Shepherd, SO, Strauss, JA, Wang, Q, Dube, JJ, Goodpaster, B, Mashek, DG and Chow, LS

Training alters the distribution of perilipin proteins in muscle following acute free fatty acid exposure.

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

Shepherd SO[1], Strauss JA[1], Wang Q[2], Dube JJ[3], Goodpaster B[4], Mashek DG[5], Chow LS[5]

[1]Research Institute for Sport & Exercise Sciences, Liverpool John Moores University

[2]Division of Biostatistics, School of Public Health, University of Minnesota

[3]Department of Biology, Chatham University

[4]Translational Research Institute for Metabolism & Diabetes, Florida Hospital

[5]Department of Medicine, University of Minnesota

Running head: Muscle lipid droplet response to lipid infusion

Address for correspondence:

Dr Sam Shepherd

Research Institute for Sport & Exercise Sciences

Liverpool John Moores University

Tom Reilly Building

Byrom Street

Liverpool

L3 3AF

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)

Introduction

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

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

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

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

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

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

Materials and Methods

Participants and ethical approval

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

Experimental protocol

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

Images of cross-sectionally orientated sections were used for determining fibre type-specific differences in the protein expression of IMTG, PLIN2, PLIN3, PLIN4, and PLIN5. Images were captured using an inverted confocal microscope (Zeiss LSM710, Carl Zeiss AG, Oberkochen, Germany) with a 40x 0.75 NA oil immersion objective. An argon laser was used to excite the Alexa Fluor 488 fluorophore and BODIPY, whilst a helium-neon laser excited the Alexa Fluor 546 and 633 fluorophores. Images to assess LD characteristics and co-localisation with either PLIN2, PLIN3, PLIN4 or PLIN5 were captured with the same system but using an 8x digital zoom. When assessing fibre-specific content of IMTG and individual PLIN proteins, fibres stained positively for myosin heavy chain type I were classified as type I fibres, whereas those with no staining were classified as type II fibres.

Image processing was undertaken using Image-Pro Plus 5.1 Software (Media Cybernetics, MD, USA). To assess fibre type distribution of IMTG and each PLIN protein, between 8 and 12 images were used per muscle section, resulting in a similar proportion of fibres being analysed for each trained (97±10 type I fibres, 118±15 type II fibres) and sedentary participant (105±17 type I fibres, 125±17 type II fibres). An intensity threshold was uniformly selected to represent a positive signal for IMTG and each PLIN protein. The content of IMTG and each PLIN protein was expressed as the positively stained area fraction relative to the total area of each muscle fibre. Images captured at 8x digital zoom were used to identify changes in LD density (number of LDs expressed relative to area) and LD size (mean area of individual LDs).

Co-localisation analysis was performed separately for each PLIN protein with IMTG, as described previously for PLIN2 and PLIN5 (Shepherd *et al.*, 2013). Briefly, a positive signal for the PLIN protein of interest and IMTG in sequential images was obtained by selecting a uniform intensity threshold (Fig. 1A-C). Based on the selected threshold, binary images were created and subsequently used for co-localisation analysis. A co-localisation map displaying the merged images was generated (Fig. 1D), with the overlapping regions subsequently extracted to a separate image (Fig. 1E). First, the total number of extracted objects in this image as a proportion of the total number of PLIN2 objects (or

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

Statistical analysis

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

Results

Baseline characteristics

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

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

Lipid droplet responses to lipid infusion

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

but this did not result in a significant overall increase in IMTG content in type II fibres. Furthermore, IMTG content was not elevated in type II fibres of sedentary participants following lipid infusion.

Lipid droplet and PLIN protein co-localisation

Lipid infusion had no effect on the protein content of any of the PLINs in either trained or sedentary participants (P>0.05; Fig. 2). We next investigated the association between LD and each PLIN protein by expressing the number of overlapping objects relative to the total number of PLIN protein objects (Fig. 1). Further, we also determined the number of LDs that either contained (PLIN+ LD) or were devoid of each PLIN protein (PLIN- LD) in response to the combined lipid and insulin infusion. The results of these analyses are detailed below.

PLIN2: At baseline, the fraction of PLIN2 co-localised with IMTG was similar between the groups in type I fibres (Trained: 0.65±0.02, Sedentary 0.68±0.02), and lipid infusion did not change this relationship. However, lipid infusion did lead to an increase in the number of PLIN2+ LDs in trained participants (+27%; P=0.01 for Bx3 vs. Bx1; Fig. 4A) but no changes occurred in the sedentary group. In contrast, the number of PLIN2- LDs was elevated by lipid infusion by a similar degree in both groups (Trained: +69%, Sedentary: +69%; P<0.05 for Bx3 vs. Bx1 for both groups; Fig. 4B). In type II fibres, the fraction of PLIN2 co-localised with IMTG was similar between the groups at baseline (Trained: 0.59±0.03, Sedentary 0.65±0.04). Lipid infusion increased PLIN2 co-localisation with IMTG from baseline (0.59±0.03) to post-infusion (0.71±0.03) in type II fibres in trained participants only (+21%; P=0.001 for Bx3 vs. Bx1). This was accounted for by a 33% significant decrease in free PLIN2 in response to lipid infusion (0.006±0.002 vs. 0.005±0.001 PLIN2 objects.μm^2 for Bx1 and Bx3, respectively; P=0.004).

PLIN3: There was no difference between trained and sedentary groups when comparing the fraction of PLIN3 co-localised with IMTG in both type I (Trained: 0.67±0.04, Sedentary 0.64±0.04) and type II fibres (Trained: 0.62±0.05, Sedentary: 0.61±0.05; P>0.05), and this relationship was unchanged by lipid

infusion. However, lipid infusion did lead to an increase in the number of PLIN3+ LDs in type I fibres of trained participants (+73%; P=0.004 for Bx3 vs. Bx1; Fig. 5A), whereas no changes occurred in the sedentary group. In contrast, in sedentary participants lipid infusion augmented the number of PLIN3- LDs (+133%; P<0.001 for Bx3 vs. Bx1; Fig. 5B) in type I fibres, with no changes in the trained group. Furthermore, no changes in the number of PLIN3+ or PLIN3- LDs occurred in type II fibres in either group in response to lipid infusion.

PLIN4: A similar fraction of PLIN4 co-localised with IMTG when comparing trained and sedentary groups at baseline in both type I (Trained: 0.74±0.05, Sedentary 0.70±0.06) and type II fibres (Trained: 0.66±0.06, Sedentary 0.66±0.07; P>0.05). Lipid infusion had no effect on this relationship, and no increase in PLIN4+ LDs was observed in either group (Fig. 6A & C). However, the number of PLIN4- LDs was elevated in type I fibres following lipid infusion in both trained (+55%; P=0.005 for Bx3 vs. Bx1) and sedentary participants (+94%; P=0.02 for Bx3 vs. Bx1; Fig. 6B).

