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1 # Training alters the distribution of perilipin proteins in muscle following
2 # acute free fatty acid exposure

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12 **Running head:** Muscle lipid droplet response to lipid infusion

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Key points summary

- The lipid droplet (LD)-associated perilipin (PLIN) proteins promote IMTG storage, but whether the abundance and association of the PLIN proteins with LDs is related to the diverse lipid storage in muscle between trained and sedentary individuals is unknown.
- We show that lipid infusion augments IMTG content in type I fibres of both trained and sedentary individuals. Most importantly, despite there being no change in PLIN protein content, lipid infusion did increase the number of LDs connected with PLIN proteins in trained individuals only.
- We conclude that trained individuals are able to redistribute the pre-existing pool of PLIN proteins to an expanded LD pool during a lipid infusion, and through this adaptation may support storage of fatty acids in IMTG.

Abstract

Because the lipid droplet (LD)-associated perilipin (PLIN) proteins promote intramuscular triglyceride (IMTG) storage, we investigated the hypothesis that differential protein content of PLINs and their distribution with LDs may be linked to the diverse lipid storage in muscle between trained and sedentary individuals. Trained (n=11) and sedentary (n=10) subjects, matched for age, sex and BMI, received either a 6-h lipid or glycerol infusion in the setting of a concurrent hyperinsulinaemic-euglycaemic clamp. Sequential muscle biopsies (0-h, 2-h, 6-h) were analysed using confocal immunofluorescence microscopy for fibre type-specific IMTG content and PLIN associations with LDs. In both groups lipid infusion increased IMTG content in type I fibres (trained: +62%, sedentary: +79%; $P<0.05$), but did not affect PLIN protein content. At baseline, PLIN2 (+65%), PLIN3 (+105%) and PLIN5 (+53%; all $P<0.05$) protein content was higher in trained compared to sedentary individuals. In trained individuals, lipid infusion increased the number of LDs associated with PLIN2 (+27%), PLIN3 (+73%) and PLIN5 (+40%; all $P<0.05$) in type I fibres. In contrast, in sedentary individuals lipid infusion only increased the number of LDs not associated with PLIN proteins. Acute FFA elevation, therefore, induces a redistribution of PLIN proteins to an expanded LD pool in trained individuals only, and this may be part of the mechanism which enables fatty acids to be stored in IMTG.

Keywords

Intramuscular triglyceride, perilipin 2, perilipin 3, perilipin 5, insulin sensitivity

Abbreviations:

Diacylglycerol (DAG)

Free fatty acid (FFA)

Glucose infusion rate (GIR)

Intramuscular triglyceride (IMTG)

Lipid droplet (LD)

Perilipin (PLIN)

111 **Introduction**

112 Large intramuscular triglyceride (IMTG) stores in skeletal muscle of sedentary individuals are strongly
113 associated with insulin resistance and an elevated risk of developing type 2 diabetes (Kelley *et al.*, 1999;
114 Goodpaster *et al.*, 2001). Even larger IMTG stores are observed in endurance-trained athletes but this
115 occurs in the presence of high insulin sensitivity, a phenomenon termed the "athlete's paradox"
116 (Goodpaster *et al.*, 2001; van Loon *et al.*, 2004). Research addressing this concept suggests that lipid
117 metabolites, such as diacylglycerols (DAGs) and ceramides (van Loon & Goodpaster, 2006; Samuel &
118 Shulman, 2012), rather than IMTG per se, are mechanistically linked to insulin resistance in sedentary
119 individuals, due to their ability to directly impair insulin signalling (Itani *et al.*, 2002; Adams *et al.*,
120 2004). Therefore, the metabolic consequences of a lipid overload seems to depend on whether fatty
121 acids taken up by muscle accumulate as DAGs and/or ceramides or are stored as IMTG, as occurs in
122 trained athletes. In support, when trained and sedentary individuals underwent an Intralipid® infusion
123 to acutely raise plasma free fatty acid (FFA) concentrations during a hyperinsulinaemic euglycaemic
124 clamp (Chow *et al.*, 2014), training status modified how the fatty acids were stored in skeletal muscle
125 (Chow *et al.*, 2014). Specifically, fatty acids reflecting the composition of the Intralipid® infusion
126 appeared in IMTG in trained individuals, whereas they accumulated in IMTG and DAGs in sedentary
127 individuals (Chow *et al.*, 2014). The mechanisms by which trained individuals preferentially
128 accumulate IMTG rather than DAGs in the setting of FFA elevation warrants further investigation.

129

130 In skeletal muscle, IMTG are stored within cytosolic lipid droplets (LD) which have over 300 proteins
131 incorporated into their phospholipid monolayer (Zhang *et al.*, 2011), the most abundant of which are
132 the perilipin (PLIN) family of proteins. Much of our knowledge of the PLIN proteins in human skeletal
133 muscle is generated from studies in which muscle samples are obtained from overnight-fasted
134 participants under resting conditions. This approach has revealed that the PLIN protein content is higher
135 in type I compared to type II muscle fibres (Shaw *et al.*, 2009; Shepherd *et al.*, 2013; Pourteymour *et*
136 *al.*, 2015), and that PLIN2 and PLIN5 are observed at both the LD and non-LD locations (Shepherd *et*
137 *al.*, 2012, 2013). Furthermore, exercise training augments protein levels of PLIN2, PLIN3 and PLIN5
138 in skeletal muscle alongside a greater IMTG content (Shaw *et al.*, 2012; Shepherd *et al.*, 2013; Shepherd

139 *et al.*, 2014). This suggests that during exercise training interventions the increase in PLIN protein
140 content is proportional to the increase in IMTG levels. It may also imply that the increase in PLIN
141 protein content plays a mechanistic role in the increased IMTG content in trained individuals. In support,
142 IMTG accumulates in muscle cells overexpressing PLIN3 (Kleinert *et al.*, 2016), and PLIN5
143 overexpression in primary human myotubes promotes IMTG storage by restricting basal lipolytic rates
144 (Laurens *et al.*, 2016). Moreover, myotubes overexpressing PLIN5 exhibit reduced DAG and ceramide
145 accumulation in response to a palmitate overload (Laurens *et al.*, 2016), and similarly when rats are fed
146 a high-fat diet, muscle-specific overexpression of PLIN2 (Bosma *et al.*, 2012a) or PLIN5 (Bosma *et al.*,
147 2012a; Bosma *et al.*, 2013) promotes IMTG storage with no accumulation of DAG. Collectively, these
148 data suggest that the PLIN proteins may play an important role in enabling excess fatty acids to be
149 stored in IMTG.
150

151 Studying the PLIN proteins under 'dynamic' conditions, where IMTG levels are altered independently
152 of changes in PLIN protein content, may provide further insight into their potential roles. In accordance,
153 we have shown that LDs containing either PLIN2 (Shepherd *et al.*, 2012) or PLIN5 (Shepherd *et al.*,
154 2012, 2013) are preferentially used during moderate-intensity exercise, thereby highlighting a potential
155 role for the PLIN proteins in the breakdown and oxidation of IMTG. Gemmink et al. (Gemmink *et al.*,
156 2016) recently reported that prolonged fasting in trained individuals augmented IMTG content and
157 increased the quantity of PLIN5 in contact with LDs, suggesting that the pre-existing PLIN5 pool is
158 redistributed across the LD pool when it expands. Furthermore, only an increase in the number and
159 size of LDs that contained PLIN5 occurred (Gemmink *et al.*, 2016), suggesting a role for PLIN5 in
160 mediating IMTG storage. This could be one mechanism for IMTG storage in muscle, which could be
161 modified by endurance training. It is yet to be investigated if a similar redistribution of other PLIN
162 proteins occurs under conditions of elevated FFA exposure during a hyperinsulinaemic euglycaemic
163 clamp.
164

165 In order to further elucidate the roles of the PLIN proteins in skeletal muscle, the present study aimed
166 to determine the effect of endurance training, compared to a sedentary condition, on the dynamic

167 behaviour of LDs and PLIN proteins during acute, moderate FFA elevation concurrent to a
168 hyperinsulinaemic-euglycaemic clamp. Specifically, we used our previously described method
169 (Shepherd *et al.*, 2012, 2013) to identify changes in LDs that either contained (PLIN+ LDs) or were
170 devoid of PLIN (PLIN- LDs) during the lipid infusion. Lipid accumulation in muscle is not uniform
171 across fibre types and therefore all analyses were performed on a fibre-type specific basis. Importantly,
172 the lipid infusion was undertaken in the setting of hyperinsulinaemia to maximise skeletal muscle fatty
173 acid uptake (Dyck *et al.*, 2001; Chabowski *et al.*, 2004), suppress lipase activity (Holm *et al.*, 2000) and
174 drive TAG synthesis (Muoio *et al.*, 1999; Dyck *et al.*, 2001). Therefore, we investigated the hypothesis
175 that in response to simultaneous infusion of Intralipid® and insulin, the increase in IMTG storage in
176 trained individuals would be accompanied by a redistribution of the cellular pool of PLIN proteins
177 across the expanded LD pool.
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195 **Materials and Methods**

196 ***Participants and ethical approval***

197 The samples used in this study were collected as part of a previous study (Chow *et al.*, 2012; Chow *et*
198 *al.*, 2014) and therefore a portion of the demographic data in Table 1 has been previously presented
199 (Chow *et al.*, 2012; Chow *et al.*, 2014). In this study, twenty one young, healthy, lean participants who
200 were either trained (n=11) or sedentary (n=10) (see Table 1 for characteristics) were included and
201 matched for sex, age (±5 yr) and BMI (±1.5 kg.m^2). The International Physical Activity Questionnaire
202 was used to classify individuals as sedentary (30 minutes or less of exercise per week) or trained (history
203 of aerobic training, preferably running, at ≥45 min/day, ≥5 days/wk) (Craig *et al.*, 2003). The study
204 protocol adhered to the Declaration of Helsinki and was approved by the University of Minnesota
205 Institutional Review Board and written, informed consent was obtained from all participants.

