Jevons, EFP, Gejl, KD, Strauss, JA, Ørtenblad, N and Shepherd, SO

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

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

Jevons EFP[1], Gejl KD[2], Strauss JA[1], Ørtenblad N[2], Shepherd SO[1].

[1]Liverpool John Moores University, Research Institute of Sport and Exercise Sciences.
[2]University of Southern Denmark, Department of Sports Science and Clinical Biomechanics, Campusvej 55, Odense M.

Address for correspondence:

Dr Sam Shepherd

Research Institute for Sport & Exercise Sciences

Liverpool John Moores University

Tom Reilly Building

Byrom Street

Liverpool

L3 3AF

Abstract

Intramuscular triglycerides (IMTG) are a key substrate during prolonged exercise, but little is known about the rate of IMTG resynthesis in the post-exercise period. We investigated the hypothesis that the distribution of the lipid droplet (LD)-associated perilipin (PLIN) proteins is linked to IMTG storage following exercise. 14 elite male triathletes (27±1 y, 66.5±1.3 $mL.kg^{-1}.min^{-1}$) completed 4 h of moderate-intensity cycling. During the first 4 h of recovery, subjects received either carbohydrate or H_2O, after which both groups received carbohydrate. Muscle biopsies collected pre and post-exercise, and 4 h and 24 h post-exercise were analysed using confocal immunofluorescence microscopy for fibre type-specific IMTG content and PLIN distribution with LDs. Exercise reduced IMTG content in type I fibres (-53%, P=0.002), with no change in type IIa fibres. During the first 4 h of recovery, IMTG content increased in type I fibres (P=0.014), but was not increased further after 24 h where it was similar to baseline levels in both conditions. During recovery the number of LDs labelled with PLIN2 (70%), PLIN3 (63%) and PLIN5 (62%; all P<0.05) all increased in type I fibres. Importantly, the increase in LDs labelled with PLIN proteins only occurred at 24 h post-exercise. In conclusion, IMTG resynthesis occurs rapidly in type I fibres following prolonged exercise in highly-trained individuals. Further, increases in IMTG content following exercise preceded an increase in the number of LDs labelled with PLIN proteins. These data, therefore, suggest that the PLIN proteins do not play a key role in post-exercise IMTG resynthesis.

Keywords:
Intramyocellular lipid, perilipin 2, perilipin 3, perilipin 5, carbohydrate restriction

Abbreviations:
Intramuscular triglyceride (IMTG)
Carbohydrate (CHO)
Lipid droplet (LD)
Perilipin (PLIN)

Introduction

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

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

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

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

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

Methods

Subjects

Fourteen elite male triathletes (27.2 ± 0.9 y, 183 ± 2 cm, 75.3 ± 1.4 kg) that had competed at national and/or international level were recruited as part of a larger study (10). Participants had been elite athletes for 4.8 ± 1.4 y and trained on average 16.4 ± 0.9 hours a week. There were no differences between experimental groups, other than VO_{2max} where the participants in the CHO condition had a significantly higher VO_{2max} (CHO: 68.3 ± 1.4 $mL.kg^{-1}.min^{-1}$, H_2O: 63.5 ± 1.8 $mL.kg^{-1}.min^{-1}$, $P < 0.05$). All participants were fully informed of any risks associated with the study before providing informed verbal and written consent. Ethical approval was approved by the ethics committee of the Region of Southern Denmark (Project ID: S-20090140) and was conducted according to the Declaration of Helsinki.

Experimental procedures

All experimental procedures have been described previously (10, 14). Briefly, participants completed 4 h of cycling at an average of 73% ± 1% HR_{max} equating to ~56% of VO_{2max} (determined via pre-experimental submaximal incremental test and VO_{2max} test) with an intended HR intensity of ~75% HR_{max}. Subjects were provided a standardised breakfast (see "Dietary Procedures" below) 90 min before completing the cycle in which they used personal equipment of their choice (i.e. bike, shoes and pedals) on mounted turbo trainers (Elite Crono Mag ElastoGel Trainer, Fontaniva, Italy). During exercise participants were only allowed to consume water (minimum of 1 mL $water.kg^{-1}.h^{-1}$). Following exercise, participants were randomly selected to receive either CHO (n = 7) or H_2O (n = 7) during the first 4 h of recovery. For the remaining 20 h period following exercise all participants consumed a CHO-rich diet. All procedures were conducted in laboratories at the Department of Sports Science and Clinical Biomechanics, University of Southern Denmark, Odense.

Dietary procedures

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

orange juice and energy bar; 82 $kJ.kg^{-1}$ bw). All calorie intake was calculated based upon the participant's body mass. During the initial 2 h recovery period following exercise, the CHO group consumed a meal consisting of pasta, chicken, vegetables and a CHO beverage (1.07 g $CHO.kg^{-1}$ $bw.h^{-1}$). and subsequently participants were provided with an energy bar and CHO beverage (1.05 g $CHO.kg^{-1}$ $bw.h^{-1}$) in the following 2 h. During this 4 h period, the H_2O group remained fasted and only consumed water. After the initial 4 h recovery period, both groups received the same standardised meals for the remaining 20 h of recovery. In addition, the H_2O group received energy corresponding to that of the CHO group during the 4 h recovery period to ensure that the total energy intake between groups was equal. Thus, the CHO group received dinner and breakfast whereas the H_2O group received lunch, an energy bar, dinner and breakfast. In total, subjects received 264 $kJ.kg^{-1}$ bw (10 g $CHO.kg^{-1}$ bw) on the first experimental day.

