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1 **Skeletal muscle lipid droplets are resynthesized before being**
2 **coated with perilipin proteins following prolonged exercise in**
3 **elite male triathletes**

4
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18 **Abstract**

19 Intramuscular triglycerides (IMTG) are a key substrate during prolonged exercise, but
20 little is known about the rate of IMTG resynthesis in the post-exercise period. We
21 investigated the hypothesis that the distribution of the lipid droplet (LD)-associated
22 perilipin (PLIN) proteins is linked to IMTG storage following exercise. 14 elite male
23 triathletes (27 ± 1 y, 66.5 ± 1.3 mL.kg⁻¹.min⁻¹) completed 4 h of moderate-intensity
24 cycling. During the first 4 h of recovery, subjects received either carbohydrate or H₂O,
25 after which both groups received carbohydrate. Muscle biopsies collected pre and post-
26 exercise, and 4 h and 24 h post-exercise were analysed using confocal
27 immunofluorescence microscopy for fibre type-specific IMTG content and PLIN
28 distribution with LDs. Exercise reduced IMTG content in type I fibres (-53%,
29 $P=0.002$), with no change in type IIa fibres. During the first 4 h of recovery, IMTG
30 content increased in type I fibres ($P=0.014$), but was not increased further after 24 h
31 where it was similar to baseline levels in both conditions. During recovery the number
32 of LDs labelled with PLIN2 (70%), PLIN3 (63%) and PLIN5 (62%; all $P<0.05$) all
33 increased in type I fibres. Importantly, the increase in LDs labelled with PLIN proteins
34 only occurred at 24 h post-exercise. In conclusion, IMTG resynthesis occurs rapidly in
35 type I fibres following prolonged exercise in highly-trained individuals. Further,
36 increases in IMTG content following exercise preceded an increase in the number of
37 LDs labelled with PLIN proteins. These data, therefore, suggest that the PLIN proteins
38 do not play a key role in post-exercise IMTG resynthesis.

39 **Keywords:**

40 Intramyocellular lipid, perilipin 2, perilipin 3, perilipin 5, carbohydrate restriction

41

42 **Abbreviations:**

43 Intramuscular triglyceride (IMTG)

44 Carbohydrate (CHO)

45 Lipid droplet (LD)

46 Perilipin (PLIN)

47 **Introduction**

48 The location of intramuscular triglyceride (IMTG)-containing lipid droplets (LD) in
49 close proximity to mitochondria underpins the importance of IMTG as a fuel source
50 during prolonged moderate-intensity exercise in trained individuals, particularly in type
51 I muscle fibres (36). Indeed, many studies report a decrease in IMTG content during
52 exercise (16, 35), but to date there has been much less focus on post-exercise IMTG
53 resynthesis. This is in contrast to the large body of research that has focused on
54 glycogen use during exercise and dietary strategies to optimise glycogen repletion
55 following exercise (5). High carbohydrate (CHO) diets, however, are reciprocally low
56 in fat (typically 2-25% of total energy intake) and markedly reduce IMTG storage (9,
57 13, 33). Indeed, post-exercise IMTG resynthesis is suppressed up to 48 h following 3 h
58 moderate-intensity cycling when consuming a high CHO diet (containing 24% fat) (36).
59 More recently though, post-exercise nutritional strategies have shifted towards CHO- or
60 calorie-restriction in an attempt to augment specific training adaptations in human
61 skeletal muscle. In this respect, limiting CHO or energy intake following glycogen-
62 depleting exercise has been shown to enhance the activation of intracellular signalling
63 pathways compatible with mitochondrial biogenesis (reviewed in 13). Typically, in
64 these studies CHO or energy provision is limited throughout exercise as well as during
65 the first few hours following exercise, after which habitual energy and macronutrient
66 intake are resumed. Whether this nutritional strategy, designed to augment skeletal
67 muscle training adaptations, can also accelerate post-exercise IMTG resynthesis, is yet
68 to be investigated.

69

70 Given the paucity of studies investigating post-exercise IMTG resynthesis, it is
71 unsurprising that the mechanisms governing the synthetic response are poorly
72 understood. In skeletal muscle, cytosolic LDs provide a storage depot for IMTG, and
73 given their large proteome (>300 proteins) (42) these LDs are now considered a highly
74 active organelle. The perilipin (PLIN) proteins are the most abundant of the LD
75 proteins in skeletal muscle, and are more highly expressed in type I compared to type II
76 muscle fibres thereby mirroring the fibre-specific distribution of IMTG (27, 28, 29, 30).
77 Moreover, exercise training typically augments both the protein levels of PLIN2, PLIN3
78 and PLIN5 alongside elevations in IMTG content (28, 30), implying that the increase in

79 PLIN protein content is mechanistically important to facilitate growth of the IMTG
80 pool. This assertion is supported by the observation that muscle-specific PLIN2 (3) or
81 PLIN5 overexpression (4) in rodents fed a high-fat diet promotes IMTG storage, which
82 may be linked to an ability of the PLIN proteins to restrict basal lipolytic rates (19).
83 Recently, Gemmink *et al.*, (11) reported that IMTG storage augmented by prolonged
84 fasting in healthy individuals coincided with an increase in the size and number of LDs
85 containing PLIN5. Because no changes occurred in the protein level of PLIN5, these
86 data suggest that a redistribution of the pre-existing PLIN5 pool occurs when the LD
87 pool expands. We recently corroborated this finding using an acute lipid infusion to
88 stimulate IMTG accretion, and demonstrated that a redistribution of PLIN3, as well as
89 PLIN5, also occurs across a growing LD pool (31). Whilst the use of both prolonged
90 fasting and lipid infusion has provided insight into the potential role of the PLIN
91 proteins in supporting IMTG storage, these experimental models do not represent the
92 normal physiological milieu; that is, they expose the muscle to excess free fatty acid
93 concentrations and stimulate IMTG accretion starting from a ‘resting’ level. This
94 physiological state, therefore, is distinct from one in which trained individuals regularly
95 use (and reduce the size of) the IMTG pool during exercise and subsequently
96 resynthesize IMTG in the post-exercise period. Investigating the PLIN proteins under
97 more physiologically dynamic conditions may therefore provide additional insight into
98 their role in skeletal muscle.

99

100 In addition to the possible mediation of IMTG storage, the PLIN proteins are suggested
101 to be important in mediating the breakdown and oxidation of IMTG. We have
102 previously shown that LDs containing either PLIN2 (29) or PLIN5 (30) are
103 preferentially used during 1 h of moderate-intensity exercise, and recently reported that
104 hormone-sensitive lipase targets LDs containing PLIN5 for breakdown during exercise
105 (39). PLIN3 is associated with fat oxidation in cultured muscle cells (8), but whether
106 PLIN3 plays a role in the breakdown and oxidation of IMTG *in vivo* is not known.
107 Therefore, we asked the question whether PLIN3-containing LDs are also preferentially
108 targeted for breakdown during exercise.

109

110 Carbohydrate consumption post-exercise will increase circulating insulin concentrations
111 which will in turn inhibit systemic lipolysis and reduce plasma free fatty acid
112 concentrations. If no energy is consumed, insulin concentrations will remain low and
113 plasma free fatty acid concentrations will be high, thus providing a source of fatty acids
114 to be used to rebuild IMTG stores. In this context, we first aimed to investigate the
115 hypothesis that post-exercise IMTG resynthesis would be accelerated under conditions
116 of acute CHO restriction in elite endurance athletes. To achieve this, CHO was ingestion
117 was restricted during the initial 4 h recovery period following prolonged moderate-
118 intensity exercise. By assessing changes in IMTG content in response to exercise and up
119 to 24 h post-exercise, this provided a physiological model to further clarify the roles of
120 the PLIN proteins in mediating IMTG utilisation and storage. In this respect, we
121 hypothesised that during exercise there would be a preference to use LDs labelled with
122 PLIN proteins, and during recovery from prolonged exercise there would be a
123 preferential increase in LDs labelled with PLIN proteins. Consequently, the secondary
124 aim of this study was to investigate changes in the distribution of PLIN proteins relative
125 to LDs during exercise and in the post-exercise period using our previously described
126 immunofluorescence microscopy methodology (31). Finally, because IMTG utilisation
127 during exercise is specific to the intermyofibrillar region of the fibre (18), we
128 determined changes in IMTG content and the PLIN LD distribution on a subcellular-
129 specific basis.