206

207 ***Experimental protocol***

208 The protocol for the study has been described in detail previously (Chow *et al.*, 2012; Chow *et al.*,
209 2014). Briefly, after undergoing assessments of aerobic exercise capacity (VO_{2max}), body composition
210 (dual energy X-ray absorptiometry) and insulin sensitivity (3 h hyperinsulinaemic euglycaemic clamp),
211 participants attended the Masonic Clinical Research Unit at the University of Minnesota on a separate
212 day and consumed a standardised evening meal (41% carbohydrate, 32% fat, 27% protein) and
213 remained on bed rest at the unit until study completion the following day. Following an overnight fast,
214 a muscle biopsy (Bx1) was obtained from the *vastus lateralis* under local anaesthesia, after which
215 participants underwent 6 h infusion of either lipid (20% Intralipid® at 90 ml.h^{-1}) or glycerol (2.25 g.100
216 ml^{-1} at 90 ml.h^{-1}). The glycerol infusion matched the glycerol content of the lipid infusion. Participants
217 received either a lipid or glycerol infusion to maintain matching between activity groups. Simultaneous
218 to the lipid or glycerol infusion, a 6 h hyperinsulinaemic euglycaemic clamp was initiated (1.5 mIU.kg
219 free fat $mass^{-1}$.min^{-1}, KPO_4 at 50 ml.h^{-1}, dextrose titrated to maintain glucose at 4.7-5.3 mmol.l^{-1}). After
220 starting the lipid or glycerol infusion, a second muscle biopsy (Bx2; 120 min) was obtained from a
221 proximal incision of the same leg, with a third biopsy (Bx3; 360 min) being obtained from the
222 contralateral leg. Each muscle biopsy was dissected free of fat and connective tissue before being

223 embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe) and frozen in liquid nitrogen-cooled
224 isopentane for immunohistochemical analyses.
225
226 ***Muscle analysis***
227 *Immunohistochemistry*
228 Serial 5 µm cryosections were cut at -30°C and transferred on to ethanol-cleaned glass slides, fixed in
229 3.7% formaldehyde and permeabilized for 5 min in 0.5% Triton-X 100, followed by washing in
230 phosphate-buffered saline (PBS) and then incubated for 1 h with primary antibodies targeting either
231 PLIN2, PLIN3, PLIN4 or PLIN5 (see antibody section for details) in combination with a myosin
232 antibody for slow-twitch fibres. After washing with PBS, sections were subsequently incubated with
233 appropriate Alexa Fluor secondary antibodies for 30 min, washed in PBS again, and then incubated
234 with BODIPY 493/503 (Invitrogen, Paisley, UK) in order to image and quantify IMTG. Cover slips
235 were mounted with Vectashield mounting medium (H-1000, Vector Laboratories, Burlingame, CA,
236 USA) and sealed with nail varnish. Fibre type-specific protein expression of PLIN2, PLIN3, PLIN4
237 and PLIN5 was assessed using the same protocol but with the omission of BODIPY 493/503 from the
238 procedure, as previously described (Shepherd *et al.*, 2012, 2013).
239
240 *Antibodies*
241 The following primary antibodies were used: guinea pig polyclonal anti-OXPAT (PLIN5) and guinea
242 pig polyclonal anti-S3-12 (PLIN4: both Progen Biotechnik, Germany), mouse monoclonal anti-
243 adipophilin (PLIN2: American Research Products, MA, USA), rabbit polyclonal anti perilipin 3/TIP-
244 47 (PLIN3: Novus Biologicals, Cambridge, UK), mouse anti-myosin antibody for slow twitch fibres
245 (A4.840-c, DSHB, developed by Dr. Blau), wheat germ agglutinin Alexa Fluor 350 conjugate
246 (Invitrogen, Paisley, UK). Appropriate Alexa Fluor secondary antibodies were obtained from
247 Invitrogen (Paisley, UK).
248
249 *Image capture, processing and data analysis*

250 Images of cross-sectionally orientated sections were used for determining fibre type-specific differences
251 in the protein expression of IMTG, PLIN2, PLIN3, PLIN4, and PLIN5. Images were captured using
252 an inverted confocal microscope (Zeiss LSM710, Carl Zeiss AG, Oberkochen, Germany) with a 40x
253 0.75 NA oil immersion objective. An argon laser was used to excite the Alexa Fluor 488 fluorophore
254 and BODIPY, whilst a helium-neon laser excited the Alexa Fluor 546 and 633 fluorophores. Images to
255 assess LD characteristics and co-localisation with either PLIN2, PLIN3, PLIN4 or PLIN5 were captured
256 with the same system but using an 8x digital zoom. When assessing fibre-specific content of IMTG
257 and individual PLIN proteins, fibres stained positively for myosin heavy chain type I were classified as
258 type I fibres, whereas those with no staining were classified as type II fibres.
259
260 Image processing was undertaken using Image-Pro Plus 5.1 Software (Media Cybernetics, MD, USA).
261 To assess fibre type distribution of IMTG and each PLIN protein, between 8 and 12 images were used
262 per muscle section, resulting in a similar proportion of fibres being analysed for each trained (97±10
263 type I fibres, 118±15 type II fibres) and sedentary participant (105±17 type I fibres, 125±17 type II
264 fibres). An intensity threshold was uniformly selected to represent a positive signal for IMTG and each
265 PLIN protein. The content of IMTG and each PLIN protein was expressed as the positively stained
266 area fraction relative to the total area of each muscle fibre. Images captured at 8x digital zoom were
267 used to identify changes in LD density (number of LDs expressed relative to area) and LD size (mean
268 area of individual LDs).
269
270 Co-localisation analysis was performed separately for each PLIN protein with IMTG, as described
271 previously for PLIN2 and PLIN5 (Shepherd *et al.*, 2013). Briefly, a positive signal for the PLIN protein
272 of interest and IMTG in sequential images was obtained by selecting a uniform intensity threshold (Fig.
273 1A-C). Based on the selected threshold, binary images were created and subsequently used for co-
274 localisation analysis. A co-localisation map displaying the merged images was generated (Fig. 1D),
275 with the overlapping regions subsequently extracted to a separate image (Fig. 1E). First, the total
276 number of extracted objects in this image as a proportion of the total number of PLIN2 objects (or

277 PLIN3, PLIN4 or PLIN5 objects, depending on the analysis performed) was used as a measure of co-
278 localisation. The number of extracted objects was expressed relative to area and therefore represents
279 the density of PLIN-associated LDs (PLIN+ LDs). In addition, the number of extracted objects was
280 subtracted from the total number of LDs, and expressed relative to area, to quantify the density of LDs
281 not associated with PLIN (PLIN- LDs). Finally, the number of extracted objects was subtracted from
282 the total number of PLIN objects (an expressed relative to area) to determine the density of "free PLIN".
283 The PLIN proteins do not always form a continuous structure around the LD, and are therefore observed
284 as several discrete structures on the LD. Consequently, it is possible that two or more extracted objects
285 are derived from the same LD leading to an overestimation of the PLIN+ LDs. This possibility was
286 accounted for during the colocalisation analysis. As previously described (Shepherd *et al.*, 2012, 2013),
287 several controls were performed to check for bleed through, non-specific secondary antibody binding
288 and autofluorescence before co-localisation analysis was performed.
289

290 ***Statistical analysis***

291 All baseline data are reported as means ± S.E.M. A two-tailed t-test was used to determine significant
292 differences at baseline between groups. Multiple group comparisons of LD, PLIN protein expression
293 and LD-PLIN co-localisation variables were performed as follows: 1) trained lipid vs. trained glycerol,
294 2) sedentary lipid vs. sedentary glycerol, 3) trained lipid vs. sedentary lipid, and 4) trained glycerol vs.
295 sedentary glycerol. Linear mixed effects models, with random intercepts to account for repeated
296 measurements within subjects, were used to examine these group differences, as well as differences
297 over the infusion (across biopsies) and between fibre types. LD, PLIN protein expression and LD-PLIN
298 co-localisation variables are reported as least square means ± S.E.M. Pairwise differences between
299 biopsies were performed using post hoc tests. Significance was set at the 0.05 level of confidence. All
300 analyses were conducted with SAS (version 9.2; SAS Institute, Cary, NC).
301
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304

305 **Results**

306 ***Baseline characteristics***

307 As expected due to matching, there was no differences between sedentary and trained participants for
308 age and BMI (Table 1). Trained participants displayed lower body fat, and higher free-fat mass, VO_{2max}
309 and glucose infusion rate (GIR) compared to sedentary participants (Table 1).