PLIN5: The fraction of PLIN5 co-localised with IMTG was similar between trained and sedentary groups at baseline in both type I (Trained: 0.64±0.04, Sedentary 0.58±0.04; P>0.05) and type II fibres (Trained: 0.63±0.03, Sedentary 0.61±0.04; P>0.05). Lipid infusion led to an increase in PLIN5 co-localised with IMTG from baseline (0.58±0.04) to post-infusion (0.71±0.03) in type I fibres of sedentary participants only (+21%; P=0.013 for Bx3 vs. Bx1). This occurred alongside a trend for a decrease in free PLIN5 in response to lipid infusion (0.011±0.002 vs. 0.008±0.002 for Bx1 and Bx3, respectively; P=0.089). Notably, however, the number of PLIN5+ LDs was only augmented by lipid infusion in type I fibres of trained participants (+40; P=0.006 for Bx3 vs. Bx1; Fig. 7A), whereas the number of PLIN5- LDs was elevated by lipid infusion only in type I fibres of sedentary participants (+123%; P=0.03 for Bx3 vs. Bx1; Fig. 7B).

Discussion

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

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

et al., 2016). As well as synthesising new LDs, expanding the size of LDs, may be an additional mechanism by which trained individuals are able to sequester excess plasma FFA into IMTG.

Under resting conditions, the expression of PLIN2 and PLIN5 is closely related to IMTG content (Minnaard *et al.*, 2009; Amati *et al.*, 2011; Peters *et al.*, 2012; Shepherd *et al.*, 2013), although not all PLIN2 or PLIN5 is associated with the IMTG pool (Shepherd *et al.*, 2012, 2013). The results of the present study now show that this partial co-localisation with IMTG also exists for PLIN3 and PLIN4. In response to acute FFA elevation, IMTG levels were increased independent of changes in PLIN protein content. We therefore investigated whether acute FFA elevation altered the fraction of each PLIN associated with IMTG, and for each PLIN protein we describe two pools of LDs: (1) PLIN-associated LDs (PLIN+ LD), and (2) LDs that do not contain PLIN (PLIN- LDs). The major novel finding of the present study was that in trained individuals, acute FFA elevation led to an increase in the number of PLIN2+, PLIN3+ and PLIN5+ LDs specifically in type I fibres. Furthermore, this occurred in the absence of a change in the fraction of any PLIN protein associated with IMTG. Gemmink et al. (2016) recently showed that in trained individuals elevating plasma FFA concentrations through prolonged fasting also led to a greater number of PLIN5+ LDs, but this was accompanied by an increase in the fraction of PLIN5 associated with IMTG. Since PLIN5 protein expression was unaltered by fasting in this study, the authors concluded that prolonged fasting led to a redistribution of cytosolic PLIN5 to the LD surface (Gemmink *et al.*, 2016). We now provide evidence that in trained individuals PLIN2, PLIN3 and PLIN5 are all redistributed across the expanded LD pool following acute FFA elevation. However, as we did not observe a change in the fraction of any PLIN protein associated with IMTG, it appears that PLIN2, PLIN3 and PLIN5 are redistributed from pre-existing PLIN+ LDs to either newly-synthesised LDs and/or pre-existing PLIN- LDs. As a result, the proportion of the expanded LD pool containing these PLIN proteins was maintained.

The redistribution of PLIN2, PLIN3 and PLIN5 in trained individuals may be an important adaptation to enable FFA storage as IMTG in LDs. In support, palmitate incorporation into IMTG is reduced when 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

group, and therefore it is possible that sedentary individuals have a reduced capacity to redistribute PLINs when the muscle LD pool expands. Knockdown of PLIN2 in cultured muscle cells leads to increased palmitate incorporation into DAG (Bosma *et al.*, 2012a), whereas rats fed a high-fat diet accumulated IMTG, with no changes in DAG, but only when PLIN2 (Bosma *et al.*, 2012a) or PLIN5 (Bosma *et al.*, 2013) was overexpressed in muscle. PLIN2 and PLIN5 therefore appear to be important in channelling fatty acids into IMTG. The lack of a redistribution and/or lower abundance of these proteins in the present study may explain our previous finding that fatty acids reflecting the composition of an Intralipid® infusion appeared in IMTG and DAGs in sedentary individuals and only IMTG in trained individuals (Chow *et al.*, 2014).

Although IMTG content was unchanged in type II fibres, LD size did increase in trained individuals following acute FFA elevation. In addition, the fraction of PLIN2 associated with IMTG was increased, and therefore PLIN2 might also be redistributed to LDs in type II fibres, possibly to ensure that the surface coverage of larger LDs is maintained. Although PLIN2 is considered to be predominantly found at the LD surface (Prats *et al.*, 2006; Wolins *et al.*, 2006; Bell *et al.*, 2008), PLIN2 has also been observed at the endoplasmic reticulum (Robenek *et al.*, 2006), and may cycle between the cytosolic and LD fractions (Robenek *et al.*, 2006; Wang *et al.*, 2009). Since there was a significant decrease in PLIN2 that was unbound to LDs (free PLIN2) following acute FFA elevation, it is possible therefore that in type II fibres PLIN2 that was not previously associated with LDs became connected to the LD pool. Similarly, in sedentary individuals acute FFA elevation led to an increase in the fraction of PLIN5 associated with LDs in type I fibres. Like the aforementioned changes in PLIN2 in type II fibres of trained individuals, it is possible that PLIN5 that was previously not associated with LDs was recruited to the LD pool in type I fibres of sedentary individuals, especially as there was a tendency for PLIN5 that was unbound to LDs to decrease. However, because in sedentary individuals only an increase in the number of PLIN5- LDs was observed, it is likely that previously unbound PLIN5 was recruited to LDs already coated with PLIN5.

Little is known about the role of PLIN4 in skeletal muscle, although we report for the first time here that PLIN4 expression is ~2-fold greater in type I compared to type II fibres, and that trained individuals exhibit higher protein expression of both PLIN4 compared to sedentary individuals. These observations are in accordance with the fibre type distribution of the other PLINs in skeletal muscle, and findings of greater PLIN2, PLIN3 and PLIN5 expression in muscle in response to chronic training (Peters *et al.*, 2012; Shaw *et al.*, 2012; Louche *et al.*, 2013; Shepherd *et al.*, 2013; Shepherd *et al.*, 2014). Despite the higher PLIN4 expression in trained individuals, we only observed an increase in PLIN4- LDs suggesting that no redistribution of PLIN4 occurred in response to acute FFA elevation. Future studies will determine the precise role of PLIN4 in human skeletal muscle.