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141 **Methods**

142 *Subjects*

143 Fourteen elite male triathletes (27.2 ± 0.9 y, 183 ± 2 cm, 75.3 ± 1.4 kg) that had
144 competed at national and/or international level were recruited as part of a larger study
145 (10). Participants had been elite athletes for 4.8 ± 1.4 y and trained on average $16.4 \pm$
146 0.9 hours a week. There were no differences between experimental groups, other than
147 VO_{2max} where the participants in the CHO condition had a significantly higher VO_{2max}
148 (CHO: 68.3 ± 1.4 mL.kg⁻¹.min⁻¹, H₂O: 63.5 ± 1.8 mL.kg⁻¹.min⁻¹, $P < 0.05$). All
149 participants were fully informed of any risks associated with the study before providing
150 informed verbal and written consent. Ethical approval was approved by the ethics
151 committee of the Region of Southern Denmark (Project ID: S-20090140) and was
152 conducted according to the Declaration of Helsinki.

153

154 *Experimental procedures*

155 All experimental procedures have been described previously (10, 14). Briefly,
156 participants completed 4 h of cycling at an average of $73\% \pm 1\%$ HR_{max} equating to
157 $\sim 56\%$ of VO_{2max} (determined via pre-experimental submaximal incremental test and
158 VO_{2max} test) with an intended HR intensity of $\sim 75\%$ HR_{max}. Subjects were provided a
159 standardised breakfast (see “Dietary Procedures” below) 90 min before completing the
160 cycle in which they used personal equipment of their choice (i.e. bike, shoes and pedals)
161 on mounted turbo trainers (Elite Crono Mag ElastoGel Trainer, Fontaniva, Italy).
162 During exercise participants were only allowed to consume water (minimum of 1 mL
163 water.kg⁻¹.h⁻¹). Following exercise, participants were randomly selected to receive either
164 CHO ($n = 7$) or H₂O ($n = 7$) during the first 4 h of recovery. For the remaining 20 h
165 period following exercise all participants consumed a CHO-rich diet. All procedures
166 were conducted in laboratories at the Department of Sports Science and Clinical
167 Biomechanics, University of Southern Denmark, Odense.

168

169 *Dietary procedures*

170 The dietary intake was controlled and corresponded to recommendations provided by
171 the American College of Sport Medicine (26). A breakfast was provided 90 min prior to
172 exercise and consisted of CHO rich foods (i.e. porridge oats, raisins, skimmed milk,

173 orange juice and energy bar; 82 kJ.kg⁻¹ bw). All calorie intake was calculated based
174 upon the participant's body mass. During the initial 2 h recovery period following
175 exercise, the CHO group consumed a meal consisting of pasta, chicken, vegetables and
176 a CHO beverage (1.07 g CHO.kg⁻¹ bw.h⁻¹). and subsequently participants were provided
177 with an energy bar and CHO beverage (1.05 g CHO.kg⁻¹ bw.h⁻¹) in the following 2 h.
178 During this 4 h period, the H₂O group remained fasted and only consumed water. After
179 the initial 4 h recovery period, both groups received the same standardised meals for the
180 remaining 20 h of recovery. In addition, the H₂O group received energy corresponding
181 to that of the CHO group during the 4 h recovery period to ensure that the total energy
182 intake between groups was equal. Thus, the CHO group received dinner and breakfast
183 whereas the H₂O group received lunch, an energy bar, dinner and breakfast. In total,
184 subjects received 264 kJ.kg⁻¹ bw (10 g CHO.kg⁻¹ bw) on the first experimental day.

185

186 *Sample collection*

187 Muscle biopsies were collected from participants from the *m. vastus lateralis* before and
188 after exercise, as well as at 4 h and 24 h post-exercise, under local anaesthetic (1%
189 lidocaine; Amgro, Copenhagen, Denmark) using a Bergstrom needle (2) with suction.
190 Biopsies were from the same region and depth on alternating legs with incisions
191 separated by ~5cm with care to avoid damage of multiple biopsies (37). Once collected,
192 samples (100-150 mg) were quickly dissected from fat and connective tissue and
193 divided into multiple pieces. They were then embedded in TissueTek (Sakura Finetek,
194 Alphen aan den Rijn, the Netherlands) and frozen in pre-cooled isopentane before being
195 stored at -80°C for later analysis.

196

197 *Immunofluorescence microscopy*

198 Five µm thick cryosections were cut at -30°C and transferred onto ethanol-cleaned glass
199 slides. From each participant pre and post-exercise, and 4 h and 24 h post-exercise
200 muscle samples were mounted on to the same slide to ensure consistency in the staining
201 process between sections. Slides were fixed in 3.7% formaldehyde solution for 1 hour,
202 followed by three rinses (each for 30 s) in doubly distilled water before
203 permeabilization in 0.5% Triton X-100 for 5 min. Following three 5 min washes in
204 phosphate buffered saline (PBS), slides were incubated for 1 h with appropriate primary

205 antibodies targeting myosin heavy chain type I and myosin heavy chain type IIa alone
206 or in combination with antibodies targeting PLIN2, PLIN3 or PLIN5 (see below for
207 details). Following this incubation period, a further three 5 min PBS washes were
208 completed before the slides were incubated with appropriate Alexa Fluor secondary
209 antibodies for 30 min. Three more 5 min washes in PBS preceded a 20 min incubation
210 with BODIPY 493/503 (Invitrogen, Paisley, UK) in order to visualize IMTG. This was
211 subsequently left to incubate for 20 min. Following a final 5 min wash in PBS solution,
212 coverslips were mounted using Vectashield (H-1000 Vector Laboratories, Burlingame,
213 CA, USA) and sealed with nail varnish.

214

215 *Antibodies*

216 For the lipid analysis the primary antibodies applied targeted myosin heavy chain type I
217 (MHCI – A4.840c) and myosin heavy chain type IIa (MCHIIa – N2.261c; both DSHB,
218 University of Iowa, USA), and visualized using the secondary antibodies goat anti-
219 mouse IgM 546 and goat anti-mouse IgG blue 405, respectively. Wheat germ
220 agglutinin Alexa Fluor 633 (Invitrogen, Paisley, UK) was used to visualize the cell
221 border. For the PLIN analysis, myosin heavy chain type I was stained alongside either a
222 mouse monoclonal anti-adipophilin (PLIN2; American Research Products, Waltham
223 MA, USA), rabbit polyclonal anti-perilipin 3/TIP-47 (PLIN3; Novus Biologicals,
224 Cambridge, UK) or guinea pig polyclonal anti-OXPAT (PLIN5; Progen Biotechnik,
225 Heidelberg, Germany) primary antibody. In this instance, the secondary antibodies
226 used were Goat Anti-Mouse IgG1 633, goat anti-rabbit IgG 633, or goat anti-guinea pig
227 IgG 633 to visualize PLIN2, PLIN3 and PLIN5, respectively (ThermoFisher Scientific,
228 Paisley, UK). Each PLIN protein was stained for individually.

229

230 *Image capture, image processing and data analysis*

231 Images of cross-sectionally orientated sections, used to investigate fibre type-specific
232 IMTG content and LD morphology, were captured using an inverted confocal
233 microscope (Zeiss LSM710; Carl Zeiss AG, Oberkochen, Germany) with a 63x 1.4 NA
234 oil immersion objective. An argon laser was used to excite the Alexa Fluor 488
235 fluorophore and BODIPY 493/503, a helium-neon laser excited the Alexa Fluor 546 and
236 633 fluorophores, and a diode laser excited the Alexa Fluor 405 fluorophore. To assess

237 fibre-specific IMTG content, fibres that were positively stained for myosin heavy chain
238 type I were classified as type I fibres, while those that were stained positively for
239 myosin heavy chain type IIa were classified as type IIa fibres. ~20 images were
240 captured per time point aiming for an even split across type I and type IIa fibres. All
241 other fibres were assumed to be type IIx fibres, and although some images were
242 captured, in this data set there was an insufficient number of type IIx fibres to perform
243 statistical analysis and therefore the results are not included. Overall ~900 images were
244 analysed, equating to 70-80 images per participant.

245

246 To investigate co-localisation between LD and PLIN proteins the same microscope and
247 magnification were utilised to obtain the digital images, but with a 4x digital zoom
248 applied on the straightest edge of an identified cell (Fig. 4). This first allowed an image
249 to be taken at the peripheral region of the cell and subsequently the field of view was
250 manually moved to the centre of the cell to generate an image of the central region of
251 the cell. There were ~10 peripheral and ~10 central images obtained for each time point
252 per participant, and each PLIN protein was investigated individually meaning there was
253 up to 240 images taken for each participant.