310

311 At baseline, all PLIN proteins displayed a fibre type-specific distribution, such that PLIN protein
312 content (expressed as % area stained; Fig. 2) was significantly greater in type I fibres compared with
313 type II fibres, irrespective of training status ($P<0.05$). Compared to sedentary participants, trained
314 participants had higher PLIN2, PLIN3, and PLIN5 protein content ($P<0.05$) in both type I and type II
315 fibres (Fig. 2). A trend for greater PLIN4 protein content in type I fibres of trained participants ($P=0.06$;
316 Fig. 2C) compared to sedentary participants was also observed, whereas no differences between groups
317 was evident for PLIN4 content in type II fibres. IMTG content was significantly greater in type I fibres
318 compared with type II fibres in both trained and sedentary participants ($P<0.001$), but overall IMTG
319 content was similar between groups (Fig. 3A).

320

321 ***Lipid droplet responses to lipid infusion***

322 During the hyperinsulinaemic euglycaemic clamp, lipid infusion elevated FFA concentrations, although
323 this occurred to a greater degree in sedentary (932±105 $\mu mol.L^{-1}$) compared to trained participants
324 (600±86 $\mu mol.L^{-1}$; $P=0.03$, Table 1). The glycerol infusion combined with the hyperinsulinaemic
325 euglycaemic clamp led to comparable suppression of FFA concentrations between groups ($P=0.91$).
326 Lipid infusion significantly increased IMTG content in type I fibres of both trained (+62%; $P=0.001$
327 for Bx3 vs. Bx1) and sedentary participants (+79%; $P=0.02$ for Bx3 vs. Bx1), with no differences
328 between groups (Fig. 3A). In trained participants, the greater IMTG content following lipid infusion
329 was attributed to an increase in both LD density (+97%; $P=0.01$ for Bx3 vs. Bx1) and LD size (+22%;
330 $P=0.03$ for Bx3 vs. Bx1), whereas in type I fibres of sedentary participant's lipid infusion only led to
331 an increase in LD density (+64%; $P=0.03$ for Bx3 vs. Bx1; Fig. 3B & C). Interestingly, lipid infusion
332 also increased LD size in type II fibres of trained participants (+64%; $P=0.03$ for Bx3 vs. Bx1; Fig. 3C),

333 but this did not result in a significant overall increase in IMTG content in type II fibres. Furthermore,
334 IMTG content was not elevated in type II fibres of sedentary participants following lipid infusion.
335
336 ***Lipid droplet and PLIN protein co-localisation***
337 Lipid infusion had no effect on the protein content of any of the PLINs in either trained or sedentary
338 participants (P>0.05; Fig. 2). We next investigated the association between LD and each PLIN protein
339 by expressing the number of overlapping objects relative to the total number of PLIN protein objects
340 (Fig. 1). Further, we also determined the number of LDs that either contained (PLIN+ LD) or were
341 devoid of each PLIN protein (PLIN- LD) in response to the combined lipid and insulin infusion. The
342 results of these analyses are detailed below.
343
344 *PLIN2:* At baseline, the fraction of PLIN2 co-localised with IMTG was similar between the groups in
345 type I fibres (Trained: 0.65±0.02, Sedentary 0.68±0.02), and lipid infusion did not change this
346 relationship. However, lipid infusion did lead to an increase in the number of PLIN2+ LDs in trained
347 participants (+27%; P=0.01 for Bx3 vs. Bx1; Fig. 4A) but no changes occurred in the sedentary group.
348 In contrast, the number of PLIN2- LDs was elevated by lipid infusion by a similar degree in both groups
349 (Trained: +69%, Sedentary: +69%; P<0.05 for Bx3 vs. Bx1 for both groups; Fig. 4B). In type II fibres,
350 the fraction of PLIN2 co-localised with IMTG was similar between the groups at baseline (Trained:
351 0.59±0.03, Sedentary 0.65±0.04). Lipid infusion increased PLIN2 co-localisation with IMTG from
352 baseline (0.59±0.03) to post-infusion (0.71±0.03) in type II fibres in trained participants only (+21%;
353 P=0.001 for Bx3 vs. Bx1). This was accounted for by a 33% significant decrease in free PLIN2 in
354 response to lipid infusion (0.006±0.002 vs. 0.005±0.001 PLIN2 objects.μm^2 for Bx1 and Bx3,
355 respectively; P=0.004).
356
357 *PLIN3:* There was no difference between trained and sedentary groups when comparing the fraction of
358 PLIN3 co-localised with IMTG in both type I (Trained: 0.67±0.04, Sedentary 0.64±0.04) and type II
359 fibres (Trained: 0.62±0.05, Sedentary: 0.61±0.05; P>0.05), and this relationship was unchanged by lipid

360 infusion. However, lipid infusion did lead to an increase in the number of PLIN3+ LDs in type I fibres
361 of trained participants (+73%; *P*=0.004 for Bx3 vs. Bx1; Fig. 5A), whereas no changes occurred in the
362 sedentary group. In contrast, in sedentary participants lipid infusion augmented the number of PLIN3-
363 LDs (+133%; *P*<0.001 for Bx3 vs. Bx1; Fig. 5B) in type I fibres, with no changes in the trained group.
364 Furthermore, no changes in the number of PLIN3+ or PLIN3- LDs occurred in type II fibres in either
365 group in response to lipid infusion.
366
367 *PLIN4:* A similar fraction of PLIN4 co-localised with IMTG when comparing trained and sedentary
368 groups at baseline in both type I (Trained: 0.74±0.05, Sedentary 0.70±0.06) and type II fibres (Trained:
369 0.66±0.06, Sedentary 0.66±0.07; *P*>0.05). Lipid infusion had no effect on this relationship, and no
370 increase in PLIN4+ LDs was observed in either group (Fig. 6A & C). However, the number of PLIN4-
371 LDs was elevated in type I fibres following lipid infusion in both trained (+55%; *P*=0.005 for Bx3 vs.
372 Bx1) and sedentary participants (+94%; *P*=0.02 for Bx3 vs. Bx1; Fig. 6B).
373
374 *PLIN5:* The fraction of PLIN5 co-localised with IMTG was similar between trained and sedentary
375 groups at baseline in both type I (Trained: 0.64±0.04, Sedentary 0.58±0.04; *P*>0.05) and type II fibres
376 (Trained: 0.63±0.03, Sedentary 0.61±0.04; *P*>0.05). Lipid infusion led to an increase in PLIN5 co-
377 localised with IMTG from baseline (0.58±0.04) to post-infusion (0.71±0.03) in type I fibres of sedentary
378 participants only (+21%; *P*=0.013 for Bx3 vs. Bx1). This occurred alongside a trend for a decrease in
379 free PLIN5 in response to lipid infusion (0.011±0.002 vs. 0.008±0.002 for Bx1 and Bx3, respectively;
380 *P*=0.089). Notably, however, the number of PLIN5+ LDs was only augmented by lipid infusion in type
381 I fibres of trained participants (+40; *P*=0.006 for Bx3 vs. Bx1; Fig. 7A), whereas the number of PLIN5-
382 LDs was elevated by lipid infusion only in type I fibres of sedentary participants (+123%; *P*=0.03 for
383 Bx3 vs. Bx1; Fig. 7B).
384
385
386

387 **Discussion**

388 This study examined the effect of training on the muscle LD pool and the association of the PLIN
389 proteins with LDs in response to acute FFA elevation (through infusion of Intralipid®) alongside a
390 hyperinsulinaemic-euglycaemic clamp. The major novel observations are that acutely elevating FFA
391 concentrations alongside hyperinsulinaemia: 1) augments IMTG content in type I fibres of both trained
392 and sedentary individuals, but that increases in the number and/or size of LDs are dependent on training
393 status, 2) increased the number of LDs associated with PLIN2, PLIN3 and PLIN5 in trained individuals,
394 and 3) led to the accumulation of LDs that were not associated with any of the PLIN proteins in
395 sedentary individuals. Together, and in line with our hypothesis, these data indicate that the ability to
396 redistribute PLIN proteins to the expanded LD pool under conditions stimulating IMTG synthesis is
397 unique to trained individuals.