The use of validated immunofluorescence microscopy techniques (Shepherd *et al.*, 2012, 2013) to examine fibre type-specific changes in LD characteristics and the associations of PLIN proteins with LDs in response to acute FFA elevation is a clear strength of this study. Applying these techniques to samples obtained under 'dynamic' conditions has provided further insight into the potential role of the PLIN proteins in muscle. Our colocalisation assays, however, only permit fibre-specific analysis of the association between LDs and a single PLIN protein. This is important to acknowledge because acute FFA elevation increased PLIN4- LDs in trained and sedentary individuals, and increased both PLIN2+ and PLIN2- LDs in trained individuals. One possibility is that PLIN- LDs are actually newly-formed LDs that have not yet acquired sufficient PLIN protein to exceed the lower detection limit of the microscope. It is also possible that PLIN4- LDs and PLIN2- LDs were in fact coated with PLIN3 and/or PLIN5. Co-localisation analysis of PLIN2 and PLIN5 in rat skeletal muscle demonstrated only a partial overlap between the two proteins (Macpherson *et al.*, 2012), suggesting that some, but not all LDs, have both of these proteins associated with them. Whether there are distinct pools of LDs in skeletal muscle that have all, some, or none of the PLIN proteins associated remains to be determined.

Although exercise capacity was significantly different between the trained and sedentary participants, the mean VO_{2max} for the trained group was lower than that reported for previously published 'trained' groups (van Loon *et al.*, 2004; Amati *et al.*, 2011), which may explain the lack of difference in baseline

IMTG between trained and sedentary participants that we observed compared with previous literature (Goodpaster *et al.*, 2001; Amati *et al.*, 2011). We consider this a strength of the study because it demonstrates that only a small increase in exercise capacity, as can be achieved using exercise training, can improve the ability to redistribute PLIN proteins to LDs during acute FFA elevation and channel fatty acids into IMTG. In addition, since we had matched for age, gender and BMI, the trained participants consistently had a higher $VO_{2\,max}$ than their matched counterparts and therefore our findings were still consistent with a training effect. A further strength of the study is the use of a glycerol infusion as a control, as this approach enabled the specific examination of the effect of acute FFA elevation in trained and sedentary individuals. Previous studies conducted in trained individuals using a saline infusion lacked such control (Matzinger *et al.*, 2002; Schenk *et al.*, 2009).

In conclusion, this study has generated novel evidence that acute FFA elevation concurrent to a hyperinsulinaemic-euglycaemic clamp does not change PLIN protein content in skeletal muscle, but rather leads to a redistribution of PLIN2, PLIN3 and PLIN5 to an expanded LD pool in trained individuals only. In contrast, no redistribution of PLIN proteins occurs in sedentary individuals. This may be part of the mechanism by which trained individuals are able to channel fatty acids into IMTG.

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.

582 **References**

583 Adams JM, II, Pratipanawatr T, Berria R, Wang E, DeFronzo RA, Sullards MC & Mandarino LJ. (2004).
584 Ceramide Content Is Increased in Skeletal Muscle From Obese Insulin-Resistant Humans.
585 *Diabetes* **53,** 25-31.

586

587 Amati F, Dube JJ, Alvarez-Carnero E, Edreira MM, Chomentowski P, Coen PM, Switzer GE, Bickel
588 PE, Stefanovic-Racic M, Toledo FG & Goodpaster BH. (2011). Skeletal muscle triglycerides,
589 diacylglycerols, and ceramides in insulin resistance: another paradox in endurance-trained
590 athletes? *Diabetes* **60,** 2588-2597.

591

592 Bell M, Wang H, Chen H, McLenithan JC, Gong DW, Yang RZ, Yu D, Fried SK, Quon MJ, Londos C
593 & Sztalryd C. (2008). Consequences of lipid droplet coat protein downregulation in liver cells:
594 abnormal lipid droplet metabolism and induction of insulin resistance. *Diabetes* **57,** 2037-2045.

595

596 Bosma M, Hesselink MK, Sparks LM, Timmers S, Ferraz MJ, Mattijssen F, van Beurden D, Schaart G,
597 de Baets MH, Verheyen FK, Kersten S & Schrauwen P. (2012a). Perilipin 2 improves insulin
598 sensitivity in skeletal muscle despite elevated intramuscular lipid levels. *Diabetes* **61,** 2679-
599 2690.

600

601 Bosma M, Minnaard R, Sparks LM, Schaart G, Losen M, de Baets MH, Duimel H, Kersten S, Bickel
602 PE, Schrauwen P & Hesselink MK. (2012b). The lipid droplet coat protein perilipin 5 also
603 localizes to muscle mitochondria. *Histochem Cell Biol* **137,** 205-216.

604

605 Bosma M, Sparks LM, Hooiveld GJ, Jorgensen JA, Houten SM, Schrauwen P, Kersten S & Hesselink
606 MK. (2013). Overexpression of PLIN5 in skeletal muscle promotes oxidative gene expression
607 and intramyocellular lipid content without compromising insulin sensitivity. *Biochim Biophys*
608 *Acta* **1831,** 844-852.

609

610 Chabowski A, Coort SL, Calles-Escandon J, Tandon NN, Glatz JF, Luiken JJ & Bonen A. (2004).
611 Insulin stimulates fatty acid transport by regulating expression of FAT/CD36 but not FABPpm.
612 *Am J Physiol Endocrinol Metab* **287,** E781-789.

613

614 Chow LS, Mashek DG, Austin E, Eberly LE, Persson XM, Mashek MT, Seaquist ER & Jensen MD.
615 (2014). Training status diverges muscle diacylglycerol accumulation during free fatty acid
616 elevation. *Am J Physiol Endocrinol Metab* **307,** E124-131.

617

618 Chow LS, Seaquist ER, Eberly LE, Mashek MT, Schimke JM, Nair KS & Mashek DG. (2012). Acute
619 free fatty acid elevation eliminates endurance training effect on insulin sensitivity. *J Clin*
620 *Endocrinol Metab* **97,** 2890-2897.

621

622 Covington JD, Galgani JE, Moro C, LaGrange JM, Zhang Z, Rustan AC, Ravussin E & Bajpeyi S.
623 (2014). Skeletal muscle perilipin 3 and coatomer proteins are increased following exercise and
624 are associated with fat oxidation. *PLoS One* **9,** e91675.

625

626 Covington JD, Noland RC, Hebert RC, Masinter BS, Smith SR, Rustan AC, Ravussin E & Bajpeyi S.
627 (2015). Perilipin 3 Differentially Regulates Skeletal Muscle Lipid Oxidation in Active,
628 Sedentary, and Type 2 Diabetic Males. *J Clin Endocrinol Metab* **100,** 3683-3692.

Craig CL, Marshall AL, Sjostrom M, Bauman AE, Booth ML, Ainsworth BE, Pratt M, Ekelund U, Yngve A, Sallis JF & Oja P. (2003). International physical activity questionnaire: 12-country reliability and validity. *Med Sci Sports Exerc* **35,** 1381-1395.

Dyck DJ, Steinberg G & Bonen A. (2001). Insulin increases FA uptake and esterification but reduces lipid utilization in isolated contracting muscle. *Am J Physiol Endocrinol Metab* **281,** E600-607.