254

255 Image processing was completed using Image-Pro Plus 5.1 software (Media
256 Cybernetics, Rockville, MD, USA). To assess IMTG content, LD morphology and
257 PLIN protein expression on a fibre type-specific basis, the fibre was first separated into
258 a peripheral region to measure subsarcolemmal LD (first 2 μm from the cell border) and
259 the central region to measure intermyofibrillar LD (remainder of the cell). This
260 approach of using a fixed 2 μm distance from the membrane to represent the
261 subsarcolemmal region has been utilised previously to examine IMTG content in
262 differing populations (35). An intensity threshold was uniformly selected to represent a
263 positive signal for IMTG. The content of IMTG was expressed as the positively stained
264 area relative to the total area of the peripheral or central region of each muscle fibre. LD
265 density was expressed as the number of LDs relative to the area of the peripheral or
266 central region. The area of individual LD's was used to calculate mean LD size in each
267 region.

268

269 Because only significant changes in IMTG content were observed in type I fibres (see
270 results), the LD and PLIN co-localisation analysis was only conducted in type I fibres.
271 Co-localisation analysis was performed separately for each PLIN protein with LDs.
272 Briefly, an intensity threshold was uniformly selected to represent a positive signal for
273 IMTG and the PLIN protein of interest. Based on the threshold selected, dual images
274 were generated and subsequently used for co-localisation analysis. The overlapping
275 objects within the images were then extracted creating a separate image of the co-
276 localised areas. This first allowed the identification of the total number of extracted
277 objects, corresponding to the total number of LDs labelled with PLIN2, 3 or 5 protein
278 (PLIN+ LD). Second, the number of extracted objects was subtracted from the total
279 number of LD in order to quantify the number of LD's with no PLIN protein associated
280 (PLIN- LD). Finally, the number of extracted objects was subtracted from the total
281 number of PLIN objects to determine the number of 'free PLIN' objects. The number of
282 objects identified through each of these analyses were expressed relative to the area of
283 interest, thus providing data on changes in the density of PLIN+ LDs, PLIN-LDs and
284 free PLIN. The peripheral region was identified within the appropriate images by
285 creating a 2 μm wide area of interest, meaning that the above analyses were only
286 conducted in this area of the image. Before conducting this analysis, numerous controls
287 were performed to check for bleed through and non-specific secondary antibody binding
288 before co-localisation analysis was conducted, as previously described (29, 30).

289

290 *Statistics*

291 Statistical analyses were performed using SPSS (SPSS; version 23, IBM, USA). Linear
292 mixed modelling was used to examine all dependent variables (IMTG content, LD
293 morphology, PLIN protein expression and co-localisation analysis) at the different time
294 points, with data separated into the two different experimental conditions (CHO and
295 H₂O) in the recovery period. All main effects and interactions were tested using a linear
296 mixed-effects model, with random intercepts to account for repeated measurements
297 within subjects to examine differences between experimental condition, fibre type and
298 subcellular region. Subsequent Bonferroni adjustment post-hoc analysis was used to
299 examine main effects and interactions. Data is presented as mean \pm SEM. Significance
300 was accepted at $P < 0.05$.

301

302 **Results**

303 *Lipid analysis:*

304 *Pre exercise IMTG content and LD morphology*

305 Before exercise, IMTG content was two-fold greater in type I compared to type IIa
306 fibres (main fibre effect; $P < 0.001$, Table 1), and IMTG content was greater in the
307 periphery of the cell (within the 2 μm border) when compared to the central region
308 (main region effect; $P = 0.025$). Overall though, the majority of IMTG was observed in
309 the central compared to the peripheral region of the cell (main region effect; $P < 0.001$,
310 Table 2). Considering the number and size of LD's, there were two-fold greater LD's in
311 type I fibres compared to type IIa fibres ($P = 0.001$). LD's in the central region tended
312 to be 12% larger than in the peripheral region across both fibre types ($P = 0.089$, Table
313 1). Thus, pre-exercise fibre type differences in IMTG content were predominantly
314 explained by differences in LD number, with LD size being similar across fibre types.

315

316 *Effect of exercise on IMTG content and LD morphology*

317 Four hours of steady state moderate-intensity exercise led to a 53% decrease in IMTG
318 content in type I fibres (fibre \times time interaction; $P = 0.002$, Fig. 1a). No significant
319 decrease in IMTG content was observed in type IIa fibres. Moreover, when examining
320 exercise-induced changes in type I fibres on a subcellular-specific basis, IMTG content
321 was reduced by 55% within the central region (time \times region interaction; $P < 0.001$),
322 whereas IMTG content was not altered in the peripheral region ($P = 0.570$).
323 Consequently, the relative distribution of IMTG across the subcellular regions
324 decreased from $\sim 87\%$ before exercise in the central region to $\sim 77\%$ after exercise, with
325 a reciprocal increase in the relative distribution of IMTG within the peripheral region
326 from $\sim 13\%$ before exercise to $\sim 23\%$ after exercise (main time effect; $P = 0.022$, Table
327 2).

328

329 When examining changes in LD morphology in response to exercise, LD number was
330 reduced by 46% in type I fibres only (fibre \times time interaction; $P = 0.043$, Fig. 1b). No
331 changes in LD number occurred in type IIa fibres ($P = 0.474$, Fig. 1b), and no changes

332 in LD size were observed in either fibre type (Fig. 1c). Thus, IMTG utilization during
333 exercise could be entirely explained by a decrease in LD number.

334

335 *Effect of recovery on IMTG content and LD morphology*

336 During recovery from prolonged exercise IMTG content increased significantly in the
337 central region of type I fibres from post-exercise to 4 h post-exercise, and from post-
338 exercise to 24 h post-exercise (time x fibre x region interaction, $P < 0.001$, Fig. 2a).
339 Post-hoc analysis revealed that the increase between 4 h and 24 h post-exercise alone
340 was not significant ($P = 0.160$). No changes in IMTG content occurred in type IIa fibres
341 (Fig. 2b). When comparing CHO and H₂O groups, IMTG content was lower post-
342 exercise in the H₂O condition compared to the CHO condition in both fibre types
343 (condition x time interaction; $P = 0.029$). In the H₂O condition, there was an increase in
344 IMTG content from post-exercise to 4 h post-exercise, and from post-exercise to 24 h
345 post-exercise ($P = 0.014$). In contrast, in the CHO condition IMTG content was not
346 significantly changed from post-exercise to 24 h post-exercise ($P = 1.000$). Importantly
347 though, by 24 h post-exercise IMTG content was statistically similar between
348 conditions ($P > 0.05$). When examining subcellular IMTG distribution during recovery,
349 IMTG in the central region increased from ~77% post-exercise to ~82% 4 h post-
350 exercise, finally returning to pre-exercise distribution by 24 h post-exercise with ~86%
351 of IMTG observed in the central region (main time effect; $P = 0.005$, Table 2). This was
352 mirrored by changes in IMTG distribution in the peripheral region decreasing from
353 ~23% after exercise to ~18% 4 h post-exercise, and finally to ~14% 24 h post-exercise
354 (main time effect; $P = 0.005$, Table 2).

355

356 When considering LD number and size, LD number increased in type I fibres from post-
357 exercise to 4 h post-exercise, and from post-exercise to 24 h post-exercise (time x fibre
358 interaction; $P = 0.028$). More specifically, LD number significantly increased in the
359 H₂O condition from post-exercise to 4 h post-exercise, and from post-exercise to 24 h
360 post-exercise (condition x time interaction; $P = 0.003$, Fig. 2c). No changes in LD
361 number occurred between 4 h post-exercise and 24 h post-exercise. Overall no
362 significant changes were observed in LD size throughout recovery ($P > 0.05$, Fig. 2e &

363 f). Thus, changes in IMTG content through recovery could be explained by increases in
364 LD number, with no differences being observed in LD size.

365

366 *PLIN analysis:*

367 Because significant changes in both IMTG content and LD morphology occurred
368 specifically in type I fibres during exercise and recovery, subsequent PLIN protein
369 content and co-localisation analysis was limited to type I fibres. Importantly, the protein
370 expression of PLIN2, PLIN3 and PLIN5 was unaltered by exercise or recovery in either
371 region in both the CHO and H₂O conditions ($P > 0.05$, Fig 5 & 6). However, there were
372 regional differences in PLIN protein expression, with the central region having greater
373 PLIN content compared to the peripheral region ($P < 0.05$, Table 3, Fig 5 & 6). As well
374 as overall protein content, we examined the co-localisation of PLIN proteins and LD by
375 expressing the number of overlapping objects relative to the total number of PLIN
376 proteins present. Further to this, we examined the number of LD's that either had PLIN
377 (PLIN+ LD), or did not have PLIN associated (PLIN- LD) and also quantified free
378 PLIN (as described previously, 30, 31). The results of these analyses are detailed below.