398

399 The first novel finding of the present study was that acute FFA elevation and insulin infusion led to an
400 increase in IMTG content that was specific to type I fibres in both trained and sedentary individuals.
401 Previously, IMTG content was not significantly elevated by acute FFA elevation when analysed in
402 whole muscle homogenates (Chow *et al.*, 2014), highlighting the importance of considering fibre type-
403 specific responses when investigating adaptations to the intramuscular lipid pool. Hyperinsulinaemia
404 will increase fatty acid uptake into muscle (Dyck *et al.*, 2001; Chabowski *et al.*, 2004), suppress
405 intramuscular lipase activity (Holm *et al.*, 2000), and enhance fatty acid triacylglycerol esterification
406 (Muoio *et al.*, 1999; Dyck *et al.*, 2001), and when combined with acute FFA elevation it is likely that
407 these conditions underpin the net increase in IMTG content. It is notable that the increase in IMTG
408 content in trained individuals was attributable to both a greater LD number and size, whereas only an
409 increase in LD number could explain the higher IMTG content following acute FFA elevation in
410 sedentary participants. An increase in LD number could be deemed advantageous since this would
411 maintain a high LD surface area to volume ratio, thereby providing a greater surface area available for
412 LD regulatory proteins (such as PLINs) to support fatty acid storage and mobilisation relative to
413 metabolic demand. A combined increase in LD number and size in trained individuals was reported in
414 a recent study using prolonged fasting to physiologically raise plasma FFA concentrations (Gemmink

415 *et al.*, 2016). As well as synthesising new LDs, expanding the size of LDs, may be an additional
416 mechanism by which trained individuals are able to sequester excess plasma FFA into IMTG.
417
418 Under resting conditions, the expression of PLIN2 and PLIN5 is closely related to IMTG content
419 (Minnaard *et al.*, 2009; Amati *et al.*, 2011; Peters *et al.*, 2012; Shepherd *et al.*, 2013), although not all
420 PLIN2 or PLIN5 is associated with the IMTG pool (Shepherd *et al.*, 2012, 2013). The results of the
421 present study now show that this partial co-localisation with IMTG also exists for PLIN3 and PLIN4.
422 In response to acute FFA elevation, IMTG levels were increased independent of changes in PLIN
423 protein content. We therefore investigated whether acute FFA elevation altered the fraction of each
424 PLIN associated with IMTG, and for each PLIN protein we describe two pools of LDs: (1) PLIN-
425 associated LDs (PLIN+ LD), and (2) LDs that do not contain PLIN (PLIN- LDs). The major novel
426 finding of the present study was that in trained individuals, acute FFA elevation led to an increase in
427 the number of PLIN2+, PLIN3+ and PLIN5+ LDs specifically in type I fibres. Furthermore, this
428 occurred in the absence of a change in the fraction of any PLIN protein associated with IMTG.
429 Gemmink et al. (2016) recently showed that in trained individuals elevating plasma FFA concentrations
430 through prolonged fasting also led to a greater number of PLIN5+ LDs, but this was accompanied by
431 an increase in the fraction of PLIN5 associated with IMTG. Since PLIN5 protein expression was
432 unaltered by fasting in this study, the authors concluded that prolonged fasting led to a redistribution of
433 cytosolic PLIN5 to the LD surface (Gemmink *et al.*, 2016). We now provide evidence that in trained
434 individuals PLIN2, PLIN3 and PLIN5 are all redistributed across the expanded LD pool following acute
435 FFA elevation. However, as we did not observe a change in the fraction of any PLIN protein associated
436 with IMTG, it appears that PLIN2, PLIN3 and PLIN5 are redistributed from pre-existing PLIN+ LDs
437 to either newly-synthesised LDs and/or pre-existing PLIN- LDs. As a result, the proportion of the
438 expanded LD pool containing these PLIN proteins was maintained.
439
440 The redistribution of PLIN2, PLIN3 and PLIN5 in trained individuals may be an important adaptation
441 to enable FFA storage as IMTG in LDs. In support, palmitate incorporation into IMTG is reduced when
442 PLIN2 is knocked-down in cultured muscle cells (Bosma *et al.*, 2012a), whereas PLIN3 overexpression

443 in muscle cells increases palmitate incorporation into IMTG (Kleinert *et al.*, 2016). Furthermore,
444 muscle-specific overexpression of PLIN2 (Bosma *et al.*, 2012a) or PLIN5 (Bosma *et al.*, 2013)
445 enhances IMTG storage in rats fed a high-fat diet. PLIN5 overexpression in primary human myotubes
446 also leads to an increase in IMTG content by restricting rates of basal lipolysis (Laurens *et al.*, 2016).
447 In this regard, there is a large body of evidence obtained in a number of different cell types
448 demonstrating that the PLIN proteins support triacylglycerol storage by regulating basal lipolytic rates
449 (reviewed in MacPherson & Peters, 2015). Through this role, PLIN5 (and PLIN2 and PLIN3) may lead
450 to enlargement of LDs, and could therefore explain the observed increase in LD size in trained
451 individuals. PLIN2 also has been observed to cluster at specific locations in the endoplasmic reticulum
452 membrane where LD biogenesis occurs (Robenek *et al.*, 2006). In trained individuals, part of the PLIN2
453 protein pool may therefore be redistributed to the membrane of the endoplasmic reticulum in response
454 to acute FFA elevation to support the synthesis of new LDs. Maintaining the proportion of the LD pool
455 that contains PLIN2, PLIN3 and PLIN5 may also be important to support mobilisation and oxidation
456 of IMTG-derived FAs when metabolic demand increases. We have shown that both PLIN2+ and
457 PLIN5+ LDs (Shepherd *et al.*, 2013) are preferentially targeted for breakdown during exercise. This is
458 in line with studies in cultured cells demonstrating that PLIN5 overexpression enhances triacylglycerol
459 hydrolysis and fat oxidation, possibly by recruiting LDs to the mitochondrial network (Wang *et al.*,
460 2011; Bosma *et al.*, 2012b; Laurens *et al.*, 2016). A positive association is also reported between PLIN3
461 expression and both whole-body fat oxidation (Covington *et al.*, 2014) and *ex vivo* palmitate oxidation
462 (Covington *et al.*, 2014; Covington *et al.*, 2015), and PLIN3 is observed in the mitochondrial fraction
463 of sedentary and endurance-trained rats (Ramos *et al.*, 2015), suggesting that PLIN3 plays a role in
464 IMTG oxidation. Taken together, these data suggest that redistributing PLIN2, PLIN3 and PLIN5 in
465 response to acute FFA elevation would confer a metabolic advantage by maintaining a metabolically
466 flexible intramuscular LD pool.
467
468 In contrast to the trained group, acute FFA elevation led to an increase in the number of PLIN2-, PLIN3-,
469 PLIN4-, and PLIN5- LDs in sedentary individuals, suggesting that no redistribution of these proteins
470 occurred. The protein content of all PLINs was lower in sedentary individuals compared to the trained

471 group, and therefore it is possible that sedentary individuals have a reduced capacity to redistribute
472 PLINs when the muscle LD pool expands. Knockdown of PLIN2 in cultured muscle cells leads to
473 increased palmitate incorporation into DAG (Bosma *et al.*, 2012a), whereas rats fed a high-fat diet
474 accumulated IMTG, with no changes in DAG, but only when PLIN2 (Bosma *et al.*, 2012a) or PLIN5
475 (Bosma *et al.*, 2013) was overexpressed in muscle. PLIN2 and PLIN5 therefore appear to be important
476 in channelling fatty acids into IMTG. The lack of a redistribution and/or lower abundance of these
477 proteins in the present study may explain our previous finding that fatty acids reflecting the composition
478 of an Intralipid® infusion appeared in IMTG and DAGs in sedentary individuals and only IMTG in
479 trained individuals (Chow *et al.*, 2014).
480
481 Although IMTG content was unchanged in type II fibres, LD size did increase in trained individuals
482 following acute FFA elevation. In addition, the fraction of PLIN2 associated with IMTG was increased,
483 and therefore PLIN2 might also be redistributed to LDs in type II fibres, possibly to ensure that the
484 surface coverage of larger LDs is maintained. Although PLIN2 is considered to be predominantly found
485 at the LD surface (Prats *et al.*, 2006; Wolins *et al.*, 2006; Bell *et al.*, 2008), PLIN2 has also been
486 observed at the endoplasmic reticulum (Robenek *et al.*, 2006), and may cycle between the cytosolic and
487 LD fractions (Robenek *et al.*, 2006; Wang *et al.*, 2009). Since there was a significant decrease in PLIN2
488 that was unbound to LDs (free PLIN2) following acute FFA elevation, it is possible therefore that in
489 type II fibres PLIN2 that was not previously associated with LDs became connected to the LD pool.
490 Similarly, in sedentary individuals acute FFA elevation led to an increase in the fraction of PLIN5
491 associated with LDs in type I fibres. Like the aforementioned changes in PLIN2 in type II fibres of
492 trained individuals, it is possible that PLIN5 that was previously not associated with LDs was recruited
493 to the LD pool in type I fibres of sedentary individuals, especially as there was a tendency for PLIN5
494 that was unbound to LDs to decrease. However, because in sedentary individuals only an increase in
495 the number of PLIN5- LDs was observed, it is likely that previously unbound PLIN5 was recruited to
496 LDs already coated with PLIN5.
497