Gemmink A, Bosma M, Kuijpers HJ, Hoeks J, Schaart G, van Zandvoort MA, Schrauwen P & Hesselink MK. (2016). Decoration of intramyocellular lipid droplets with PLIN5 modulates fasting-induced insulin resistance and lipotoxicity in humans. *Diabetologia* **59,** 1040-1048.

Goodpaster BH, He J, Watkins S & Kelley DE. (2001). Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *J Clin Endocrinol Metab* **86,** 5755-5761.

Holm C, Osterlund T, Laurell H & Contreras JA. (2000). Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annu Rev Nutr* **20,** 365-393.

Itani SI, Ruderman NB, Schmieder F & Boden G. (2002). Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes* **51,** 2005-2011.

Kelley DE, Goodpaster B, Wing RR & Simoneau JA. (1999). Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* **277,** E1130-1141.

Kleinert M, Parker BL, Chaudhuri R, Fazakerley DJ, Serup A, Thomas KC, Krycer JR, Sylow L, Fritzen AM, Hoffman NJ, Jeppesen J, Schjerling P, Ruegg MA, Kiens B, James DE & Richter EA. (2016). mTORC2 and AMPK differentially regulate muscle triglyceride content via Perilipin 3. *Mol Metab* **5,** 646-655.

Laurens C, Bourlier V, Mairal A, Louche K, Badin PM, Mouisel E, Montagner A, Marette A, Tremblay A, Weisnagel JS, Guillou H, Langin D, Joanisse DR & Moro C. (2016). Perilipin 5 fine-tunes lipid oxidation to metabolic demand and protects against lipotoxicity in skeletal muscle. *Sci Rep* **6,** 38310.

Louche K, Badin PM, Montastier E, Laurens C, Bourlier V, de Glisezinski I, Thalamas C, Viguerie N, Langin D & Moro C. (2013). Endurance exercise training up-regulates lipolytic proteins and reduces triglyceride content in skeletal muscle of obese subjects. *J Clin Endocrinol Metab* **98,** 4863-4871.

Macpherson RE, Herbst EA, Reynolds EJ, Vandenboom R, Roy BD & Peters SJ. (2012). Subcellular localization of skeletal muscle lipid droplets and PLIN family proteins (OXPAT and ADRP) at rest and following contraction (in rat soleus muscle). *Am J Physiol Regul Integr Comp Physiol* **302,** R29-R36.

MacPherson RE & Peters SJ. (2015). Piecing together the puzzle of perilipin proteins and skeletal muscle lipolysis. *Appl Physiol Nutr Metab* **40,** 641-651.

Matzinger O, Schneiter P & Tappy L. (2002). Effects of fatty acids on exercise plus insulin-induced glucose utilization in trained and sedentary subjects. *Am J Physiol Endocrinol Metab* **282,** E125-131.

Minnaard R, Schrauwen P, Schaart G, Jorgensen JA, Lenaers E, Mensink M & Hesselink MK. (2009). Adipocyte differentiation-related protein and OXPAT in rat and human skeletal muscle: involvement in lipid accumulation and type 2 diabetes mellitus. *J Clin Endocrinol Metab* **94,** 4077-4085.

Muoio DM, Dohm GL, Tapscott EB & Coleman RA. (1999). Leptin opposes insulin's effects on fatty acid partitioning in muscles isolated from obese ob/ob mice. *Am J Physiol* **276,** E913-921.

Peters SJ, Samjoo IA, Devries MC, Stevic I, Robertshaw HA & Tarnopolsky MA. (2012). Perilipin family (PLIN) proteins in human skeletal muscle: the effect of sex, obesity, and endurance training. *Appl Physiol Nutr Metab* **37,** 724-735.

Pourteymour S, Lee S, Langleite TM, Eckardt K, Hjorth M, Bindesboll C, Dalen KT, Birkeland KI, Drevon CA, Holen T & Norheim F. (2015). Perilipin 4 in human skeletal muscle: localization and effect of physical activity. *Physiological reports* **3**.

Prats C, Donsmark M, Qvortrup K, Londos C, Sztalryd C, Holm C, Galbo H & Ploug T. (2006). Decrease in intramuscular lipid droplets and translocation of HSL in response to muscle contraction and epinephrine. *J Lipid Res* **47,** 2392-2399.

Ramos SV, Turnbull PC, MacPherson RE, LeBlanc PJ, Ward WE & Peters SJ. (2015). Changes in mitochondrial perilipin 3 and perilipin 5 protein content in rat skeletal muscle following endurance training and acute stimulated contraction. *Exp Physiol* **100,** 450-462.

Robenek H, Hofnagel O, Buers I, Robenek MJ, Troyer D & Severs NJ. (2006). Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis. *J Cell Sci* **119,** 4215-4224.

Samuel VT & Shulman GI. (2012). Mechanisms for insulin resistance: common threads and missing links. *Cell* **148,** 852-871.

Schenk S, Harber MP, Shrivastava CR, Burant CF & Horowitz JF. (2009). Improved insulin sensitivity after weight loss and exercise training is mediated by a reduction in plasma fatty acid mobilization, not enhanced oxidative capacity. *J Physiol* **587,** 4949-4961.

Shaw CS, Shepherd SO, Wagenmakers AJ, Hansen D, Dendale P & van Loon LJ. (2012). Prolonged exercise training increases intramuscular lipid content and perilipin 2 expression in type I muscle fibers of patients with type 2 diabetes. *Am J Physiol Endocrinol Metab* **303,** E1158-1165.

Shaw CS, Sherlock M, Stewart PM & Wagenmakers AJ. (2009). Adipophilin distribution and colocalization with lipid droplets in skeletal muscle. *Histochem Cell Biol* **131,** 575-581.

724 Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ &
725 Shaw CS. (2012). Preferential utilization of perilipin 2-associated intramuscular triglycerides
726 during 1 h of moderate-intensity endurance-type exercise. *Exp Physiol* **97,** 970-980.

727
728 Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJ &
729 Shaw CS. (2013). Sprint interval and traditional endurance training increase net intramuscular
730 triglyceride breakdown and expression of perilipin 2 and 5. *J Physiol* **591,** 657-675.

731
732 Shepherd SO, Cocks M, Tipton KD, Witard OC, Ranasinghe AM, Barker TA, Wagenmakers AJ &
733 Shaw CS. (2014). Resistance training increases skeletal muscle oxidative capacity and net
734 intramuscular triglyceride breakdown in type I and II fibres of sedentary males. *Exp Physiol* **99,**
735 894-908.

736
737 van Loon LJ & Goodpaster BH. (2006). Increased intramuscular lipid storage in the insulin-resistant
738 and endurance-trained state. *Pflugers Arch* **451,** 606-616.

739
740 van Loon LJC, Koopman R, Manders R, van der Weegen W, van Kranenburg GP & Keizer HA. (2004).
741 Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary
742 men and highly trained endurance athletes. *Am J Physiol Endocrinol Metab* **287,** E558-565.