379

380 *Effect of exercise on PLIN protein and LD co-localisation*

381 Exercise induced a 62% decrease in the fraction of PLIN2 co-localised with IMTG from
382 pre to post-exercise within the central region (time x region interaction; $P < 0.05$, Table
383 4), although post-hoc analysis revealed that there was also a trend for a decrease of 21%
384 within the peripheral region ($P = 0.060$). Exercise reduced the number of PLIN2+ LD in
385 both the peripheral (-27%; $P = 0.006$) and central region (-71%, $P = 0.001$, Fig. 7a).
386 Further to this, the number of PLIN2- LD was also significantly reduced by exercise,
387 which again occurred within both the peripheral (-36%, $P = 0.003$) and central region (-
388 82%, $P < 0.001$, Fig. 7b). Free PLIN2 increased by 36% from pre to post-exercise (Pre
389 exercise 0.024 ± 0.005 , post-exercise 0.034 ± 0.005 , $P = 0.012$).

390

391 When examining PLIN3, exercise caused a significant decrease in the fraction of PLIN3
392 co-localised with LD's within the central region only (-51%, time x region interaction;
393 $P < 0.05$, Table 4). Accordingly, the number of PLIN3+ LD's significantly decreased
394 by 67% from pre to post-exercise (main effect of time; $P < 0.001$, Fig. 7c). The number

395 of PLIN3- LD's were also reduced by exercise, with a decrease of 56% in the central
396 region and 30% in the peripheral region, specific to the CHO condition (main effect of
397 time; $P = 0.004$, Fig. 7d). Free PLIN3 was unaffected by exercise (pre exercise $0.032 \pm$
398 0.004 , post-exercise 0.031 ± 0.006 , $P = 0.699$).

399

400 The fraction of PLIN5 co-localised with LD decreased significantly in response to
401 exercise in the central region only (-58%, time x region interaction; $P < 0.001$, Table 4).

402 The number of PLIN5+ LD's decreased by 38% in response to exercise (main effect of
403 time; $P = 0.007$, Fig. 7e), and there tended to be a decrease in the number of PLIN5-
404 LD's ($P = 0.071$, Fig. 7f). Free PLIN5 increased by 20% from pre to post exercise (pre
405 exercise 0.034 ± 0.004 , post-exercise 0.041 ± 0.006 , $P = 0.021$).

406

407 *Effect of recovery from prolonged exercise on PLIN protein and LD co-localisation*

408 The fraction of PLIN2 co-localised with LD significantly increased throughout
409 recovery, specifically within the central region by 58% from post-exercise to 24 h post-
410 exercise (time x region interaction; $P < 0.001$, Table 5). When considering condition,
411 the increased co-localisation between PLIN2 and LD's occurred primarily in the H₂O
412 condition from post-exercise to 24 h post-exercise (time x condition interaction; $P =$
413 0.001). PLIN2+ LD's increased throughout the recovery period in the central region
414 only from post-exercise to 24 h post exercise (time x region interaction; $P = 0.001$, Fig.
415 8a). Overall the number of PLIN2+ LD's was 63% greater in the peripheral region
416 compared to the central region across all time points (main effect of region; $P < 0.05$).
417 On the other hand, PLIN2- LD's were unchanged during recovery ($P = 0.611$) and did
418 not differ between conditions ($P = 0.940$). Though when considering region, the number
419 of PLIN2- LD were greater in the peripheral region throughout recovery (main effect of
420 region; $P < 0.05$, Fig. 8b). Free PLIN2 was unaffected throughout the recovery period in
421 both conditions (post-exercise 0.032 ± 0.005 , post 4 h 0.025 ± 0.005 , post 24 h $0.026 \pm$
422 0.005 , $P = 0.699$).

423

424 The fraction of PLIN3 co-localised with LD's increased throughout recovery (Table 5)
425 in the central region by 49% from post to 24 h post-exercise (time x region interaction;
426 $P < 0.05$). The number of PLIN3+ LD's increased by 63% from post to 24 h post

427 exercise in the central region ($P = 0.014$), whereas in the peripheral region there was no
428 significant difference from post to 24 h post-exercise ($P = 0.597$, Fig. 8c). In addition,
429 there was a significant difference between regions (main effect of region; $P < 0.05$) with
430 the number of PLIN3+ LDs being ~23% greater in the peripheral region than the central
431 region throughout recovery. Condition had no effect on PLIN3+ LD's during recovery
432 ($P = 0.296$). The number of PLIN3- LD's was significantly different between conditions
433 post-exercise, with the H₂O condition having 68% more PLIN3- LD's than the CHO
434 condition ($P = 0.039$). Overall though, the number of PLIN3- LD did not change during
435 recovery ($P = 0.259$, Fig. 8d). When examining region, the number of PLIN3- LD's was
436 greater in the central region compared to the peripheral region throughout recovery ($P <$
437 0.05). Free PLIN3 was unchanged throughout the recovery period (post-exercise 0.032
438 ± 0.006 , post 4 h 0.034 ± 0.005 , post 24 h 0.033 ± 0.004 , $P = 0.787$).

439

440 The fraction of PLIN5 co-localised with LD's increased significantly in the central
441 region only from post to 24 h post exercise (59%, $P < 0.05$), though was unaffected by
442 condition ($P = 0.167$). There was a significant increase in the number of PLIN5+ LD's
443 in the central region from post to 24 h post-exercise (62%, $P = 0.002$), and in the
444 peripheral region but only from post to 4 h post exercise (20%, $P = 0.016$, Fig. 8e). On
445 the other hand, the number of PLIN5- LD's was unchanged during recovery ($P =$
446 0.780), though PLIN5- LD's were significantly greater in the peripheral region than in
447 the central ($P < 0.05$, Fig. 8f). Free PLIN5 decreases significantly throughout recovery
448 (post-exercise 0.041 ± 0.006 , post 4 h 0.033 ± 0.004 , post 24 h 0.027 ± 0.003 , $P =$
449 0.008).

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459 **Discussion**

460 The present study aimed to investigate the effect of acute CHO restriction on IMTG
461 resynthesis following prolonged exercise, and at the same time explore the dynamic
462 behaviour of LDs and PLIN proteins in order to further clarify the role of these proteins
463 in skeletal muscle. We report for the first time that IMTG resynthesis occurs rapidly in
464 the central region of type I fibres during the first 4 h of recovery following prolonged
465 exercise in highly-trained individuals. With regards to the PLIN proteins, two novel
466 observations were made: 1) during prolonged exercise LD's that had both PLIN
467 associated (PLIN+ LD's) or not associated (PLIN- LD's) were reduced, and 2) during
468 recovery from prolonged exercise only the number of PLIN+ LD's were increased at 24
469 h post-exercise. Given that significant IMTG resynthesis was apparent by 4 h post-
470 exercise, these data together indicate that the PLIN proteins do not play a key role in
471 post-exercise IMTG resynthesis, but are instead re-distributed to the newly-expanded
472 LD pool during recovery.

473

474 In order to investigate post-exercise IMTG resynthesis, we first aimed to reduce IMTG
475 content using 4 h moderate-intensity cycling. As expected, this exercise bout led to a
476 substantial decrease in IMTG content specific to type I fibres, in line with other studies
477 which have also investigated IMTG utilisation using cycling protocols lasting ≥ 3 h (36,
478 34). Moreover, the decrease in IMTG content occurred within the central region of the
479 cell primarily due to a reduction in LD number. This is in line with a recent study
480 employing transmission electron microscopy to demonstrate decreases in LD volume
481 fraction and LD number, but not LD size, in the intermyofibrillar region of muscle
482 fibres in the arms, but not legs, of elite cross-country skiers in response to 1 h of
483 exhaustive exercise (18). This is also in agreement with data showing a 40% decrease in
484 intermyofibrillar lipid content following 1 h of moderate intensity cycling exercise,
485 whilst subsarcolemmal lipid content did not change (6). Our data now extend the
486 observed preferential utilisation of the intermyofibrillar IMTG pool to prolonged
487 cycling, and highlight the capacity for immunofluorescence microscopy-based analysis
488 to detect changes in IMTG content in specific subcellular compartments.