498 Little is known about the role of PLIN4 in skeletal muscle, although we report for the first time here
499 that PLIN4 expression is ~2-fold greater in type I compared to type II fibres, and that trained individuals
500 exhibit higher protein expression of both PLIN4 compared to sedentary individuals. These observations
501 are in accordance with the fibre type distribution of the other PLINs in skeletal muscle, and findings of
502 greater PLIN2, PLIN3 and PLIN5 expression in muscle in response to chronic training (Peters *et al.*,
503 2012; Shaw *et al.*, 2012; Louche *et al.*, 2013; Shepherd *et al.*, 2013; Shepherd *et al.*, 2014). Despite the
504 higher PLIN4 expression in trained individuals, we only observed an increase in PLIN4- LDs
505 suggesting that no redistribution of PLIN4 occurred in response to acute FFA elevation. Future studies
506 will determine the precise role of PLIN4 in human skeletal muscle.
507
508 The use of validated immunofluorescence microscopy techniques (Shepherd *et al.*, 2012, 2013) to
509 examine fibre type-specific changes in LD characteristics and the associations of PLIN proteins with
510 LDs in response to acute FFA elevation is a clear strength of this study. Applying these techniques to
511 samples obtained under 'dynamic' conditions has provided further insight into the potential role of the
512 PLIN proteins in muscle. Our colocalisation assays, however, only permit fibre-specific analysis of the
513 association between LDs and a single PLIN protein. This is important to acknowledge because acute
514 FFA elevation increased PLIN4- LDs in trained and sedentary individuals, and increased both PLIN2+
515 and PLIN2- LDs in trained individuals. One possibility is that PLIN- LDs are actually newly-formed
516 LDs that have not yet acquired sufficient PLIN protein to exceed the lower detection limit of the
517 microscope. It is also possible that PLIN4- LDs and PLIN2- LDs were in fact coated with PLIN3 and/or
518 PLIN5. Co-localisation analysis of PLIN2 and PLIN5 in rat skeletal muscle demonstrated only a partial
519 overlap between the two proteins (Macpherson *et al.*, 2012), suggesting that some, but not all LDs, have
520 both of these proteins associated with them. Whether there are distinct pools of LDs in skeletal muscle
521 that have all, some, or none of the PLIN proteins associated remains to be determined.
522
523 Although exercise capacity was significantly different between the trained and sedentary participants,
524 the mean VO_{2max} for the trained group was lower than that reported for previously published 'trained'
525 groups (van Loon *et al.*, 2004; Amati *et al.*, 2011), which may explain the lack of difference in baseline

526 IMTG between trained and sedentary participants that we observed compared with previous literature
527 (Goodpaster *et al.*, 2001; Amati *et al.*, 2011). We consider this a strength of the study because it
528 demonstrates that only a small increase in exercise capacity, as can be achieved using exercise training,
529 can improve the ability to redistribute PLIN proteins to LDs during acute FFA elevation and channel
530 fatty acids into IMTG. In addition, since we had matched for age, gender and BMI, the trained
531 participants consistently had a higher $VO_{2\,max}$ than their matched counterparts and therefore our findings
532 were still consistent with a training effect. A further strength of the study is the use of a glycerol infusion
533 as a control, as this approach enabled the specific examination of the effect of acute FFA elevation in
534 trained and sedentary individuals. Previous studies conducted in trained individuals using a saline
535 infusion lacked such control (Matzinger *et al.*, 2002; Schenk *et al.*, 2009).
536
537 In conclusion, this study has generated novel evidence that acute FFA elevation concurrent to a
538 hyperinsulinaemic-euglycaemic clamp does not change PLIN protein content in skeletal muscle, but
539 rather leads to a redistribution of PLIN2, PLIN3 and PLIN5 to an expanded LD pool in trained
540 individuals only. In contrast, no redistribution of PLIN proteins occurs in sedentary individuals. This
541 may be part of the mechanism by which trained individuals are able to channel fatty acids into IMTG.
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Additional information

Competing interests

The authors have no conflicts of interest to disclose.

Author contributions

SOS, QW and LSC: conception and design of the experiments. SOS, JAS, QW, DGM and LSC: collection, analysis and interpretation of data. SOS, JAS, QW, JJD, BG, DGM and LSC: drafting and revising the manuscript. All authors have read an approved the final submission.

Funding

This work was supported by the National Institutes of Health [5K12-RR-023247-02, DK-50456, UL1 TR000135 (Mayo CTSA)], the Minnesota Medical Foundation, the Pennock Family Foundation, the University of Minnesota (CTSA: NIH UL1TR000114), and the Metabolic Studies Core of the Minnesota Obesity Center.

Acknowledgements

The antibody against myosin (human slow twitch fibres, A4.840) used in the study was developed by Dr. Blau and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The authors would like to thank Professor Anton Wagenmakers (Liverpool John Moores University) for critical review and editing of the manuscript drafts.

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767 **Tables**

Table 1. *Baseline characteristics of trained and sedentary participants*

	Trained (n=11)	Sedentary (n=10)	*P* value
Sex (males/females)	6/5	4/6	0.98
Age (y)	23 ± 1	21 ± 1	0.26
BMI ($kg.m^{-2}$)	22.2 ± 0.6	21.3 ± 0.6	0.31
FFM (kg)	50.8 ± 3.7	40.9 ± 2.3	0.04
Body fat (%)	19.9 ± 2.0	27.4 ± 3.5	0.07
VO_{2max} ($ml.kg^{-1}.min^{-1}$)	47.8 ± 2.0	38.0 ± 1.6	<0.01
Baseline GIR (μmol glucose infused.kg $FFM^{-1}.min^{-1}$)	66.1 ± 4.7	48.3 ± 5.7	0.03
FFA at end of 6 hr lipid infusion ($\mu mol.L^{-1}$)	600 ± 86	932 ± 105	0.03

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769 Values are means ± S.E.M. BMI, body mass index; FFM, free fat mass; GIR, glucose infusion rate;

770 FFA, free fatty acids.

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786 **Figure Legends**

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788 **Figure 1.** Representative immunofluorescence images co-stained for IMTG and PLIN5 that were used
789 for colocalisation analysis from a trained participant. Images for colocalisation analysis were obtained
790 at 8x zoom from the central region of a cell (indicated by the white box; A). IMTG were stained with
791 BODIPY 493/503 (green; B), PLIN5 was stained red (C) and the subsequent merged images (D) were
792 used to calculate colocalisation. Image E shows the extracted overlying area and was used to calculate
793 the relative association of PLIN5 with IMTG, and determine the number of PLIN5+ and PLIN5- LDs.
794 Note that PLIN5 is associated with the majority, but not all, LDs. Images were obtained at 8x zoom
795 (white bar = 5 μm), except for *A* (2x zoom; white bar = 25 μm). All images were obtained with the
796 same resolution (1024 x 1024 pixels). The same method was used for colocalisation analysis for PLIN2,
797 PLIN3 and PLIN4.