743
744 Wang H, Hu L, Dalen K, Dorward H, Marcinkiewicz A, Russell D, Gong D, Londos C, Yamaguchi T,
745 Holm C, Rizzo MA, Brasaemle D & Sztalryd C. (2009). Activation of hormone-sensitive lipase
746 requires two steps, protein phosphorylation and binding to the PAT-1 domain of lipid droplet
747 coat proteins. *J Biol Chem* **284,** 32116-32125.

748
749 Wang H, Sreenevasan U, Hu H, Saladino A, Polster BM, Lund LM, Gong DW, Stanley WC & Sztalryd
750 C. (2011). Perilipin 5, lipid droplet associated protein provides physical and metabolic linkage
751 to mitochondria. *J Lipid Res* **52,** 2159-2168.

752
753 Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, Varma V, Yao-Borengasser
754 A, Rasouli N, Kern PA, Finck BN & Bickel PE. (2006). OXPAT/PAT-1 Is a PPAR-Induced
755 Lipid Droplet Protein That Promotes Fatty Acid Utilization. *Diabetes* **55,** 3418-3428.

756
757 Zhang H, Wang Y, Li J, Yu J, Pu J, Li L, Zhang S, Peng G, Yang F & Liu P. (2011). Proteome of
758 skeletal muscle lipid droplet reveals association with mitochondria and apolipoprotein a-I. *J*
759 *Proteome Res* **10,** 4757-4768.

760

761

762

763

764

765

766

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

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

786 **Figure Legends**

787

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.

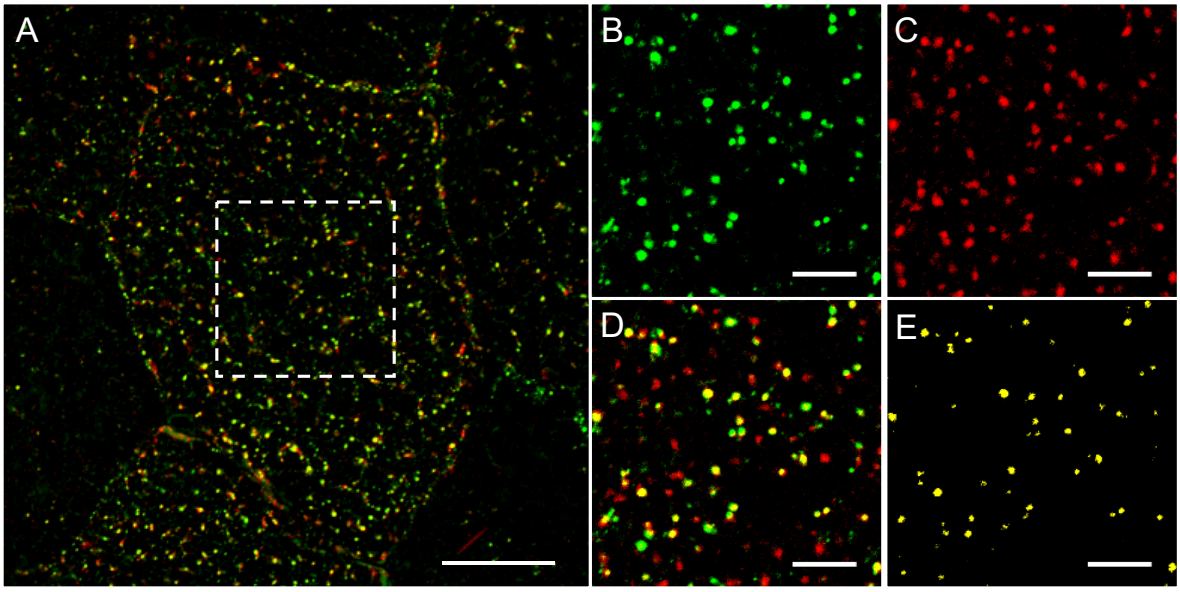
798

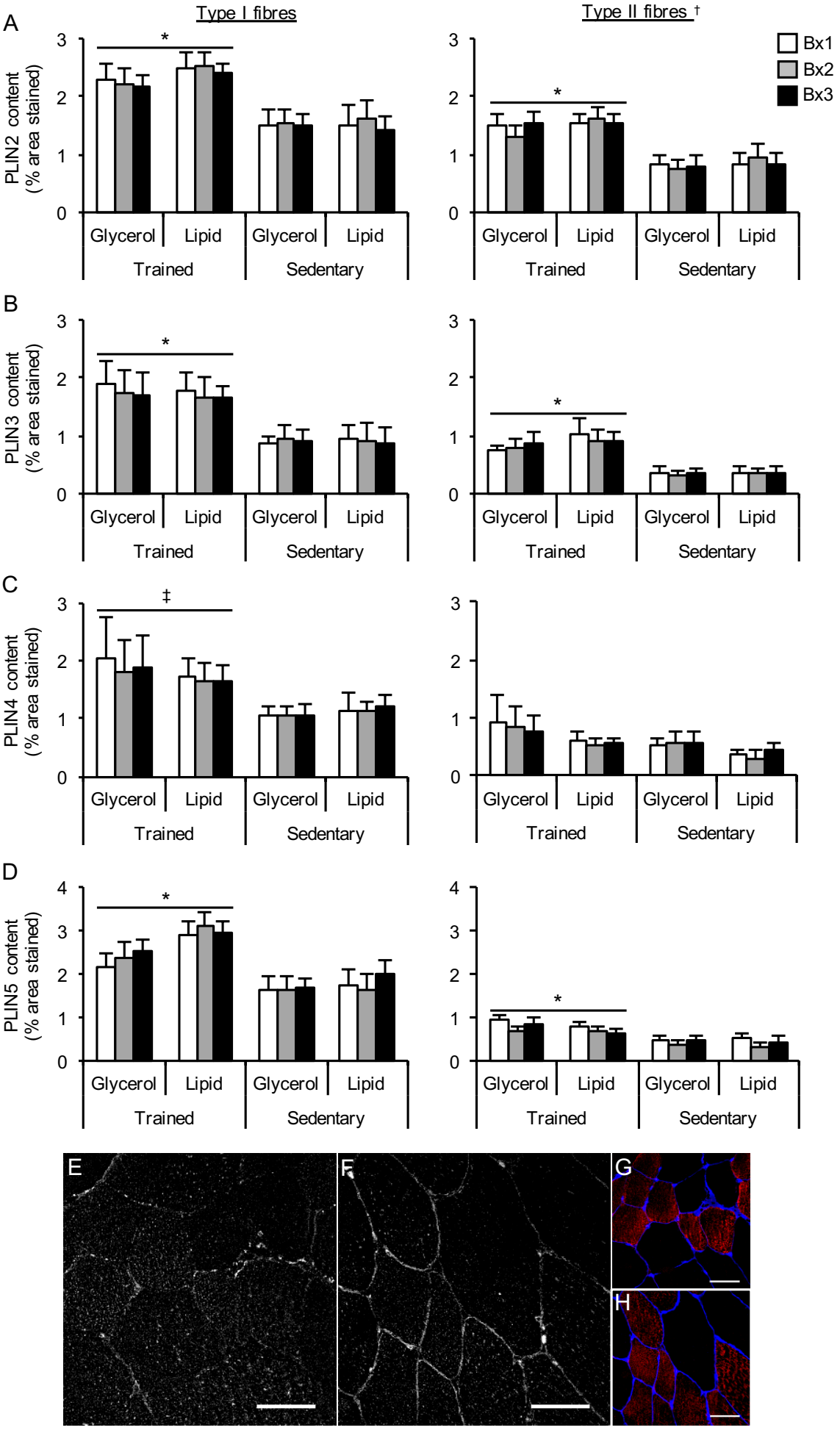
799 **Figure 2.** A 6-h Intralipid infusion did not alter fibre-specific PLIN2 (A), PLIN3 (B), PLIN4 (C) and
800 PLIN5 (D) protein expression in trained and sedentary individuals. Fibre type-specific content of
801 PLIN2, PLIN3 (E), PLIN4 (F) and PLIN5 was quantified from immunofluorescence images, where
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.