489

490 In the present study, we aimed to identify if restricting CHO in the post-exercise
491 recovery period would augment the rate of IMTG resynthesis. On first inspection, the
492 data revealed that the rate of IMTG resynthesis was greatest when only H₂O, and not
493 CHO, was ingested during the first 4 h of recovery from prolonged exercise. This was
494 expected, since CHO ingestion would increase circulating insulin concentrations
495 thereby inhibit systemic lipolysis and reducing free fatty acid availability to the muscle.
496 However, it is important to state that there was a significant difference in post-exercise
497 IMTG content between conditions, despite the experimental treatment only being
498 implemented in the post-exercise period. Since, in this case, the starting IMTG values
499 are different between groups, this precludes our ability to draw a firm conclusion as to
500 whether acute CHO restriction can truly accelerate IMTG resynthesis. In this regard, it
501 should be noted that in the study by Gejl *et al.*, (10) from which these muscle samples
502 were derived, a small, albeit non-significant, difference in glycogen utilisation was
503 observed in the CHO condition (527 mmol/kg dw, 73% reduction) compared to the H₂O
504 condition (421 mmol/kg dw, 63% reduction). Further to this, Gejl *et al.*, (10) also noted
505 a slightly greater exercise intensity in the CHO condition (74% vs 71% HR_{max} in the
506 H₂O condition), although again this was not a significant difference. We believe that
507 the combination of the small differences in exercise intensity and glycogen utilisation
508 between the groups may explain, at least partly, the lower IMTG utilisation within the
509 CHO condition in the present study. However, despite the differences in IMTG content
510 between conditions at the post-exercise time point, we did observe an increase in IMTG
511 content during the first 4 h of recovery from prolonged exercise independent of
512 experimental group. Importantly, this increase in IMTG content was sustained, but not
513 improved on, at 24 h post-exercise. Furthermore, IMTG content at 24 h post-exercise
514 had returned to baseline levels. Thus together, these data demonstrate that IMTG
515 resynthesis occurs quickly following exercise, at least in highly-trained individuals.
516 Furthermore, this time-course of IMTG resynthesis is the first of its kind to be described
517 in the literature, and importantly provides a dynamic model of IMTG utilisation during
518 exercise and post-exercise resynthesis that can be used to investigate the potential
519 mechanisms underpinning these process.
520

521 When investigating changes in IMTG content during the recovery period in more detail,
522 we observed that the increase in IMTG content occurred specifically in type I fibres and
523 within the central region of the fibre. Therefore, not only are intermyofibrillar LDs
524 targeted for breakdown during exercise, we now report for the first time that this
525 subcellular region is an important site for IMTG resynthesis in the post-exercise period.
526 Corresponding to the exercise-induced decreases in LD number, the post-exercise
527 resynthesis of IMTG was driven by increases in LD number rather than LD size. This
528 could be considered to be an advantage as an increase in LD number would provide a
529 greater LD surface area available for the interaction of lipolytic enzymes and regulatory
530 proteins (i.e. PLIN proteins) with IMTG.

531

532 Both IMTG content and PLIN protein expression exhibit a fibre-specific distribution,
533 and therefore are closely related such that PLIN2, PLIN3 and PLIN5 content is directly
534 associated with IMTG content, at least under resting conditions (1, 22, 23, 30). By
535 employing subcellular-specific analysis, we are now able to demonstrate an apparent
536 uncoupling of this relationship, since IMTG content is greatest in the peripheral region
537 of the fibre, whereas the PLIN proteins are expressed to a greater extent in the central
538 region of the cell. Importantly though, when considering the relative distribution, the
539 majority of IMTG and PLIN proteins are observed in the central region. This would
540 support the hypothesis that the PLIN proteins play a key role in the utilisation and
541 resynthesis of the IMTG pool, given that changes in IMTG content during exercise and
542 recovery were specific to the central region. Critically, we observed changes in IMTG
543 content during exercise that occurred in the absence of changes in PLIN protein
544 expression, which is in line with previous research (29, 30), and we extend this
545 observation to the post-exercise recovery period too. This provided the basis to
546 investigate changes in the LD distribution of each PLIN protein under the dynamic state
547 of exercise and recovery in order to further understand the role of these proteins within
548 skeletal muscle.

549

550 As reported previously, exercise reduced the number of PLIN2+ LDs and PLIN5+ LDs
551 (29, 30), and we now report that the number of PLIN3+ LDs also decreases in response
552 to exercise. However, in contrast to our previous studies demonstrating preferential use

553 of PLIN+ LDs in response to 1 h of exercise (29, 30), we also observed an exercise-
554 induced decrease in the number of PLIN2- and PLIN3- LDs, and PLIN5- LDs also
555 tended to decline. This is likely due to the more prolonged bout of exercise (4 h)
556 employed here than in our previous studies (1 h) (29, 30), combined with the elite level
557 endurance-trained population studied who notoriously exhibit high rates of IMTG
558 utilisation during exercise (34, 26). Given the decrease in PLIN2+ and PLIN5+ LDs
559 during exercise, combined with no change in PLIN2 and PLIN5 protein expression, it
560 was no surprise to observe an increase in the quantity of (free) PLIN2 and PLIN5 not
561 bound to LDs following exercise. In contrast, the quantity of PLIN3 not bound to LDs
562 was unchanged in response to exercise. Studies in cultured non-muscle cells have
563 demonstrated that PLIN3 is recruited from the cytosolic fraction to LDs upon lipid-
564 loading (32, 40, 41), suggesting that PLIN3 cycles between the cytosol and LD pool
565 depending on the metabolic state of the cell. Our data now indicates that this ‘cycling’
566 may be an important function of PLIN3 to support IMTG utilisation during exercise. In
567 our model, we speculate that PLIN3 may cycle from each LD that is used and be
568 recruited to a PLIN3- LD (and possibly PLIN2- and PLIN5- LDs) to subsequently
569 support continued breakdown of the IMTG pool during exercise.

570

571 During recovery, we observed an increase in PLIN and LD co-localisation for all PLIN
572 proteins within the central region of type I fibres at 24 h post-exercise. Consequently,
573 the number of PLIN2+, PLIN3+ and PLIN5+ LDs all increased during recovery, but
574 there was no change in the number of PLIN- LDs. Given that there was no change in
575 the expression of the PLIN proteins during recovery, these data suggest that the pre-
576 existing PLIN protein pool was redistributed across the expanded LD pool during
577 recovery. This corroborates previous studies reporting a redistribution of the PLIN
578 proteins in response to prolonged fasting (11) or a lipid infusion (31). In order to
579 determine the location from which the redistributed PLIN proteins originated, it is
580 important to not only consider LDs either labelled with PLIN or not, but also the
581 cytosolic pool of PLIN proteins. In this regard, when examining the distribution of
582 PLIN2 and PLIN3 throughout recovery increases in PLIN2+ and PLIN3+ LDs occurred
583 in the absence of a change in the quantity of cytosolic PLIN2 or PLIN3. This suggests
584 there is a redistribution of PLIN2 and PLIN3 from pre-existing PLIN2+ or PLIN3+ LD

585 to either newly-synthesised LD and/or pre-existing PLIN- LDs. In contrast, PLIN5+
586 LDs were increased throughout the recovery period with a corresponding decrease in
587 the quantity of cytosolic PLIN5. Therefore, unlike PLIN2 and PLIN3, it is the cytosolic
588 pool of PLIN5 that is redistributed to either newly-synthesised LDs and/or pre-existing
589 PLIN- LDs occurred during recovery, underpinning the increased fraction of LDs
590 labelled with PLIN5 at 24 h post-exercise.

591

592 Previous studies in cultured cells and rodent models have implicated the PLIN proteins
593 in supporting fatty acid incorporation into, and storage as, IMTG in LDs (3, 4, 17, 19).
594 The preferential increase in PLIN+ LDs observed during recovery would theoretically
595 support this concept. However, by obtaining muscle samples at both 4 h and 24 h post-
596 exercise we are able to report for the first time a separation in the time-course between
597 growth of the IMTG pool (at 4 h post-exercise) and increases in coating of LDs with
598 PLIN proteins (at 24 h post-exercise). This suggests that the PLIN proteins don't
599 necessarily play a role in IMTG storage in LD's per se. Rather, the coverage of newly-
600 synthesised LD with PLIN proteins at 24 h post-exercise may be an adaptive response
601 to regulate mobilisation and oxidation of IMTG-derived free fatty acids depending on
602 metabolic demand. In this respect, there is a large evidence-base generated in a number
603 of cell types supporting a role for the PLIN proteins in restricting lipolysis under basal
604 conditions (21). Both PLIN3 and PLIN5 may also play a role in IMTG oxidation.
605 Under stimulated conditions, PLIN5 overexpression in cultured cells augments
606 triacylglycerol hydrolysis and fat oxidation (19), through recruitment of LDs to the
607 mitochondrial network (38). We also recently reported that hormone-sensitive lipase is
608 targeted to PLIN5+ LDs in response to exercise (39). Whole-body fat oxidation (7) and
609 *ex vivo* palmitate oxidation (7, 8) are both positively associated with PLIN3 expression,
610 and PLIN3 is expressed in the mitochondrial fraction of sedentary and endurance-
611 trained rats (25). Based on our data, we assert that a redistribution of the PLIN proteins
612 in the post-exercise period is an important adaptation to preserve the flexibility of the
613 intramuscular LD pool to respond appropriately to changes in metabolic demand.