Sample collection

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

Immunofluorescence microscopy

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

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

Antibodies

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

Image capture, image processing and data analysis

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

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

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

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

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

Statistics

Statistical analyses were performed using SPSS (SPSS; version 23, IBM, USA). Linear mixed modelling was used to examine all dependent variables (IMTG content, LD morphology, PLIN protein expression and co-localisation analysis) at the different time points, with data separated into the two different experimental conditions (CHO and H_2O) in the recovery period. All main effects and interactions were tested using a linear mixed-effects model, with random intercepts to account for repeated measurements within subjects to examine differences between experimental condition, fibre type and subcellular region. Subsequent Bonferroni adjustment post-hoc analysis was used to examine main effects and interactions. Data is presented as mean ± SEM. Significance was accepted at $P < 0.05$.

Results

Lipid analysis:

Pre exercise IMTG content and LD morphology

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

Effect of exercise on IMTG content and LD morphology

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

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

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

Effect of recovery on IMTG content and LD morphology

During recovery from prolonged exercise IMTG content increased significantly in the central region of type I fibres from post-exercise to 4 h post-exercise, and from post-exercise to 24 h post-exercise (time x fibre x region interaction, $P < 0.001$, Fig. 2a). Post-hoc analysis revealed that the increase between 4 h and 24 h post-exercise alone was not significant ($P = 0.160$). No changes in IMTG content occurred in type IIa fibres (Fig. 2b). When comparing CHO and H_2O groups, IMTG content was lower post-exercise in the H_2O condition compared to the CHO condition in both fibre types (condition x time interaction; $P = 0.029$). In the H_2O condition, there was an increase in IMTG content from post-exercise to 4 h post-exercise, and from post-exercise to 24 h post-exercise ($P = 0.014$). In contrast, in the CHO condition IMTG content was not significantly changed from post-exercise to 24 h post-exercise ($P = 1.000$). Importantly though, by 24 h post-exercise IMTG content was statistically similar between conditions ($P > 0.05$). When examining subcellular IMTG distribution during recovery, IMTG in the central region increased from ~77% post-exercise to ~82% 4 h post-exercise, finally returning to pre-exercise distribution by 24 h post-exercise with ~86% of IMTG observed in the central region (main time effect; $P = 0.005$, Table 2). This was mirrored by changes in IMTG distribution in the peripheral region decreasing from ~23% after exercise to ~18% 4 h post-exercise, and finally to ~14% 24 h post-exercise (main time effect; $P = 0.005$, Table 2).

When considering LD number and size, LD number increased in type I fibres from post-exercise to 4 h post-exercise, and from post-exercise to 24 h post-exercise (time x fibre interaction; P = 0.028). More specifically, LD number significantly increased in the H_2O condition from post-exercise to 4 h post-exercise, and from post-exercise to 24 h post-exercise (condition x time interaction; $P = 0.003$, Fig. 2c). No changes in LD number occurred between 4 h post-exercise and 24 h post-exercise. Overall no significant changes were observed in LD size throughout recovery ($P > 0.05$, Fig. 2e &

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

PLIN analysis:

Because significant changes in both IMTG content and LD morphology occurred specifically in type I fibres during exercise and recovery, subsequent PLIN protein content and co-localisation analysis was limited to type I fibres. Importantly, the protein expression of PLIN2, PLIN3 and PLIN5 was unaltered by exercise or recovery in either region in both the CHO and H_2O conditions ($P > 0.05$, Fig 5 & 6). However, there were regional differences in PLIN protein expression, with the central region having greater PLIN content compared to the peripheral region ($P < 0.05$, Table 3,Fig 5 & 6). As well as overall protein content, we examined the co-localisation of PLIN proteins and LD by expressing the number of overlapping objects relative to the total number of PLIN proteins present. Further to this, we examined the number of LD's that either had PLIN (PLIN+ LD), or did not have PLIN associated (PLIN- LD) and also quantified free PLIN (as described previously, 30, 31). The results of these analyses are detailed below.

Effect of exercise on PLIN protein and LD co-localisation

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

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

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

The fraction of PLIN5 co-localised with LD decreased significantly in response to exercise in the central region only (-58%, time x region interaction; $P < 0.001$, Table 4). The number of PLIN5+ LD's decreased by 38% in response to exercise (main effect of time; $P = 0.007$, Fig. 7e), and there tended to be a decrease in the number of PLIN5- LD's ($P = 0.071$, Fig. 7f). Free PLIN5 increased by 20% from pre to post exercise (pre exercise 0.034 ± 0.004, post-exercise 0.041 ± 0.006, $P = 0.021$).

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

The fraction of PLIN2 co-localised with LD significantly increased throughout recovery, specifically within the central region by 58% from post-exercise to 24 h post-exercise (time x region interaction; $P < 0.001$, Table 5). When considering condition, the increased co-localisation between PLIN2 and LD's occurred primarily in the H_2O condition from post-exercise to 24 h post-exercise (time x condition interaction; $P = 0.001$). PLIN2+ LD's increased throughout the recovery period in the central region only from post-exercise to 24 h post exercise (time x region interaction; $P = 0.001$, Fig. 8a). Overall the number of PLIN2+ LD's was 63% greater in the peripheral region compared to the central region across all time points (main effect of region; $P < 0.05$). On the other hand, PLIN2- LD's were unchanged during recovery ($P = 0.611$) and did not differ between conditions ($P = 0.940$). Though when considering region, the number of PLIN2- LD were greater in the peripheral region throughout recovery (main effect of region; $P < 0.05$, Fig. 8b). Free PLIN2 was unaffected throughout the recovery period in both conditions (post-exercise 0.032 ± 0.005, post 4 h 0.025 ± 0.005, post 24 h 0.026 ± 0.005, $P = 0.699$).

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

exercise in the central region ($P = 0.014$), whereas in the peripheral region there was no significant difference from post to 24 h post-exercise ($P = 0.597$, Fig. 8c). In addition, there was a significant difference between regions (main effect of region; $P < 0.05$) with the number of PLIN3+ LDs being ~23% greater in the peripheral region than the central region throughout recovery. Condition had no effect on PLIN3+ LD's during recovery ($P = 0.296$). The number of PLIN3- LD's was significantly different between conditions post-exercise, with the H_2O condition having 68% more PLIN3- LD's than the CHO condition ($P = 0.039$). Overall though, the number of PLIN3- LD did not change during recovery ($P = 0.259$, Fig. 8d). When examining region, the number of PLIN3- LD's was greater in the central region compared to the peripheral region throughout recovery ($P < 0.05$). Free PLIN3 was unchanged throughout the recovery period (post-exercise 0.032 ± 0.006, post 4 h 0.034 ± 0.005, post 24 h 0.033 ± 0.004, $P = 0.787$).