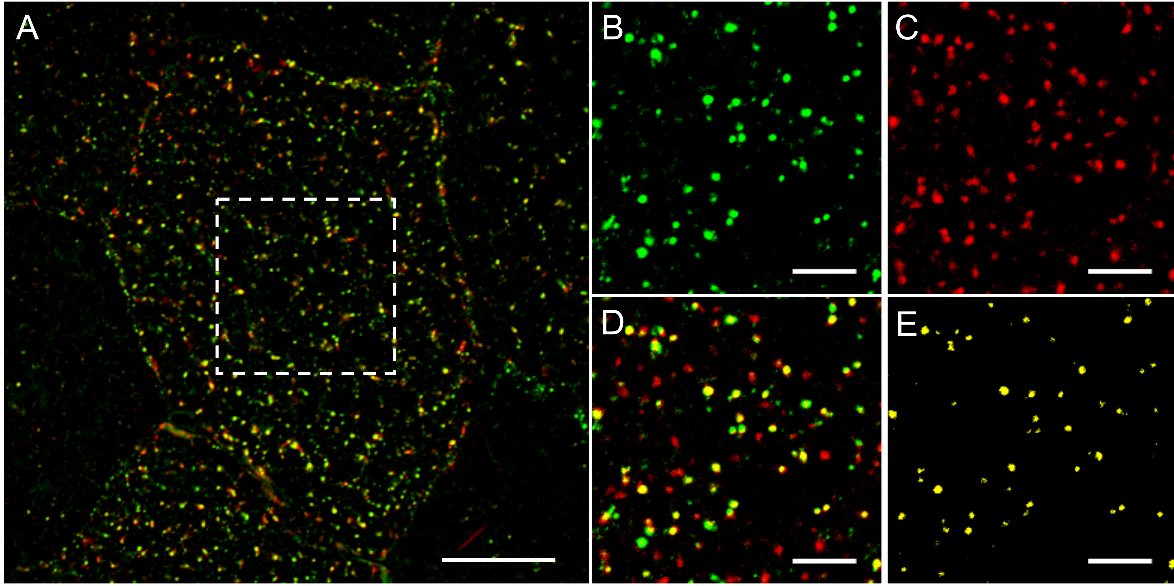
798

799 **Figure 2.** A 6-h Intralipid infusion did not alter fibre-specific PLIN2 (A), PLIN3 (B), PLIN4 (C) and
800 PLIN5 (D) protein expression in trained and sedentary individuals. Fibre type-specific content of
801 PLIN2, PLIN3 (E), PLIN4 (F) and PLIN5 was quantified from immunofluorescence images, where
802 myosin heavy chain I (MHC I) (stained red) was combined with wheat germ agglutinin Alexa Fluor
803 350 (WGA) to identify the cell border (stained blue) in skeletal muscle (G & H). Positively stained
804 fibres (red) are type I fibres, all other fibres are assumed to be type II fibres. White bars represent 50
805 μm. *Significantly different compared to sedentary group ($P<0.05$). †Significantly different than type
806 I fibres ($P<0.001$). There was a trend ($P=0.06$) for a difference in PLIN4 content between trained and
807 sedentary participants in type I fibres only.

808

809 **Figure 3.** A 6-h Intralipid infusion alters fibre-specific IMTG content (A), LD density (B) and LD size
810 (C). Fibre type-specific content of IMTG was quantified from immunofluorescence images of muscle
811 sections obtained at baseline (Bx1; D), and after 120 min (Bx2; E) and 360 min (Bx3; F) of lipid or
812 glycerol infusion. Panels G-I are corresponding images of myosin heavy chain I (MHC I) (stained red)
813 in combination with wheat germ agglutinin Alexa Fluor 350 (WGA) to identify the cell border (stained

814 blue) in skeletal muscle. Positively stained fibres (red) are type I fibres, all other fibres are assumed to
815 be type II fibres. White bars represent 50 µm. *Significantly different from Bx1 given fitness category
816 and infusion status (P<0.05). †Significantly different than type I fibres (P<0.001).
817
818 **Figure 4.** A 6-h lipid infusion alters the number of LDs with PLIN2 associated (PLIN2+ LDs) or not
819 associated (PLIN2- LDs) differently between trained and sedentary individuals. Analysis was
820 performed in type I (A, B) and type II fibres (C, D). *Significantly different from Bx1 given fitness
821 category and infusion status (P<0.05). †Significantly different between glycerol and lipid infusion
822 within fitness category for equivalent biopsies.
823
824 **Figure 5.** A 6-h lipid infusion alters the number of LDs with PLIN3 associated (PLIN3+ LDs) or not
825 associated (PLIN3- LDs) differently between trained and sedentary individuals. Analysis was
826 performed in type I (A, B) and type II fibres (C, D). *Significantly different from Bx1 given fitness
827 category and infusion status (P<0.05). †Significantly different between glycerol and lipid infusion
828 within fitness category for equivalent biopsies.
829
830 **Figure 6.** A 6-h lipid infusion does not alter the number of LDs with PLIN4 associated (PLIN3+ LDs)
831 but does increase the number of LDs without PLIN4 associated (PLIN3- LDs) in trained and sedentary
832 individuals. Analysis was performed in type I (A, B) and type II fibres (C, D). *Significantly different
833 from Bx1 given fitness category and infusion status (P<0.05).
834
835 **Figure 7.** A 6-h lipid infusion alters the number of LDs with PLIN5 associated (PLIN5+ LDs) or not
836 associated (PLIN5- LDs) differently between trained and sedentary individuals. Analysis was
837 performed in type I (A, B) and type II fibres (C, D). *Significantly different from Bx1 given fitness
838 category and infusion status (P<0.05). †Significantly different between glycerol and lipid infusion
839 within fitness category for equivalent biopsies.


A
B
C
D
E

Type I fibres

Type II fibres †

Bx1
Bx2
Bx3

A

PLIN2 content (% area stained)

3 2 1 0

*

Glycerol Lipid Glycerol Lipid

Trained Sedentary

B

PLIN3 content (% area stained)

3 2 1 0

*

Glycerol Lipid Glycerol Lipid

Trained Sedentary

C

PLIN4 content (% area stained)

3 2 1 0

‡

Glycerol Lipid Glycerol Lipid

Trained Sedentary

D

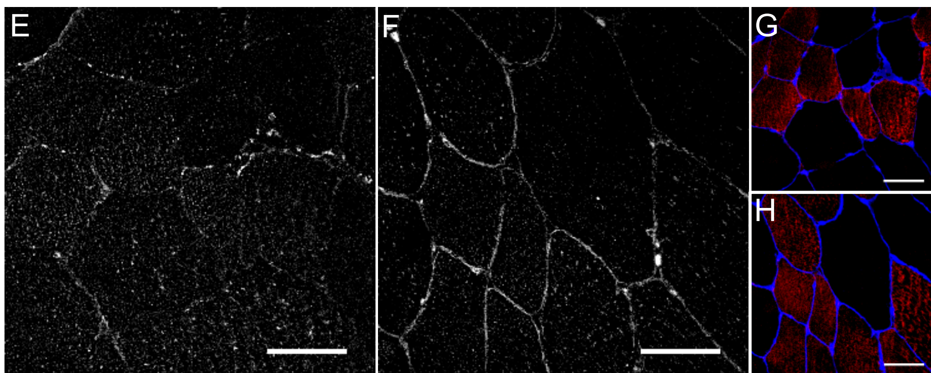
PLIN5 content (% area stained)

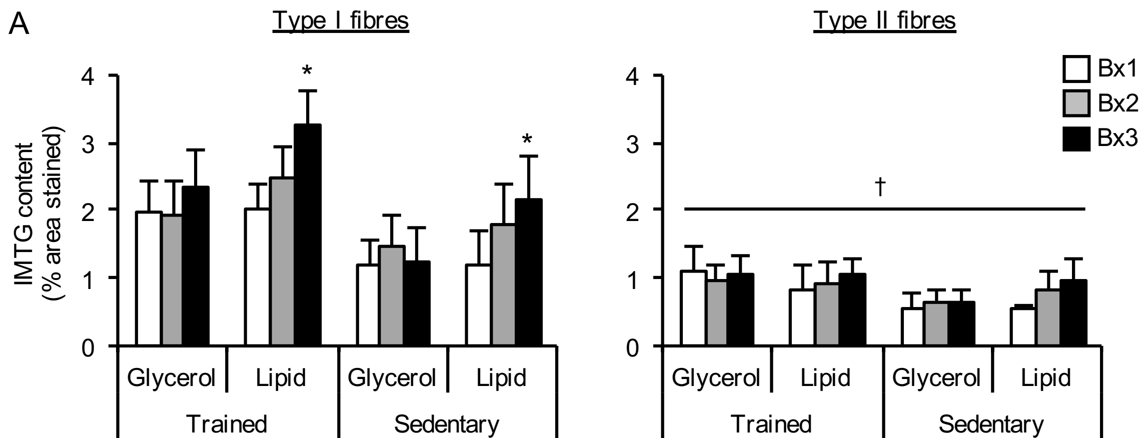
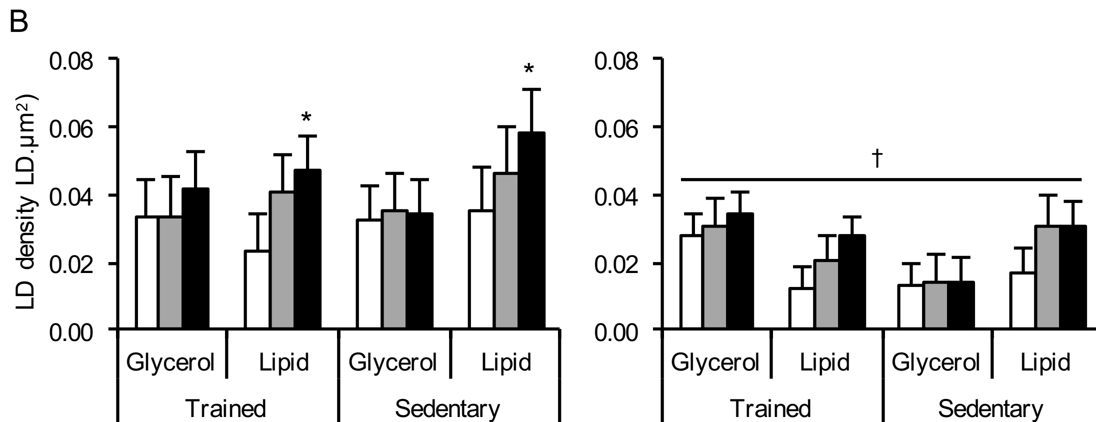
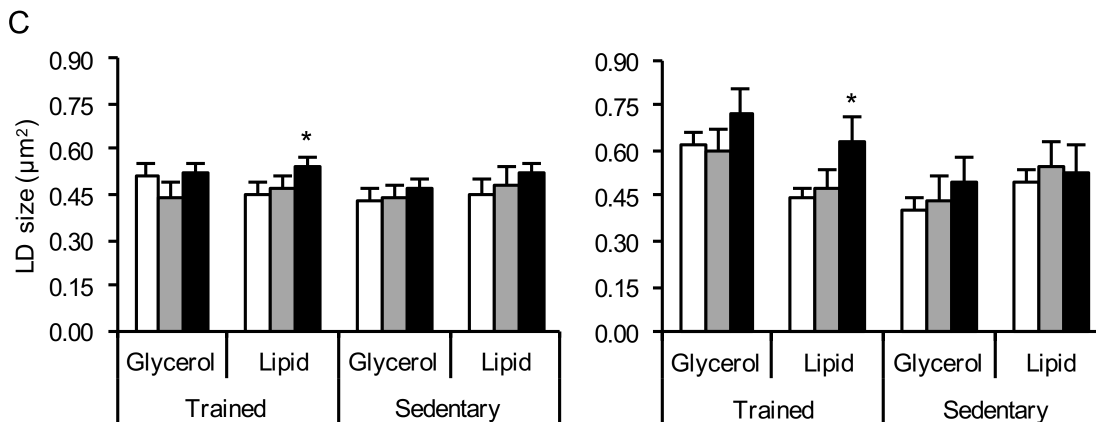
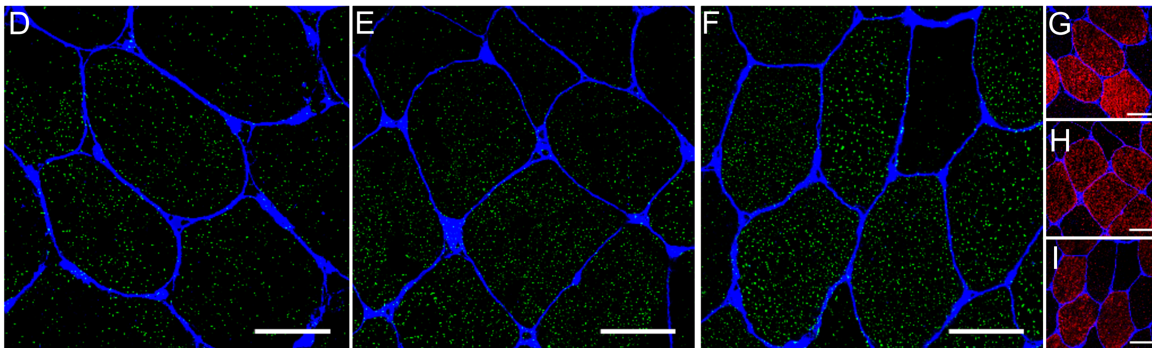
4 3 2 1 0