614

615 A strength of the present study is the use of validated immunofluorescence microscopy
616 techniques to examine fibre-type specific changes in IMTG content and LD

617 morphology, as well as the associations of PLIN proteins with LDs (29, 30, 31).
618 However, the co-localisation assays only enable examination of the association between
619 LDs and a single PLIN protein. A partial overlap between PLIN2 and PLIN5 has been
620 recorded in rat skeletal muscle (20), and both PLIN2 and PLIN5 can be found on the
621 same LD in human skeletal muscle (12). Thus, it is likely that LD's will have more
622 than one PLIN protein associated with the LD surface, meaning that decreases in PLIN-
623 LD we observed during exercise could actually be labelled with an alternative PLIN
624 protein. Alternatively, the observed decrease in PLIN- LD's could be newly-formed
625 LDs that have insufficient PLIN protein associated with the phospholipid monolayer to
626 surpass the lower detection limit of the microscope. In the same context, objects
627 quantified as free PLIN could also be small LDs which do not exceed the lower limits
628 of detection, although it has been established, at least in cultured cells, that cytosolic
629 pools (i.e. non-LD bound) of PLIN proteins do exist (40). We also acknowledge that
630 future work should determine whether PLIN4 plays a role in IMTG utilisation and/or
631 resynthesis, given that PLIN4 is highly expressed, at least at the mRNA level, in
632 skeletal muscle of healthy individuals (24).

633

634 In conclusion, this study demonstrates that IMTG resynthesis occurs rapidly in the
635 central region of type I fibres following prolonged exercise in highly-trained
636 individuals. Whilst our previous report of LDs labelled with PLIN proteins being
637 preferentially utilised (29, 30) is not substantiated when exercise is >1 h in duration, our
638 data do highlight a novel role of PLIN3 in supporting IMTG utilisation. Moreover,
639 during recovery from prolonged exercise the IMTG pool appears to first be
640 resynthesized, after which PLIN2, PLIN3 and PLIN5 are redistributed to the newly-
641 synthesised LD pool. Given the disparity in the time-course between growth of the
642 IMTG pool and coating of LDs with PLIN proteins, our data do not support a role for
643 the PLIN proteins in mediating IMTG resynthesis.

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649 ***Competing interests:***

650 The authors declare they have no competing interests.

651

652 ***Author contributions:***

653 KDG and NØ: design of original study and data collection. EFPJ and SOS: analysis and
654 interpretation of data. EFPJ, KDG, JAS, NØ and SOS: drafting and revising the
655 manuscript.

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837 **Tables**

838

839 **Table 1.** Pre-exercise IMTG content and LD morphology.

840

	Type I fibres		Type II fibres		<i>P</i> value	
	Peripheral	Central	Peripheral	Central	Fibre type	Region
IMTG content (% area stained)	4.63 ± 1.96*	3.93 ± 1.65*	2.42 ± 1.34	1.94 ± 0.91	0.001	0.025
LD size (µm ²)	0.285 ± 0.049	0.321 ± 0.056	0.269 ± 0.062	0.301 ± 0.063	0.500	0.089
LD number (LD.µm ⁻²)	0.152 ± 0.057*	0.116 ± 0.036*	0.084 ± 0.043	0.061 ± 0.023	0.001	0.260

841

842 IMTG content and LD number are expressed relative to the area of the peripheral or

843 central region. Data are means ± S.E.M. * Significantly greater in type I fibres (*P* <

844 0.05).

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861 **Table 2.** Relative distribution of IMTG between subcellular regions in response to
 862 exercise and during recovery.

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		% of IMTG			
		Type I fibres		Type IIa fibres	
		Peripheral	Central*	Peripheral	Central*
Pre		12 ± 1	88 ± 1	14 ± 1	86 ± 1
Post	CHO	20 ± 4 [†]	80 ± 4 [†]	19 ± 2 [†]	81 ± 2 [†]
	Water	23 ± 4 [†]	77 ± 4 [†]	25 ± 8 [†]	75 ± 8 [†]
Post 4 h	CHO	15 ± 2	85 ± 2	16 ± 3	84 ± 3
	Water	22 ± 7	78 ± 7	19 ± 6	81 ± 6
Post 24 h	CHO	13 ± 2	87 ± 2	15 ± 3	85 ± 2
	Water	11 ± 3	89 ± 3	16 ± 4	84 ± 4

864

865 Data are means ± S.E.M. * Significant effect of region across all time points ($P < 0.05$).

866 † Significantly different from all other time-points within the same condition ($P < 0.05$).

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883 **Table 3.** Relative distribution of PLIN proteins between subcellular regions in type I
 884 fibres.

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		% of PLIN					
		PLIN2		PLIN3		PLIN5	
		Peripheral	Central*	Peripheral	Central*	Peripheral	Central*
Pre		13 ± 3	87 ± 9	9 ± 2	91 ± 9	12 ± 1	88 ± 1
Post	CHO	13 ± 3	87 ± 3	10 ± 2	90 ± 2	12 ± 3	88 ± 2
	Water	12 ± 2	88 ± 2	12 ± 3	88 ± 3	25 ± 2	75 ± 2
Post 4 h	CHO	13 ± 2	87 ± 2	11 ± 2	90 ± 1	8 ± 1	92 ± 1
	Water	11 ± 2	75 ± 8	11 ± 2	74 ± 9	19 ± 3	81 ± 4
Post 24 h	CHO	13 ± 2	87 ± 2	11 ± 2	90 ± 2	8 ± 14	92 ± 1
	Water	10 ± 2	78 ± 9	9 ± 2	78 ± 9	19 ± 3	81 ± 3

886

887 Data are means ± S.E.M. * Significant effect of region across all time points ($P < 0.05$).

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905 **Table 4.** Changes in PLIN co-localisation with lipid droplets between subcellular
906 regions in response to exercise in type I fibres.

907

Time Point	Region	PLIN2	PLIN3	PLIN5
Pre	Peripheral	0.61 ± 0.12	0.57 ± 0.06	0.53 ± 0.09
	Central	0.64 ± 0.11	0.53 ± 0.09	0.64 ± 0.10
Post	Peripheral	0.48 ± 0.20	0.51 ± 0.12	0.50 ± 0.17
	Central	0.24 ± 0.15*	0.26 ± 0.12*	0.27 ± 0.09*

908

909 Data are means ± S.E.M. * Significant decreases from pre to post-exercise ($P < 0.05$).

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932 **Table 5.** Changes in PLIN co-localisation with lipid droplets between subcellular
 933 regions during recovery in type I fibres.

934

Time Point	Condition	Region	PLIN2	PLIN3	PLIN5
Post	CHO	Peripheral	0.58 ± 0.22	0.53 ± 0.19	0.42 ± 0.24
		Central	0.31 ± 0.17	0.26 ± 0.09	0.23 ± 0.12
	H ₂ O	Peripheral	0.46 ± 0.08	0.49 ± 0.06	0.53 ± 0.06
		Central	0.22 ± 0.11	0.36 ± 0.07	0.23 ± 0.07
Post 4 h	CHO	Peripheral	0.71 ± 0.13	0.54 ± 0.07	0.47 ± 0.23
		Central	0.48 ± 0.16	0.35 ± 0.08	0.40 ± 0.20
	H ₂ O	Peripheral	0.49 ± 0.22	0.62 ± 0.12	0.54 ± 0.11
		Central	0.33 ± 0.26	0.45 ± 0.24	0.41 ± 0.21
Post 24 h	CHO	Peripheral	0.62 ± 0.17	0.58 ± 0.06	0.48 ± 0.22
		Central	0.57 ± 0.21	0.49 ± 0.16*	0.58 ± 0.25*
	H ₂ O	Peripheral	0.62 ± 0.17	0.56 ± 0.08	0.49 ± 0.06
		Central	0.54 ± 0.21*	0.50 ± 0.11*	0.57 ± 0.21*

935

936 Data are means ± S.E.M. * Significant increases from post-exercise ($P < 0.05$).

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950 **Figure Legends:**

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952 **Figure 1. Fibre type and subcellular-specific changes in IMTG content and LD**
953 **morphology in response to prolonged exercise.**

954 IMTG content (a) LD number (b) and LD size (c) in peripheral and central subcellular
955 regions before (pre) and after (post) exercise in type I and type IIa muscle fibres. IMTG
956 content and LD number in each region was normalized to total cell area. *Significant
957 decreases in IMTG content from pre to post exercise in type I fibres only within the
958 central region ($P < 0.05$). †Significant decreases in LD number from pre to post exercise
959 in type I fibres ($P = 0.043$). Values are means \pm S.E.M.