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

Discussion

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

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

In the present study, we aimed to identify if restricting CHO in the post-exercise recovery period would augment the rate of IMTG resynthesis. On first inspection, the data revealed that the rate of IMTG resynthesis was greatest when only H_2O, and not CHO, was ingested during the first 4 h of recovery from prolonged exercise. This was expected, since CHO ingestion would increase circulating insulin concentrations thereby inhibit systemic lipolysis and reducing free fatty acid availability to the muscle. However, it is important to state that there was a significant difference in post-exercise IMTG content between conditions, despite the experimental treatment only being implemented in the post-exercise period. Since, in this case, the starting IMTG values are different between groups, this precludes our ability to draw a firm conclusion as to whether acute CHO restriction can truly accelerate IMTG resynthesis. In this regard, it should be noted that in the study by Gejl *et al.,* (10) from which these muscle samples were derived, a small, albeit non-significant, difference in glycogen utilisation was observed in the CHO condition (527 mmol/kg dw, 73% reduction) compared to the H_2O condition (421 mmol/kg dw, 63% reduction). Further to this, Gejl *et al.,* (10) also noted a slightly greater exercise intensity in the CHO condition (74% vs 71% HR_{max} in the H_2O condition), although again this was not a significant difference. We believe that the combination of the small differences in exercise intensity and glycogen utilisation between the groups may explain, at least partly, the lower IMTG utilisation within the CHO condition in the present study. However, despite the differences in IMTG content between conditions at the post-exercise time point, we did observe an increase in IMTG content during the first 4 h of recovery from prolonged exercise independent of experimental group. Importantly, this increase in IMTG content was sustained, but not improved on, at 24 h post-exercise. Furthermore, IMTG content at 24 h post-exercise had returned to baseline levels. Thus together, these data demonstrate that IMTG resynthesis occurs quickly following exercise, at least in highly-trained individuals. Furthermore, this time-course of IMTG resynthesis is the first of its kind to be described in the literature, and importantly provides a dynamic model of IMTG utilisation during exercise and post-exercise resynthesis that can be used to investigate the potential mechanisms underpinning these process.

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

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

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

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

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

585 to either newly-synthesised LD and/or pre-existing PLIN- LDs. In contrast, PLIN5+
586 LDs were increased throughout the recovery period with a corresponding decrease in
587 the quantity of cytosolic PLIN5. Therefore, unlike PLIN2 and PLIN3, it is the cytosolic
588 pool of PLIN5 that is redistributed to either newly-synthesised LDs and/or pre-existing
589 PLIN- LDs occurred during recovery, underpinning the increased fraction of LDs
590 labelled with PLIN5 at 24 h post-exercise.
591
592 Previous studies in cultured cells and rodent models have implicated the PLIN proteins
593 in supporting fatty acid incorporation into, and storage as, IMTG in LDs (3, 4, 17, 19).
594 The preferential increase in PLIN+ LDs observed during recovery would theoretically
595 support this concept. However, by obtaining muscle samples at both 4 h and 24 h post-
596 exercise we are able to report for the first time a separation in the time-course between
597 growth of the IMTG pool (at 4 h post-exercise) and increases in coating of LDs with
598 PLIN proteins (at 24 h post-exercise). This suggests that the PLIN proteins don't
599 necessarily play a role in IMTG storage in LD's per se. Rather, the coverage of newly-
600 synthesised LD with PLIN proteins at 24 h post-exercise may be an adaptive response
601 to regulate mobilisation and oxidation of IMTG-derived free fatty acids depending on
602 metabolic demand. In this respect, there is a large evidence-base generated in a number
603 of cell types supporting a role for the PLIN proteins in restricting lipolysis under basal
604 conditions (21). Both PLIN3 and PLIN5 may also play a role in IMTG oxidation.
605 Under stimulated conditions, PLIN5 overexpression in cultured cells augments
606 triacylglycerol hydrolysis and fat oxidation (19), through recruitment of LDs to the
607 mitochondrial network (38). We also recently reported that hormone-sensitive lipase is
608 targeted to PLIN5+ LDs in response to exercise (39). Whole-body fat oxidation (7) and
609 *ex vivo* palmitate oxidation (7, 8) are both positively associated with PLIN3 expression,
610 and PLIN3 is expressed in the mitochondrial fraction of sedentary and endurance-
611 trained rats (25). Based on our data, we assert that a redistribution of the PLIN proteins
612 in the post-exercise period is an important adaptation to preserve the flexibility of the
613 intramuscular LD pool to respond appropriately to changes in metabolic demand.
614
615 A strength of the present study is the use of validated immunofluorescence microscopy
616 techniques to examine fibre-type specific changes in IMTG content and LD

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

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

Competing interests:

The authors declare they have no competing interests.

Author contributions:

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

Funding:

The original study was supported by The Danish Ministry of Culture. EFPJ is part-funded by the Tom Reilly Memorial Fund at Liverpool John Moores University.

Acknowledgements:

The antibodies against myosin heavy chain type I (human slow twitch fibres, A4.840) and myosin heavy chain type IIa (human fast twitch fibres, N2.261) used in the study were 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.

References:

1. **Amati F, Dube JJ, Alvarez-Carnero E, Edreira MM, Chomentowski P, Coen PM, Switzer GE, Bickel PE, Stefanovic-Racic M, Toledo FGS, and Goodpaster BH**. Skeletal Muscle Triglycerides, Diacylglycerols, and Ceramides in Insulin Resistance Another Paradox in Endurance-Trained Athletes? *Diabetes* 60: 2588-2597, 2011.

