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

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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 H_2O,

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.

Introduction

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

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

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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}, H_2O: 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 H_2O ($n = 7$) 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 H$_2$O 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 H$_2$O 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 H$_2$O 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; Amgros, 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 H_2O) 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 ± SEM. Significance
300 was accepted at $P < 0.05$.

301

Results

Lipid analysis:

Pre exercise IMTG content and LD morphology

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

Effect of exercise on IMTG content and LD morphology

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

When examining changes in LD morphology in response to exercise, LD number was reduced by 46% in type I fibres only (fibre × time interaction; $P = 0.043$, Fig. 1b). No 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 H_2O groups, IMTG content was lower post-

342 exercise in the H_2O condition compared to the CHO condition in both fibre types

343 (condition x time interaction; $P = 0.029$). In the H_2O 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 H_2O 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 H_2O 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 ±

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 H_2O

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 ±

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 H_2O 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 $\pm$ 0.006, post 4 h 0.034 $\pm$ 0.005, post 24 h 0.033 $\pm$ 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$\pm$ 0.006, post 4 h 0.033 $\pm$ 0.004, post 24 h 0.027 $\pm$ 0.003, $P =$

449 0.008).

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Discussion

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

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

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 H_2O, 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 H_2O
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　H_2O 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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837   **Tables**

838

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

840

	Type I fibres		Type II fibres		*P* 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

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

		% 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

Data are means ± S.E.M. * Significant effect of region across all time points ($P < 0.05$).
† Significantly different from all other time-points within the same condition ($P < 0.05$).

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

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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.

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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*

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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.

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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_2O	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_2O	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_2O	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*

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

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Figure Legends:

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

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

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

IMTG content (a, b), LD number (c, d) and LD size (e, f) in peripheral and central
subcellular regions during recovery in type I and type IIa fibres. IMTG content and LD
number in each region was normalized to total cell area. *IMTG content at post-
exercise significantly lower in H_2O vs. CHO ($P = 0.029$). #Significant increase from
post-exercise in the H_2O condition only in type 1 fibres ($P < 0.05$). Values are means ±
S.E.M.

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

Sections were co-stained for IMTG (stained using Bodipy 493/503; green), fibre type
(not shown), and wheat germ agglutinin Alex Fluor 350 (WGA) in order to identify the
cell border (stained blue). Images depict IMTG content in type I fibres at pre and post-
exercise, and 4 h and 24 h post-exercise in the H_2O and CHO condition. White bars
represent 30 µm.

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

Confocal immunofluorescence microscopy images were obtained at 8x digital zoom
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 ± S.E.M.
1005
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 ± S.E.M.
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1) a)