960

961 **Figure 2. Fibre type and subcellular-specific changes in IMTG content and LD**
962 **morphology during recovery from prolonged exercise.**

963 IMTG content (a, b), LD number (c, d) and LD size (e, f) in peripheral and central
964 subcellular regions during recovery in type I and type IIa fibres. IMTG content and LD
965 number in each region was normalized to total cell area. *IMTG content at post-
966 exercise significantly lower in H₂O vs. CHO ($P = 0.029$). #Significant increase from
967 post-exercise in the H₂O condition only in type 1 fibres ($P < 0.05$). Values are means \pm
968 S.E.M.

969

970 **Figure 3. Representative immunofluorescence images of IMTG in response to and**
971 **in recovery from prolonged exercise.**

972 Sections were co-stained for IMTG (stained using Bodipy 493/503; green), fibre type
973 (not shown), and wheat germ agglutinin Alex Fluor 350 (WGA) in order to identify the
974 cell border (stained blue). Images depict IMTG content in type I fibres at pre and post-
975 exercise, and 4 h and 24 h post-exercise in the H₂O and CHO condition. White bars
976 represent 30 μ m.

977

978 **Figure 4. Representative colocalisation images of IMTG and PLIN5 visualized**
979 **using immunofluorescence microscopy.**

980 Confocal immunofluorescence microscopy images were obtained at 8x digital zoom
981 from the central and peripheral region of each cell, as indicated by the two white boxes

982 (A). IMTG were stained with Bodipy 493/503 (green; B), PLIN5 was stained in red
983 (C), and the subsequent co-localisation map (D). The overlapping area of LD and
984 PLIN5 was extracted (D) and used to calculate the fraction of PLIN5 co-localising with
985 LD, and the number of PLIN5+ and PLIN5- LD. The white dotted line in images B-E
986 represents the 2 μm area that was analysed when images at the peripheral region were
987 obtained. White bars represent 25 μm (A) and 5 μm (B-E). The same co-localisation
988 analysis was repeated for PLIN2 and PLIN3.

989

990 **Figure 5. PLIN protein expression in response to exercise.**

991 No significant changes in overall PLIN2 (a), PLIN3 (b) and PLIN5 (c) content in
992 response to exercise ($P > 0.05$).

993

994 **Figure 6. PLIN protein expression content during recovery.**

995 No significant changes in overall PLIN2 (a), PLIN3 (b) and PLIN5 (c) content during
996 recovery in either experimental condition ($P > 0.05$).

997

998 **Figure 7. Subcellular-specific changes in the number of PLIN+ and PLIN- LDs in**
999 **type I fibres in response to prolonged exercise.**

1000 The effect of exercise on a) PLIN2+ LD, b) PLIN2- LD, c) PLIN3+ LD, d) PLIN3- LD,
1001 e) PLIN5+ LD and f) PLIN5- LD. *Significant decrease in PLIN2+ LD and PLIN2- LD
1002 in both peripheral and central regions (time x region interaction effect, $P < 0.05$).
1003 #Significant decrease in PLIN3+ LD, PLIN3- LD and PLIN5+ LD in response to
1004 exercise (main effect of time, $P < 0.05$). Values are means \pm S.E.M.

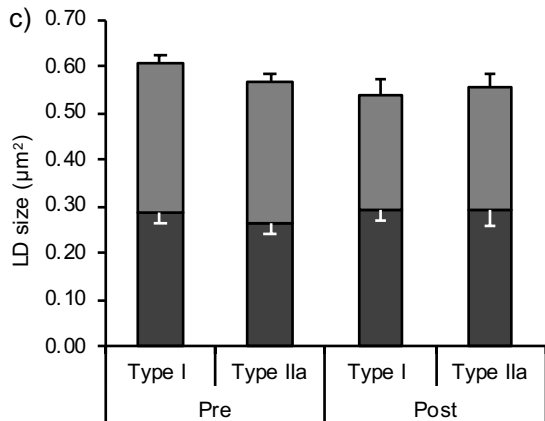
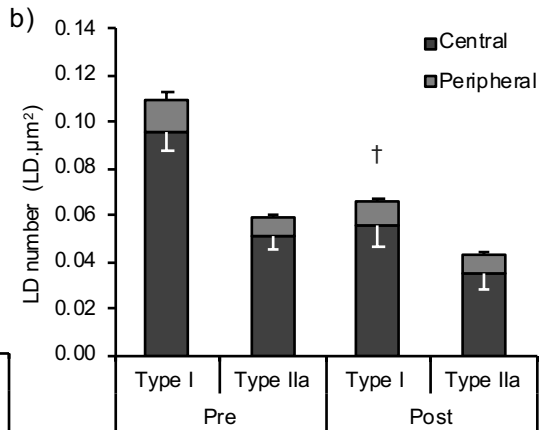
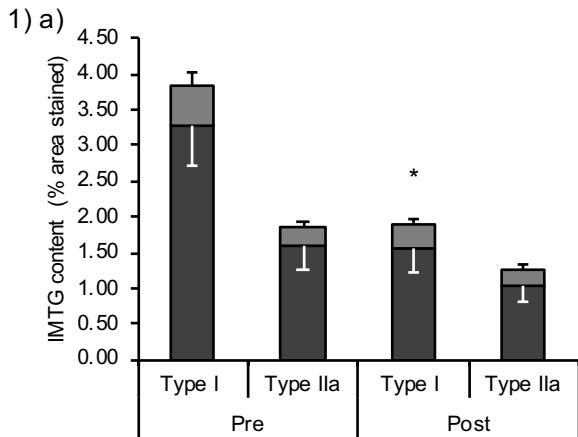
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1006 **Figure 8. Subcellular-specific changes in the number of PLIN+ and PLIN- LDs in**
1007 **type I fibres during recovery from prolonged exercise.**

1008 The effect of recovery on a) PLIN2+ LD, b) PLIN2- LD, c) PLIN3+ LD, d) PLIN3- LD,
1009 e) PLIN5+ LD and f) PLIN5- LD. *Significant increase during recovery from post-
1010 exercise to 24 h post-exercise ($P < 0.05$) with no difference between conditions. Values
1011 are means \pm S.E.M.