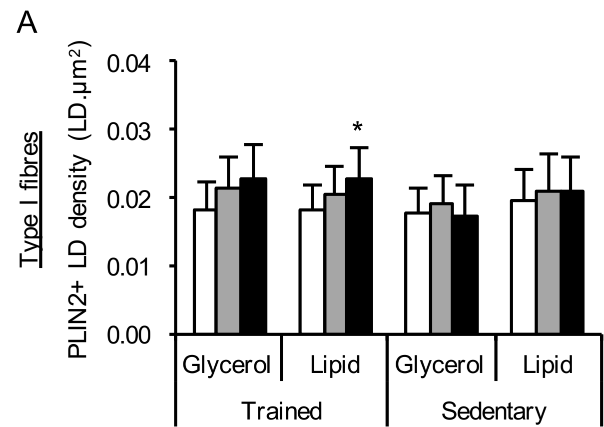
*

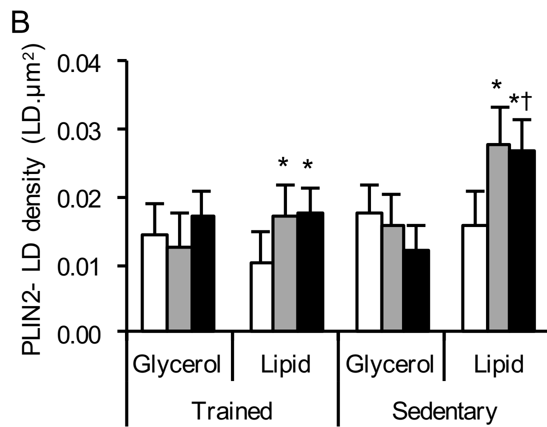
Glycerol Lipid Glycerol Lipid

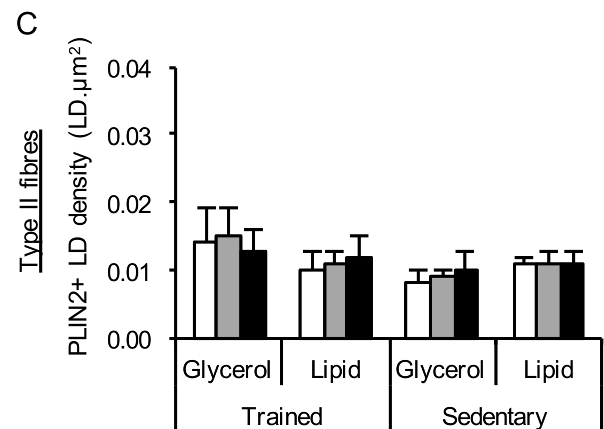
Trained Sedentary

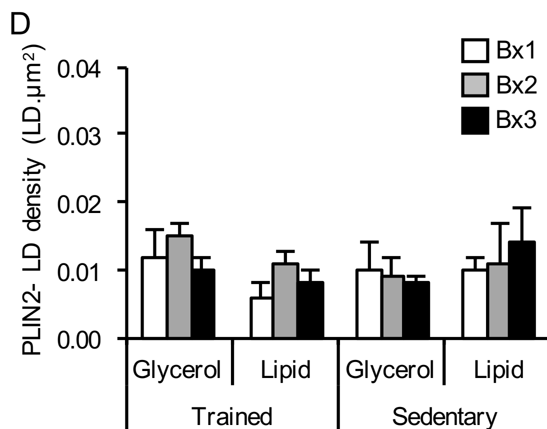


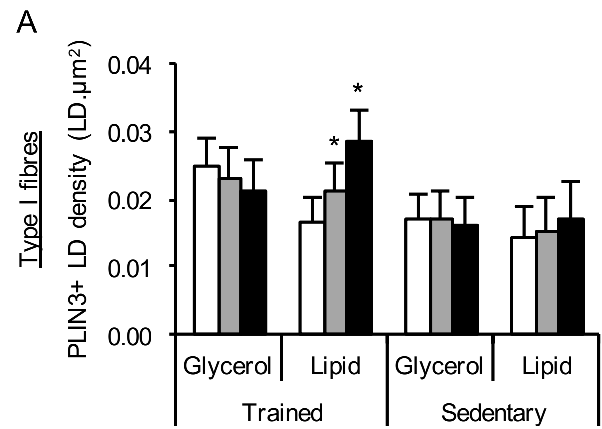
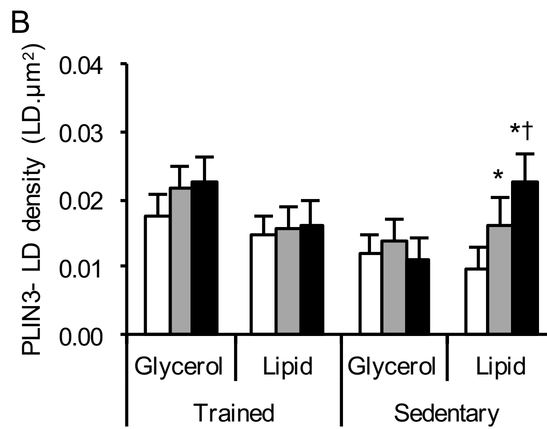
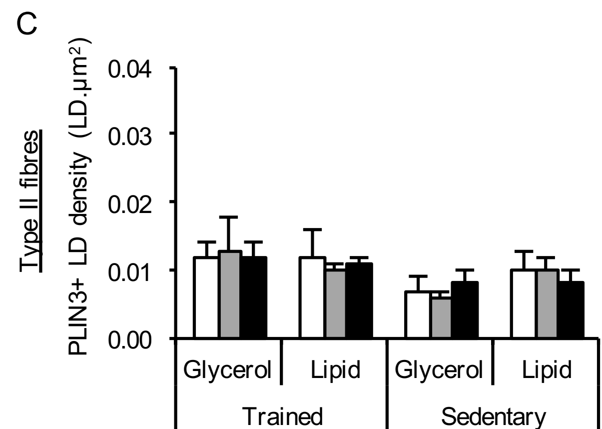
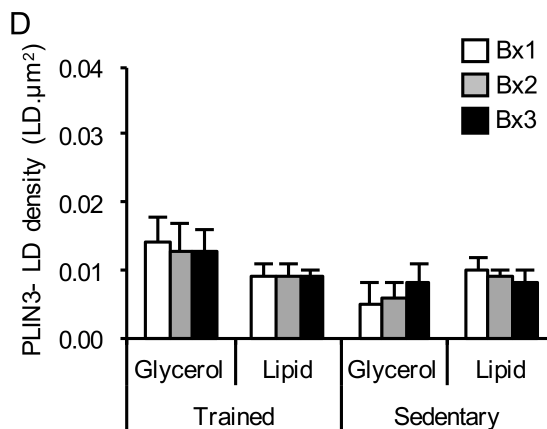