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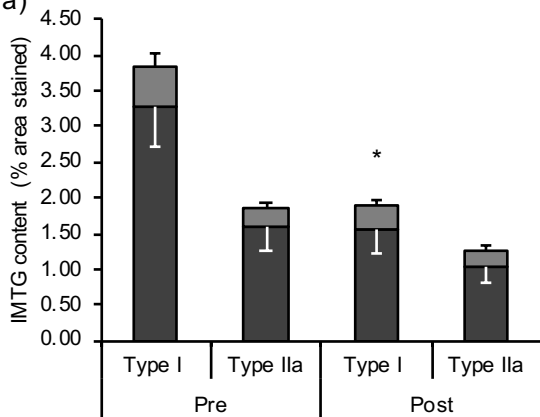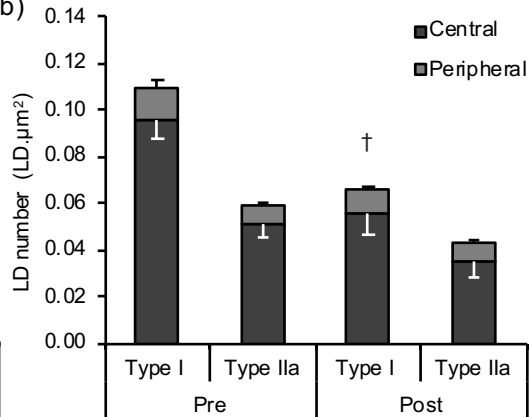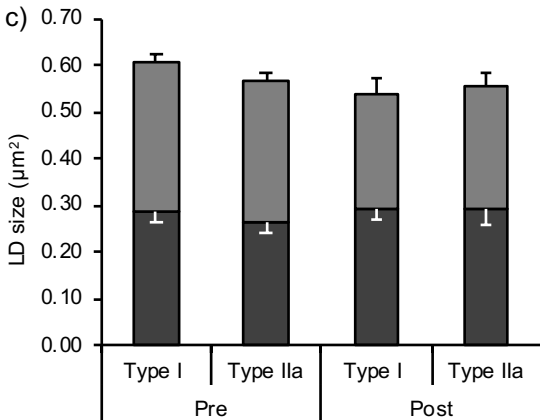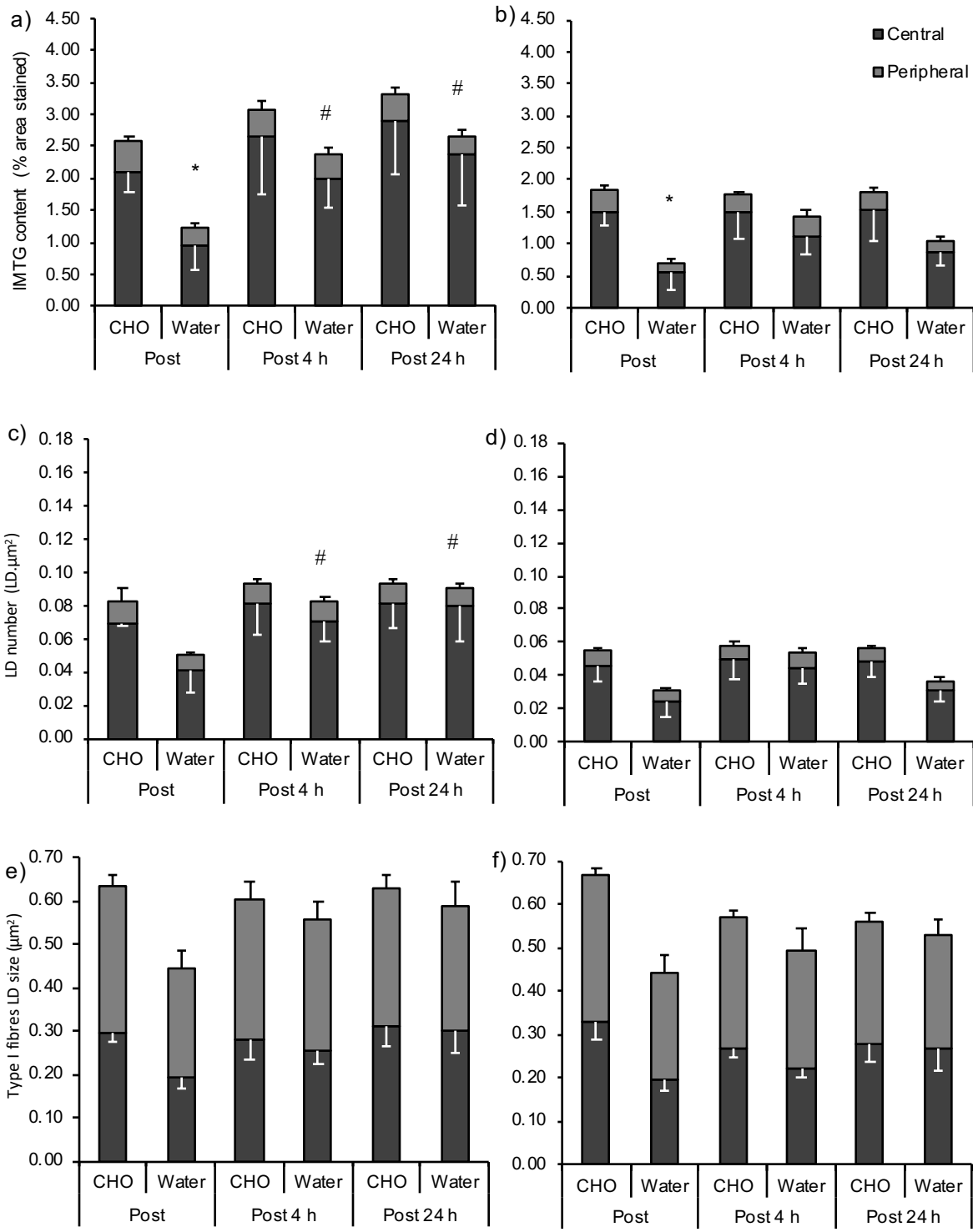
	Pre	Post	Post 4 h	Post 24 h
Water				
CHO				

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