2. **Bergstrom J**. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research *Scandinavian journal of clinical and laboratory investigation* 35: 609 - 616., 1975.

3. **Bosma M, Minnaard R, Sparks LM, Schaart G, Losen M, de Baets MH, Duimel H, Kersten S, Bickel PE, Schrauwen P, and Hesselink MKC**. The lipid droplet coat protein perilipin 5 also localizes to muscle mitochondria. *Histochem Cell Biol* 137: 205-216, 2012.

4. **Bosma M, Sparks LM, Hooiveld GJ, Jorgensen JA, Houten SM, Schrauwen P, Kersten S, and Hesselink MKC**. Overexpression of PLIN5 in skeletal muscle promotes oxidative gene expression and intramyocellular lipid content without compromising insulin sensitivity. *Biochim Biophys Acta Mol Cell Biol Lipids* 1831: 844-852, 2013.

5. **Burke LM, Hawley JA, Wong SHS, and Jeukendrup AE**. Carbohydrates for training and competition. *J Sports Sci* 29: S17-S27, 2011.

6. **Chee C, Shannon CE, Burns A, Selby AL, Wilkinson D, Smith K, Greenhaff PL, and Stephens FB**. Relative Contribution of Intramyocellular Lipid to Whole-Body Fat Oxidation Is Reduced With Age but Subsarcolemmal Lipid Accumulation and Insulin Resistance Are Only Associated With Overweight Individuals. *Diabetes* 65: 840-850, 2016.

7. **Covington JD, Galgani JE, Moro C, LaGrange JM, Zhang ZY, Rustan AC, Ravussin E, and Bajpeyi S**. Skeletal Muscle Perilipin 3 and Coatomer Proteins Are Increased following Exercise and Are Associated with Fat Oxidation. *PLoS One* 9: 8, 2014.

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

9. **Coyle EF, Jeukendrup AE, Oseto MC, Hodgkinson BJ, and Zderic TW**. Low-fat diet alters intramuscular substrates and reduces lipolysis and fat oxidation during exercise. *Am J Physiol-Endocrinol Metab* 280: E391-E398, 2001.

10. **Gejl KD, Hvid LG, Frandsen U, Jensen K, Sahlin K, and Ortenblad N**. Muscle Glycogen Content Modifies SR Ca2+ Release Rate in Elite Endurance Athletes. *Med Sci Sports Exerc* 46: 496-505, 2014.

11. **Gemmink A, Bosma M, Kuijpers HJH, Hoeks J, Schaart G, van Zandvoort M, Schrauwen P, and Hesselink MKC**. Decoration of intramyocellular lipid droplets with PLIN5 modulates fasting-induced insulin resistance and lipotoxicity in humans. *Diabetologia* 59: 1040-1048, 2016.

12. **Gemmink A, Daemen S, Kuijpers HJH, Schaart G, Duimel H, Lopez-Iglesias C, van Zandvoort M, Knoops K, and Hesselink MKC**. Super-resolution microscopy localizes perilipin 5 at lipid droplet-mitochondria interaction sites and at lipid droplets juxtaposing to perilipin 2. *Biochim Biophys Acta Mol Cell Biol Lipids* 1863: 1423-1432, 2018.

13. **Impey SG, Hearris MA, Hammond KM, Bartlett JD, Louis J, Close GL, and Morton JP**. Fuel for the Work Required: A Theoretical Framework for Carbohydrate Periodization and the Glycogen Threshold Hypothesis. *Sports Med* 48: 1031-1048, 2018.

14. **Jensen L, Gejl KD, Ortenblad N, Nielsen JL, Bech RD, Nygaard T, Sahlin K, and Frandsen U**. Carbohydrate restricted recovery from long term endurance exercise does not

affect gene responses involved in mitochondrial biogenesis in highly trained athletes. *Physiol Rep* 3: 13, 2015.

15. **Johnson NA, Stannard SR, Mehalski K, Trenell MI, Sachinwalla T, Thompson CH, and Thompson MW**. Intramyocellular triacylglycerol in prolonged cycling with high- and low-carbohydrate availability. *J Appl Physiol* 94: 1365-1372, 2003.

16. **Kiens B**. Skeletal muscle lipid metabolism in exercise and insulin resistance. *Physiol Rev* 86: 205-243, 2006.

17. **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, and Richter EA**. mTORC2 and AMPK differentially regulate muscle triglyceride content via Perilipin 3. *Mol Metab* 5: 646-655, 2016.

18. **Koh HCE, Nielsen J, Saltin B, Holmberg HC, and Ortenblad N**. Pronounced limb and fibre type differences in subcellular lipid droplet content and distribution in elite skiers before and after exhaustive exercise. *J Physiol-London* 595: 5781-5795, 2017.

19. **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, and Moro C**. Perilipin 5 fine-tunes lipid oxidation to metabolic demand and protects against lipotoxicity in skeletal muscle. *Sci Rep* 6: 12, 2016.

20. **MacPherson REK, Herbst EAF, Reynolds EJ, Vandenboom R, Roy BD, and Peters SJ**. 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, 2012.

21. **MacPherson REK, and Peters SJ**. Piecing together the puzzle of perilipin proteins and skeletal muscle lipolysis. *Appl Physiol Nutr Metab* 40: 641-651, 2015.

22. **Minnaard R, Schrauwen P, Schaart G, Jorgensen JA, Lenaers E, Mensink M, and Hesselink MKC**. 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, 2009.

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

24. **Pourteymour S, Lee S, Langleite TM, Eckardt K, Hjorth M, Bindesboll C, Dalen KT, Birkeland AI, Drevon CA, Holen T, and Norheim F**. Perilipin 4 in human skeletal muscle: localization and effect of physical activity. *Physiol Rep* 3: 15, 2015.

