds (Post-HVT) represent individual values; white (Pre-HVT) and grey (Post-HVT) bars represent
726 mean values. $^{\#}$ main effect of exercise ($P < 0.05$) *vs.* Rest; * $P < 0.05$ *vs.* Rest of the same group; † $P <$
727 0.05 *vs.* same time point of Post-HVT trial by two-way ANOVA with repeated measures followed by
728 Tukey's honestly significant difference post-hoc test. To more clearly depict fold-changes in post-
729 exercise values from potentially different Rest values in the untrained and trained state, an inset has been
730 added to each main figure (note, significant differences between trained and untrained values at Rest are
731 not reported in insets as these values are both normalized to 1).