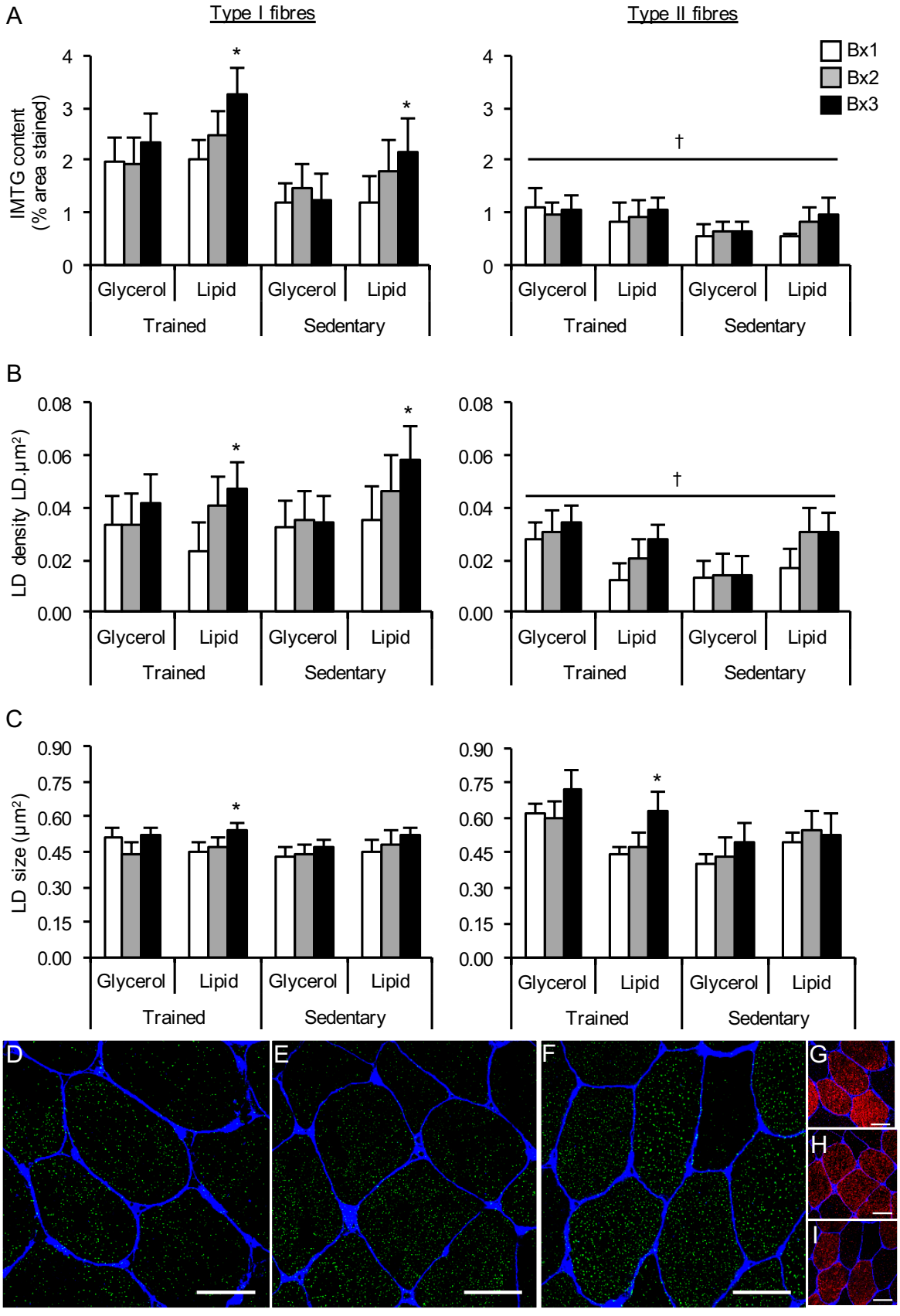
808

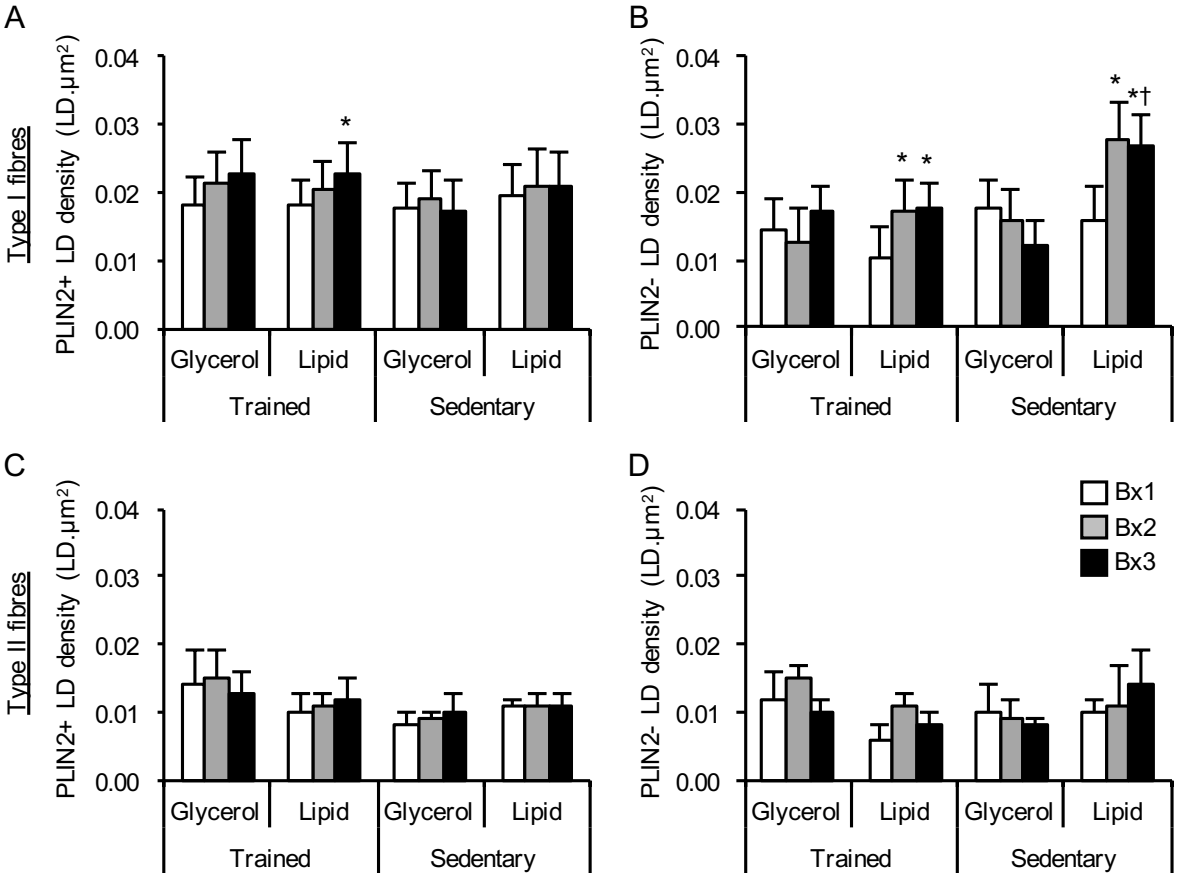
809 **Figure 3.** A 6-h Intralipid infusion alters fibre-specific IMTG content (A), LD density (B) and LD size
810 (C). Fibre type-specific content of IMTG was quantified from immunofluorescence images of muscle
811 sections obtained at baseline (Bx1; D), and after 120 min (Bx2; E) and 360 min (Bx3; 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