25. **Ramos SV, Turnbull PC, MacPherson REK, LeBlanc PJ, Ward WE, and Peters SJ**. 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, 2015.

26. **Rodriguez NR, DiMarco NM, Langley S, Denny S, Hager MH, Manore MM, Myers E, Meyer N, Stevens J, Webber JA, Benedict R, Booth M, Chuey P, Erdman KA, Ledoux M, Petrie H, Lynch P, Mansfield E, Barr S, Benardot D, Berning J, Coggan A, Roy B, Vislocky LM, Amer Dietet A, Amer Coll Sports M, and Dietitians C**. Nutrition and Athletic Performance. *Med Sci Sports Exerc* 41: 709-731, 2009.

27. **Shaw C, Sherlock M, Stewart P, and Wagenmakers A**. Adipophilin distribution and colocalisation with lipid droplets in skeletal muscle. *Histochem Cell Biol* 131: 575-581, 2009.

28. **Shaw CS, Shepherd SO, Wagenmakers AJM, Hansen D, Dendale P, and van Loon LJC**. 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-E1165, 2012.

29. **Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJM, and Shaw CS**. Preferential utilization of perilipin 2-associated intramuscular triglycerides during 1 h of moderate-intensity endurance-type exercise. *Exp Physiol* 97: 970-980, 2012.

30. **Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, Burniston JG, Wagenmakers AJM, and Shaw CS**. Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5. *J Physiol-London* 591: 657-675, 2013.

31. **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. *J Physiol-London* 595: 5587-5601, 2017.

32. **Skinner JR, Shew TM, Schwartz DM, Tzekov A, Lepus CM, Abumrad NA, and Wolins NE**. Diacylglycerol Enrichment of Endoplasmic Reticulum or Lipid Droplets Recruits Perilipin 3/TIP47 during Lipid Storage and Mobilization. *J Biol Chem* 284: 30941-30948, 2009.

33. **Starling RD, Trappe TA, Parcell AC, Kerr CG, Fink WJ, and Costill DL**. Effects of diet on muscle triglyceride and endurance performance. *J Appl Physiol* 82: 1185-1189, 1997.

34. **Stellingwerff T, Boon H, Jonkers RAM, Senden JM, Spriet LL, Koopman R, and van Loon LJC**. Significant intramyocellular lipid use during prolonged cycling in endurance-

792 trained males as assessed by three different methodologies. *Am J Physiol-Endocrinol*
793 *Metab* 292: E1715-E1723, 2007.

794 35. **van Loon LJC**. Intramyocellular triacylglycerol as a substrate source during exercise. *Proc*
795 *Nutr Soc* 63: 301-307, 2004.

796 36. **van Loon LJC, Schrauwen-Hinderling VB, Koopman R, Wagenmakers AJM,**
797 **Hesselink MKC, Schaart G, Kooi ME, and Saris WHM**. Influence of prolonged endurance
798 cycling and recovery diet on intramuscular triglyceride content in trained males. *Am J Physiol-*
799 *Endocrinol Metab* 285: E804-E811, 2003.

800 37. **Vissing K, Andersen JL, and Schjerling P**. Are exercise-induced genes induced by
801 exercise? *Faseb J* 18: 94-+, 2004.

802 38. **Wang H, Sreenevasan U, Hu H, Saladino A, Polster BM, Lund LM, Gong DW, Stanley**
803 **WC, and Sztalryd C**. Perilipin 5, a lipid droplet-associated protein, provides physical and
804 metabolic linkage to mitochondria. *J Lipid Res* 52: 2159-2168, 2011.

805 39. **Whytock KL, Shepherd SO, Wagenmakers AJM, and Strauss JA**. Hormone-sensitive
806 lipase preferentially redistributes to lipid droplets associated with perilipin-5 in human skeletal
807 muscle during moderate-intensity exercise. *J Physiol-London* 596: 2077-2090, 2018.

808 40. **Wolins NE, Quaynor BK, Skinner JR, Schoenfish MJ, Tzekov A, and Bickel P**. S3-12,
809 adipophilin, and TIP47 package lipid in adipocytes. *J Biol Chem* 280: 19146-19155, 2005.

810 41. **Wolins NE, Rubin D, and Brasaemle DL**. TIP47 associates with lipid droplets. *J Biol*
811 *Chem* 276: 5101-5108, 2001.

42. **Zhang HN, Wang Y, Li J, Yu JH, Pu J, Li LH, Zhang HC, Zhang SY, Peng G, Yang FQ, and Liu PS**. Proteome of Skeletal Muscle Lipid Droplet Reveals Association with Mitochondria and Apolipoprotein A-I. *J Proteome Res* 10: 4757-4768, 2011.

837 **Tables**

838

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

840

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

841

842 IMTG content and LD number are expressed relative to the area of the peripheral or
843 central region. Data are means ± S.E.M. * Significantly greater in type I fibres ($P <$
844 0.05).

845

846

847

848

849

850

851

852

853

854

855

856

857

858

859

860

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

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

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

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

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

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

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

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

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

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

Time Point	Condition	Region	PLIN2	PLIN3	PLIN5
Post	CHO	Peripheral	0.58 ± 0.22	0.53 ± 0.19	0.42 ± 0.24
		Central	0.31 ± 0.17	0.26 ± 0.09	0.23 ± 0.12
	H_2O	Peripheral	0.46 ± 0.08	0.49 ± 0.06	0.53 ± 0.06
		Central	0.22 ± 0.11	0.36 ± 0.07	0.23 ± 0.07
Post 4 h	CHO	Peripheral	0.71 ± 0.13	0.54 ± 0.07	0.47 ± 0.23
		Central	0.48 ± 0.16	0.35 ± 0.08	0.40 ± 0.20
	H_2O	Peripheral	0.49 ± 0.22	0.62 ± 0.12	0.54 ± 0.11
		Central	0.33 ± 0.26	0.45 ± 0.24	0.41 ± 0.21
Post 24 h	CHO	Peripheral	0.62 ± 0.17	0.58 ± 0.06	0.48 ± 0.22
		Central	0.57 ± 0.21	0.49 ± 0.16*	0.58 ± 0.25*
	H_2O	Peripheral	0.62 ± 0.17	0.56 ± 0.08	0.49 ± 0.06
		Central	0.54 ± 0.21*	0.50 ± 0.11*	0.57 ± 0.21*

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

Figure Legends:

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

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

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

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

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

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

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

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

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

Figure 5. PLIN protein expression in response to exercise.