A
Type I fibres
Type II fibres
Bx1
Bx2
Bx3
IMTG content
(% area stained)
Glycerol
Lipid
Trained
Sedentary

B
LD density LD.μm²)
Glycerol
Lipid
Trained
Sedentary

C
LD size (μm²)
Glycerol
Lipid
Trained
Sedentary

D
E
F
G
H
I


A
Type I fibres
PLIN2+ LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
*
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary

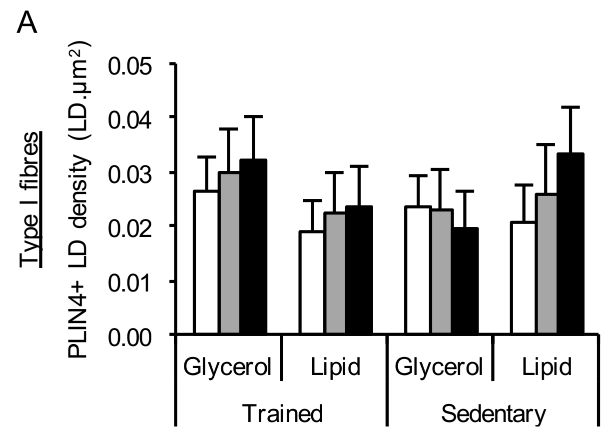

B
PLIN2- LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
*
*
*
*†
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary


C
Type II fibres
PLIN2+ LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary

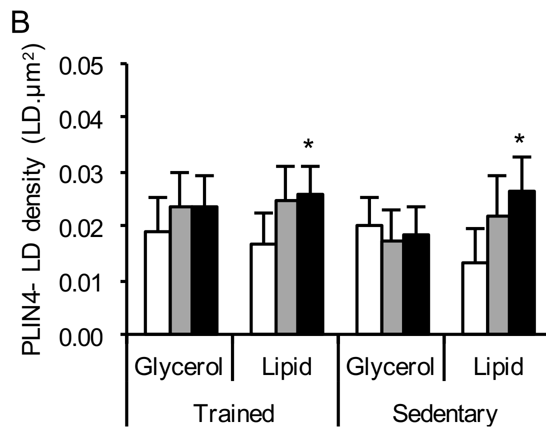

D
PLIN2- LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
Bx1
Bx2
Bx3
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary


A
Type I fibres
PLIN3+ LD density (LD.μm²)
0.04
0.03
0.02
0.01
0.00
*
*
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary

B
PLIN3- LD density (LD.μm²)
0.04
0.03
0.02
0.01
0.00
*†
*
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary

C
Type II fibres
PLIN3+ LD density (LD.μm²)
0.04
0.03
0.02
0.01
0.00
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary

D
PLIN3- LD density (LD.μm²)
0.04
0.03
0.02
0.01
0.00
Bx1
Bx2
Bx3
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary

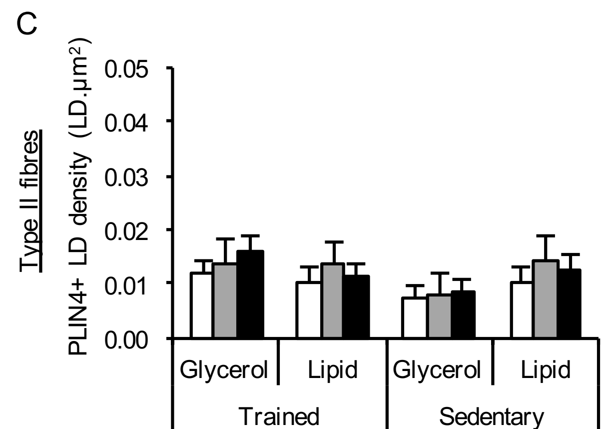
A



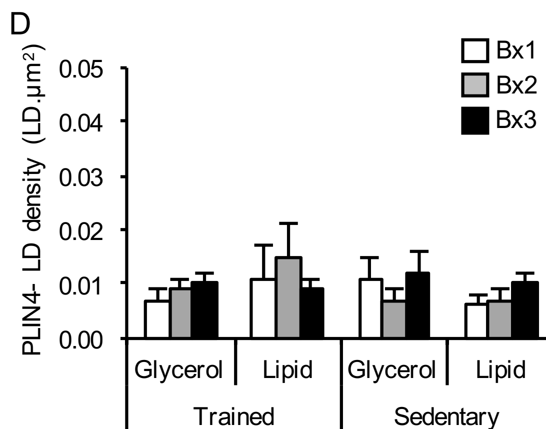
B

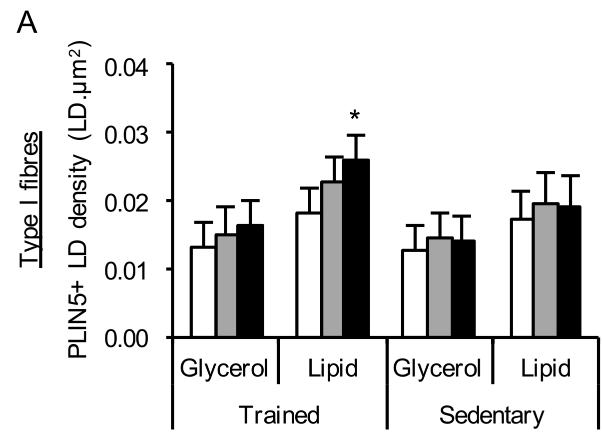


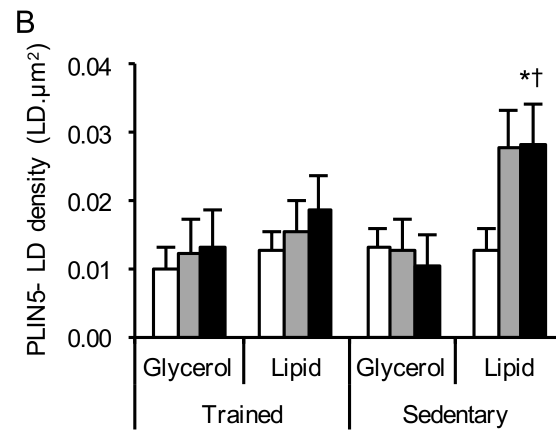
C

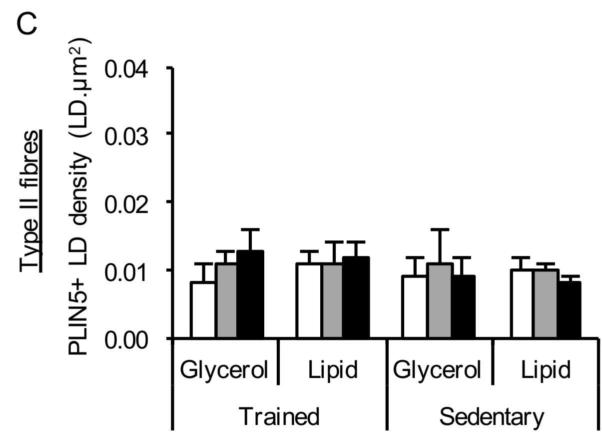


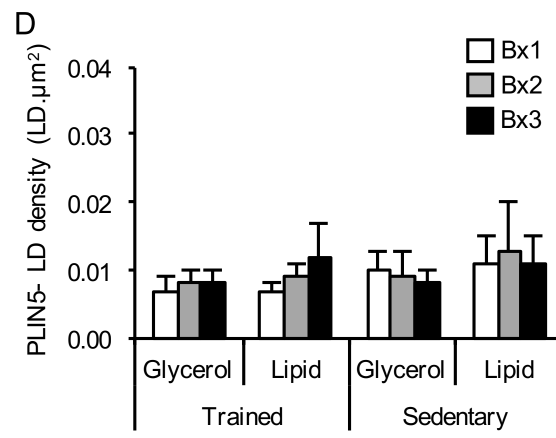
D




A
Type I fibres
PLIN5+ LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
*
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary


B
PLIN5- LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
*†
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary


C
Type II fibres
PLIN5+ LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary


D
Bx1
Bx2
Bx3
PLIN5- LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
Glycerol
Lipid
Glycerol
Lipid
Trained
Sedentary