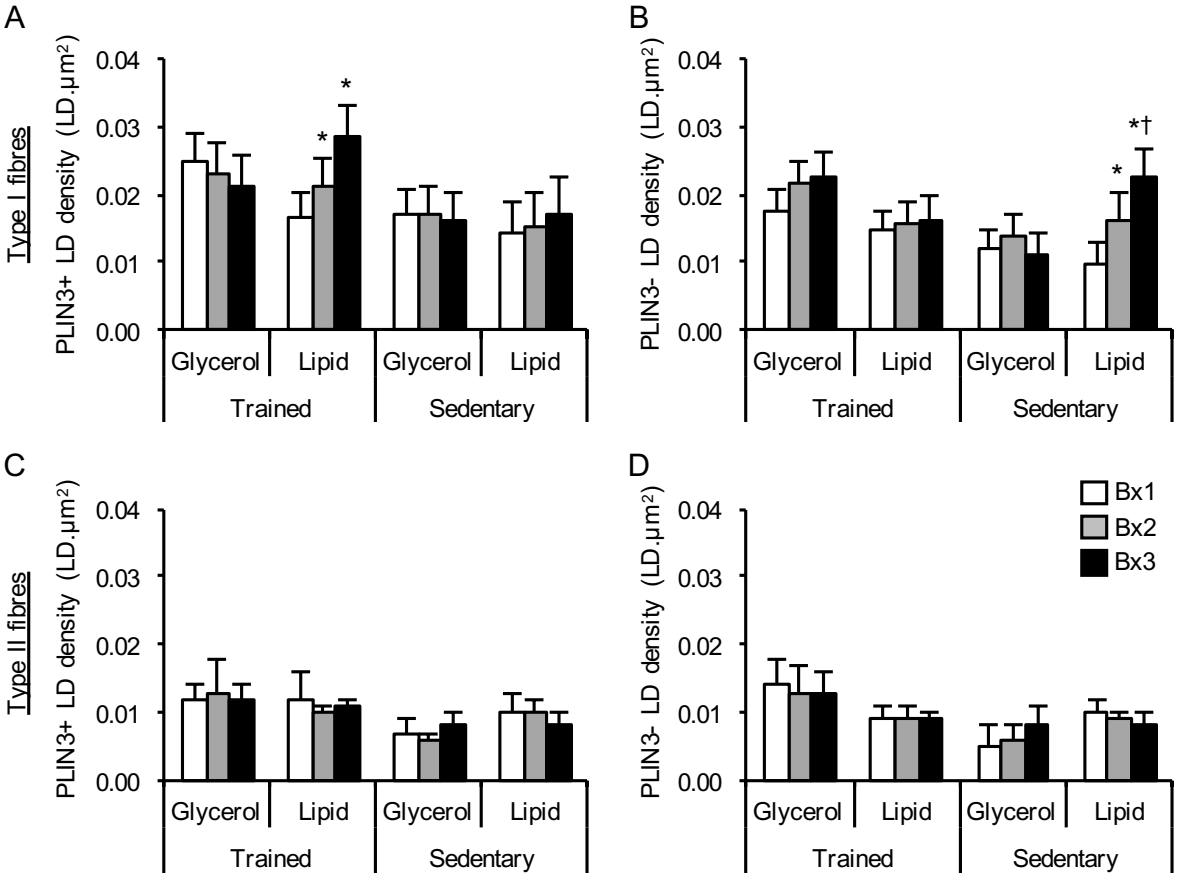
814 blue) in skeletal muscle. Positively stained fibres (red) are type I fibres, all other fibres are assumed to
815 be type II fibres. White bars represent 50 μm. *Significantly different from Bx1 given fitness category
816 and infusion status (P<0.05). †Significantly different than type I fibres (P<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.