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

Figure 6. PLIN protein expression content during recovery.

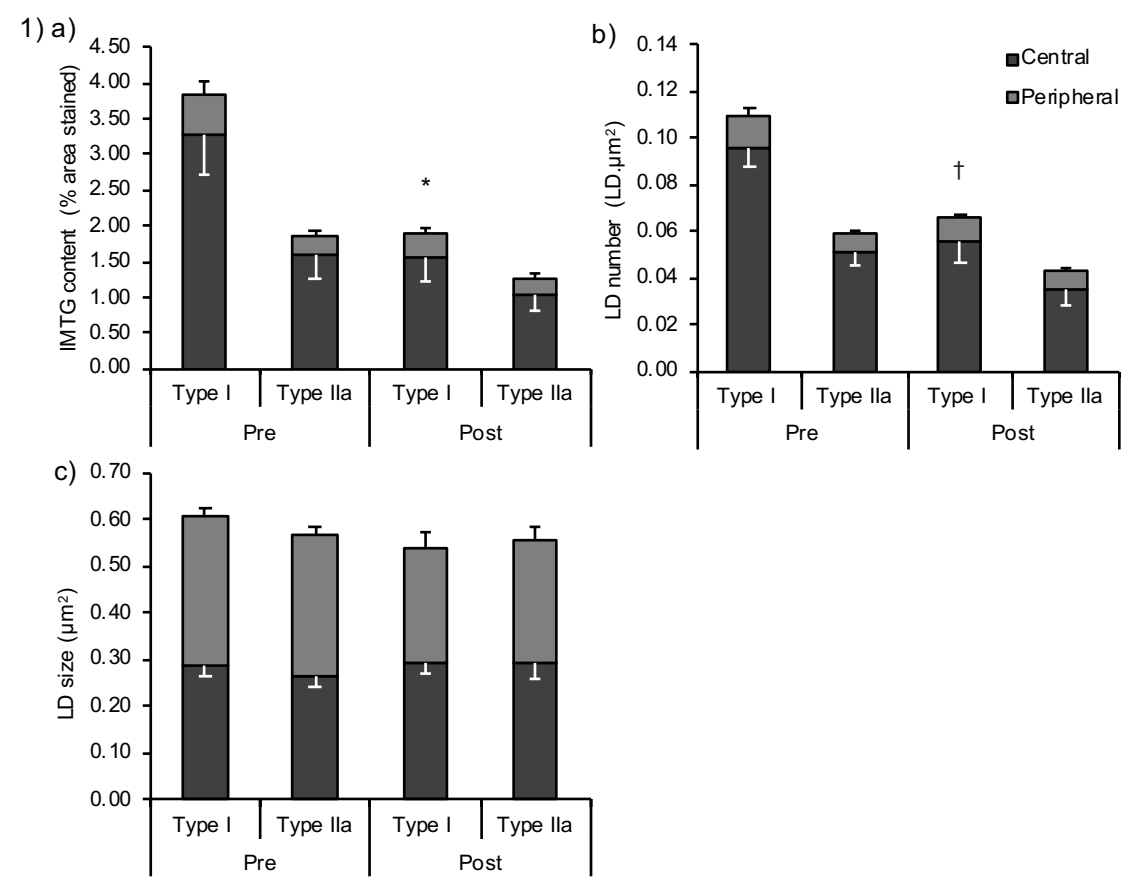
No significant changes in overall PLIN2 (a), PLIN3 (b) and PLIN5 (c) content during recovery in either experimental condition ($P > 0.05$).