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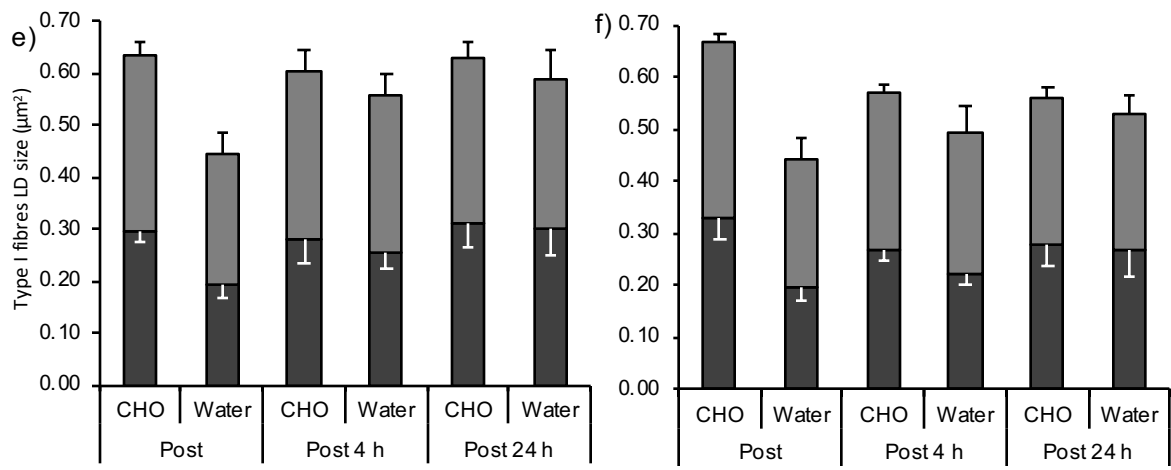
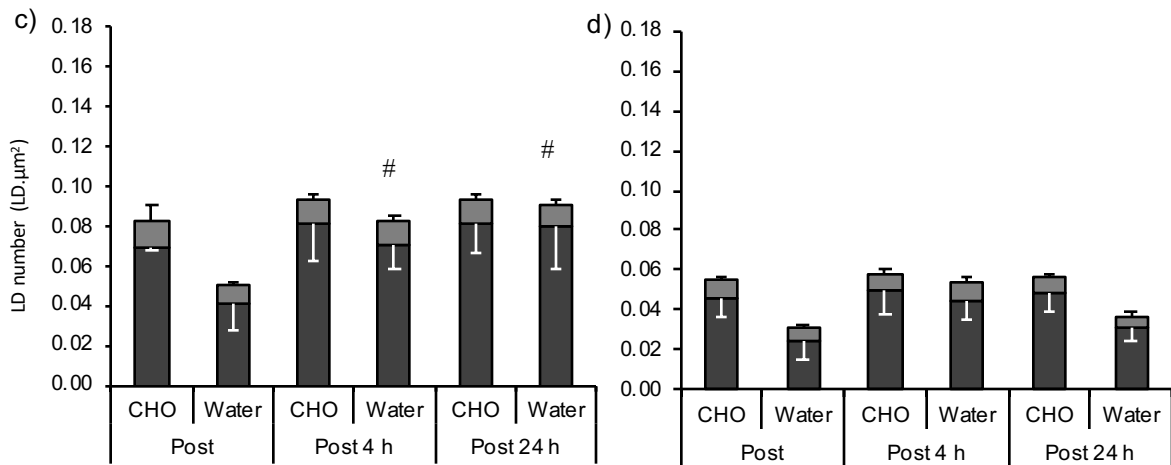
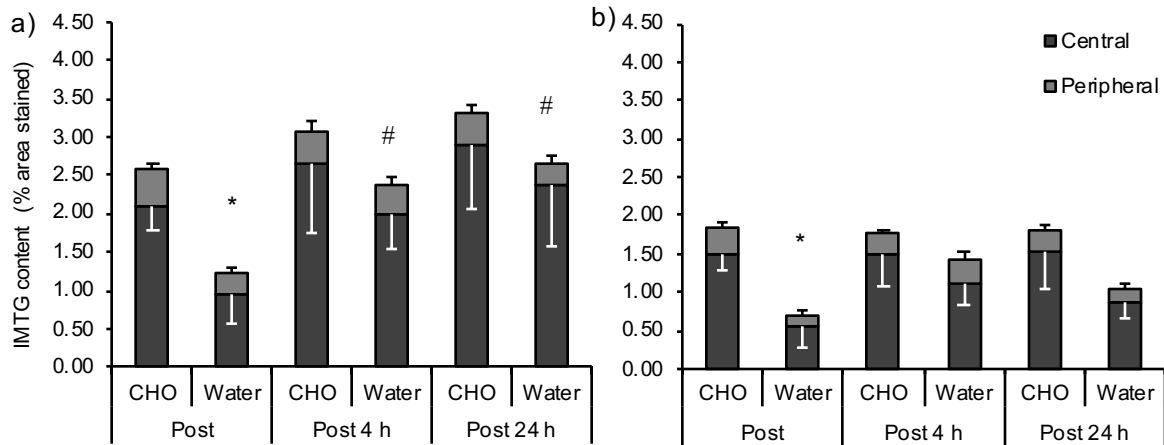
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2)

Type I fibres

Type IIa fibres



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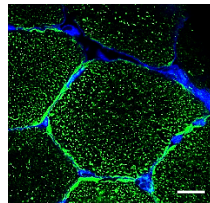
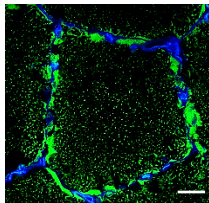
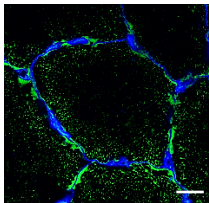
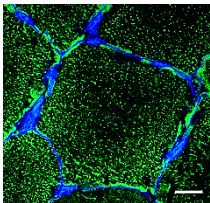
Pre

Post

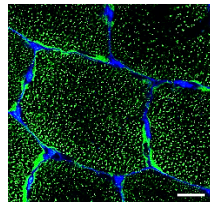
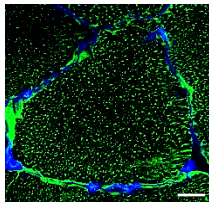
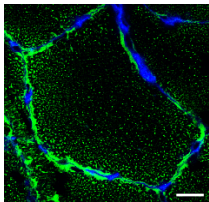
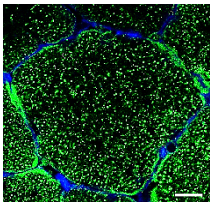
Post 4 h

Post 24 h

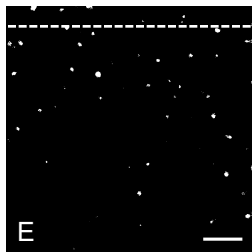
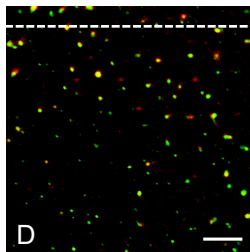
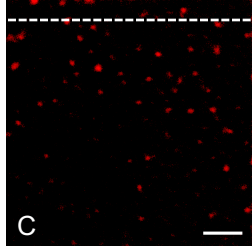
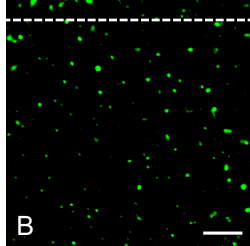
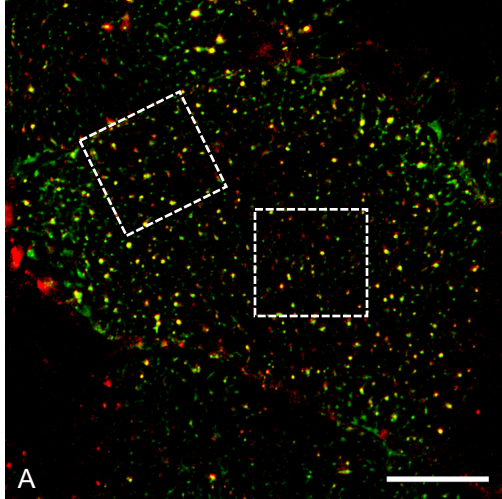
Water

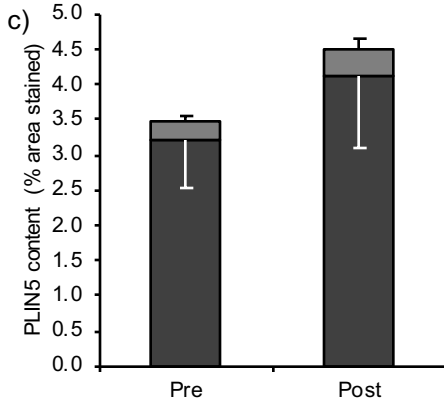
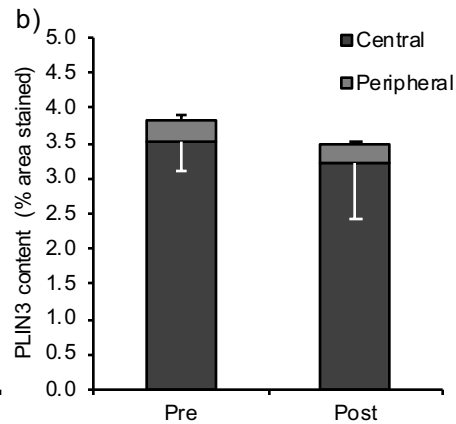
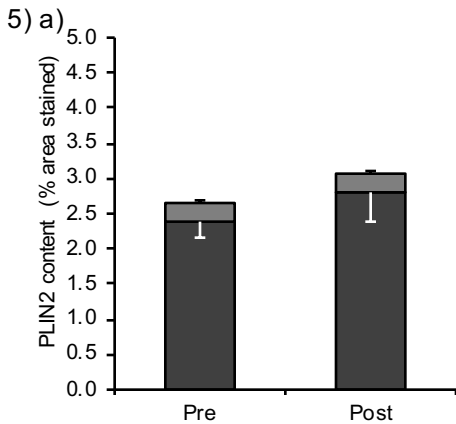


CHO



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