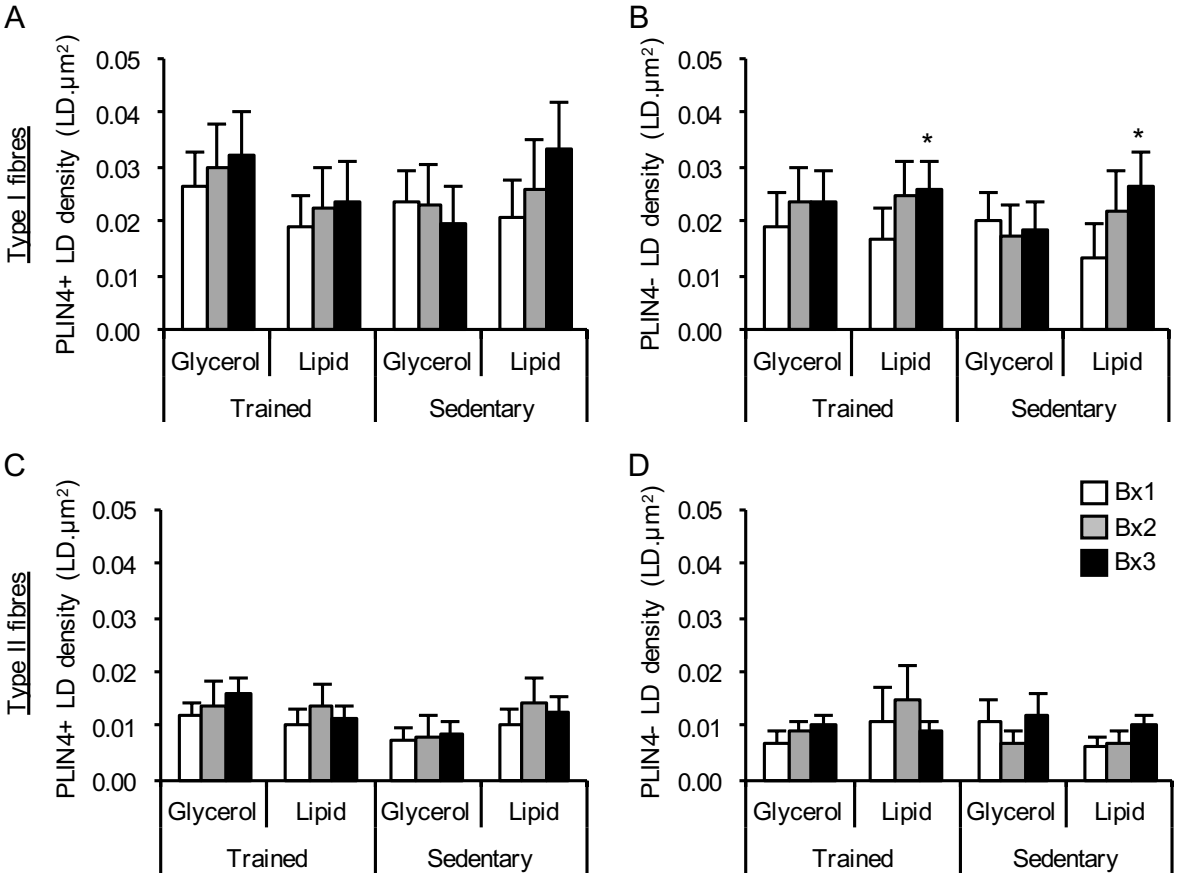

A
B
C
D
E

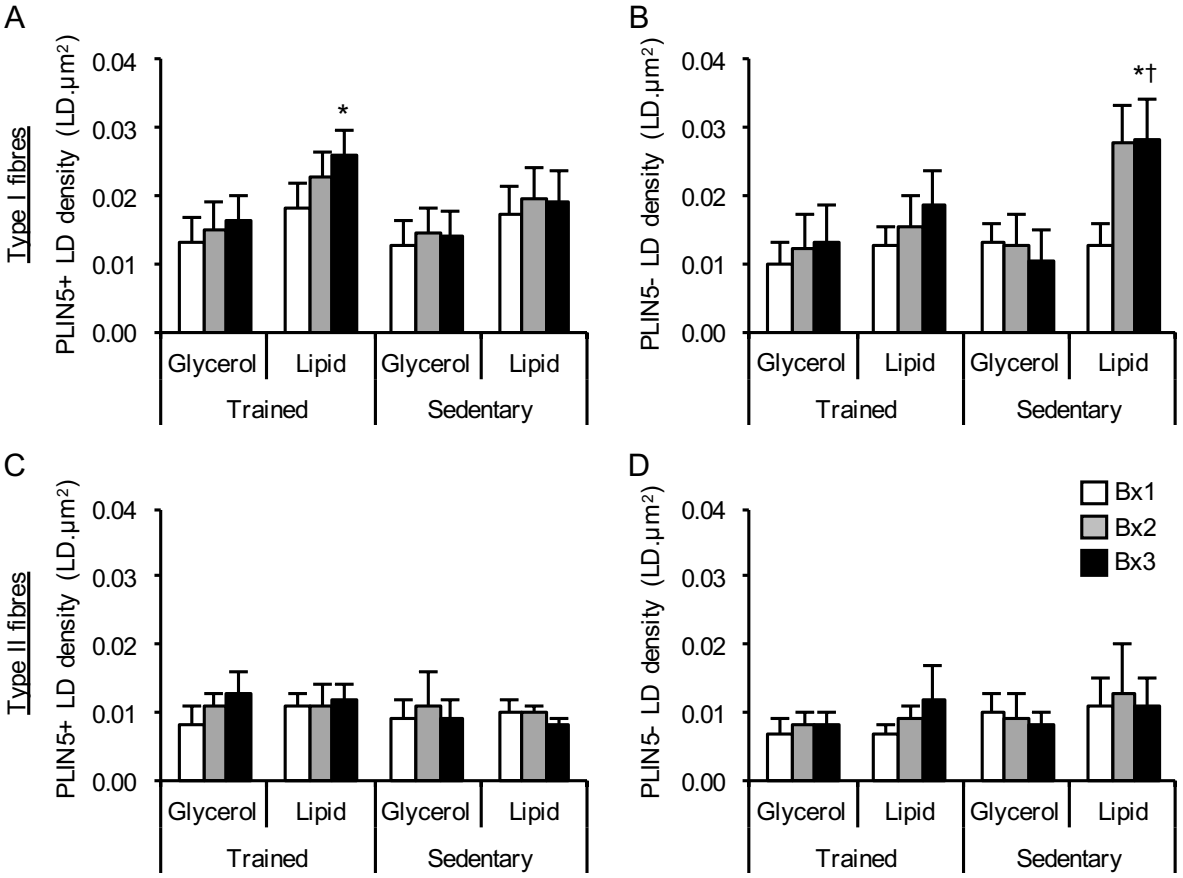

A
Type I fibres
Type II fibres †
Bx1
Bx2
Bx3
PLIN2 content (% area stained)
B
PLIN3 content (% area stained)
C
PLIN4 content (% area stained)
D
PLIN5 content (% area stained)
Glycerol
Lipid
Trained
Sedentary
*
‡
E
F
G
H


A
Type I fibres
Type II fibres
Bx1
Bx2
Bx3
IMTG content (% area stained)
Glycerol
Lipid
Trained
Sedentary
B
LD density LD.µm²
C
LD size (µm²)
D
E
F
G
H
I


A
B
C
D
Type I fibres
Type II fibres
PLIN2+ LD density (LD.µm²)
PLIN2- LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
Glycerol
Lipid
Trained
Sedentary
Bx1
Bx2
Bx3
*
*†


A
B
C
D
Type I fibres
Type II fibres
PLIN3+ LD density (LD.µm²)
PLIN3- LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
Glycerol
Lipid
Trained
Sedentary
Bx1
Bx2
Bx3
*
*†


A
B
C
D
Type I fibres
Type II fibres
PLIN4+ LD density (LD.µm²)
PLIN4- LD density (LD.µm²)
0.05
0.04
0.03
0.02
0.01
0.00
Glycerol
Lipid
Trained
Sedentary
*
Bx1
Bx2
Bx3


A
B
C
D
Type I fibres
Type II fibres
PLIN5+ LD density (LD.µm²)
PLIN5- LD density (LD.µm²)
0.04
0.03
0.02
0.01
0.00
*
*†
Glycerol
Lipid
Trained
Sedentary
Bx1
Bx2
Bx3