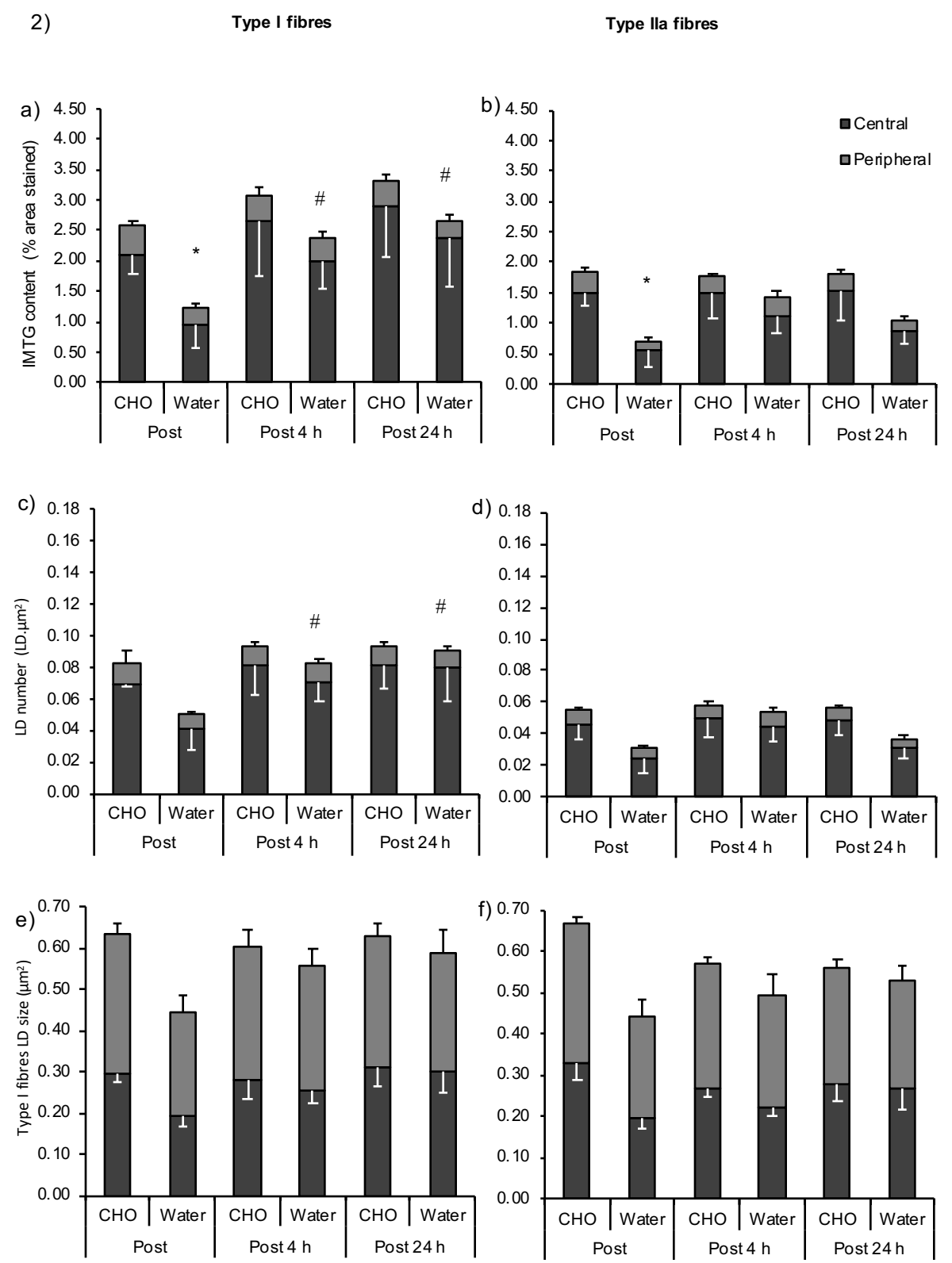
Figure 7. Subcellular-specific changes in the number of PLIN+ and PLIN- LDs in type I fibres in response to prolonged exercise.

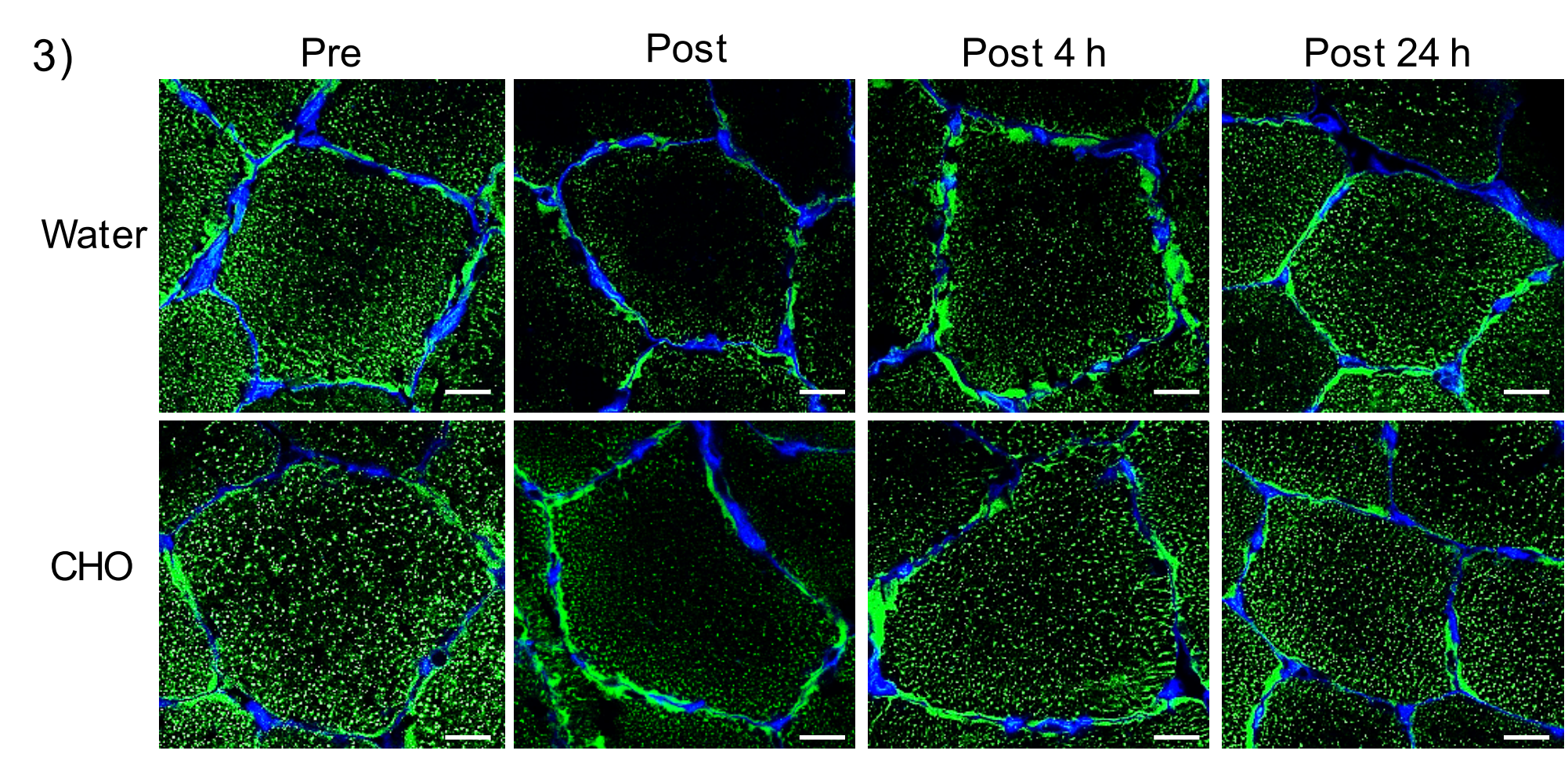
The effect of exercise on a) PLIN2+ LD, b) PLIN2- LD, c) PLIN3+ LD, d) PLIN3- LD, e) PLIN5+ LD and f) PLIN5- LD. *Significant decrease in PLIN2+ LD and PLIN2- LD in both peripheral and central regions (time x region interaction effect, $P < 0.05$). #Significant decrease in PLIN3+ LD, PLIN3- LD and PLIN5+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

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

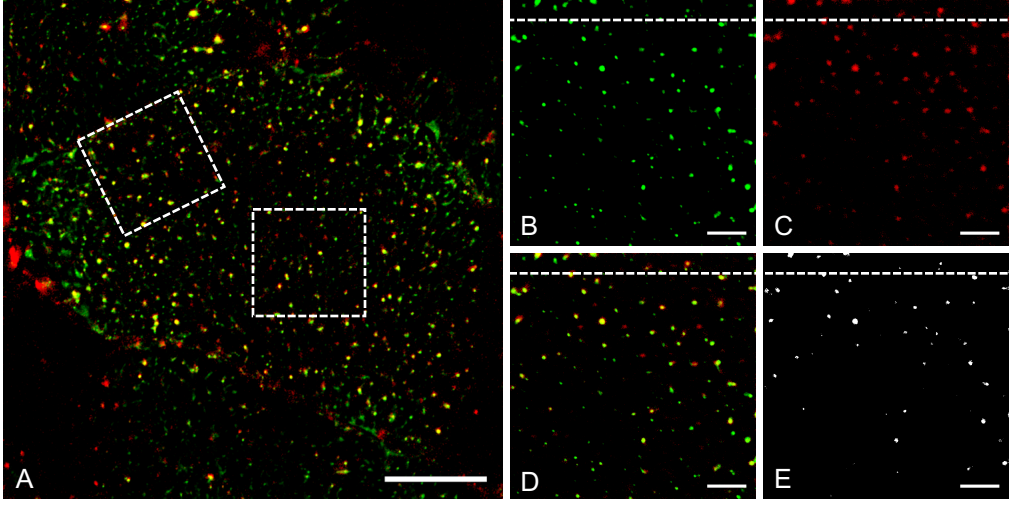
The effect of recovery on a) PLIN2+ LD, b) PLIN2- LD, c) PLIN3+ LD, d) PLIN3- LD, e) PLIN5+ LD and f) PLIN5- LD. *Significant increase during recovery from post-exercise to 24 h post-exercise ($P < 0.05$) with no difference between conditions. Values are means ± S.E.M.


1) a)
IMTG content (% area stained)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
Type I
Type IIa
Type I
Type IIa
Pre
Post
b)
LD number (LD.µm²)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Central
Peripheral
†
Type I
Type IIa
Type I
Type IIa
Pre
Post
c)
LD size (µm²)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Type I
Type IIa
Type I
Type IIa
Pre
Post

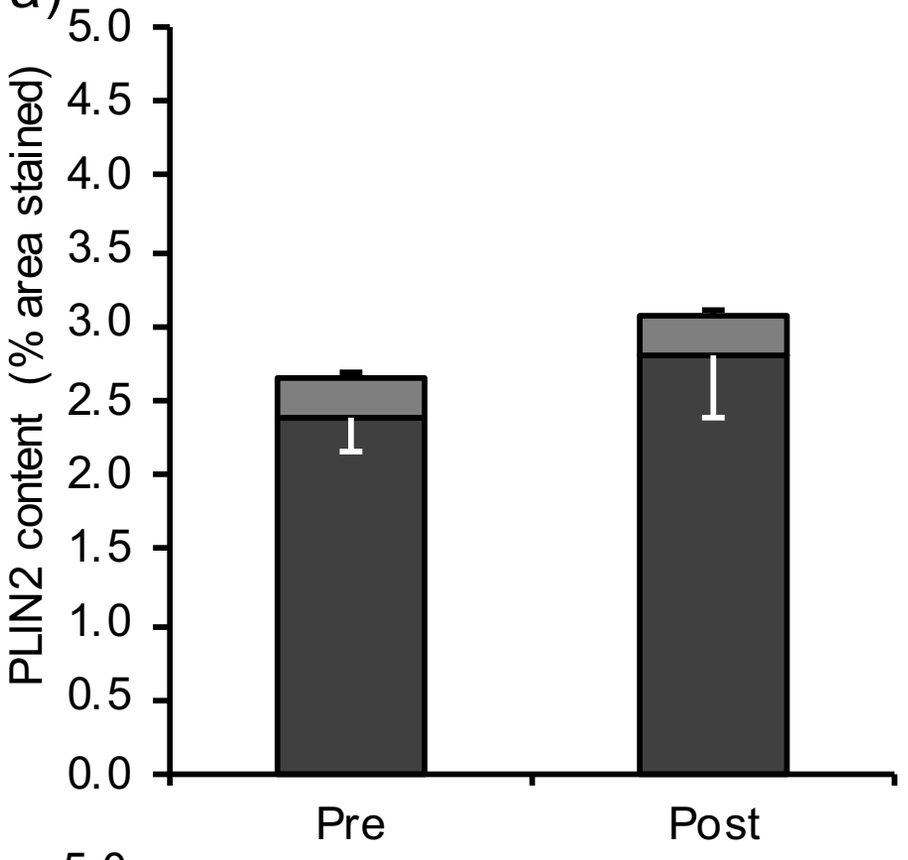

2)
Type I fibres
Type IIa fibres
a)
b)
c)
d)
e)
f)
IMTG content (% area stained)
LD number (LD.µm²)
Type I fibres LD size (µm²)
Central
Peripheral
CHO
Water
Post
Post 4 h
Post 24 h
*
#


3)
Pre
Post
Post 4 h
Post 24 h
Water
CHO

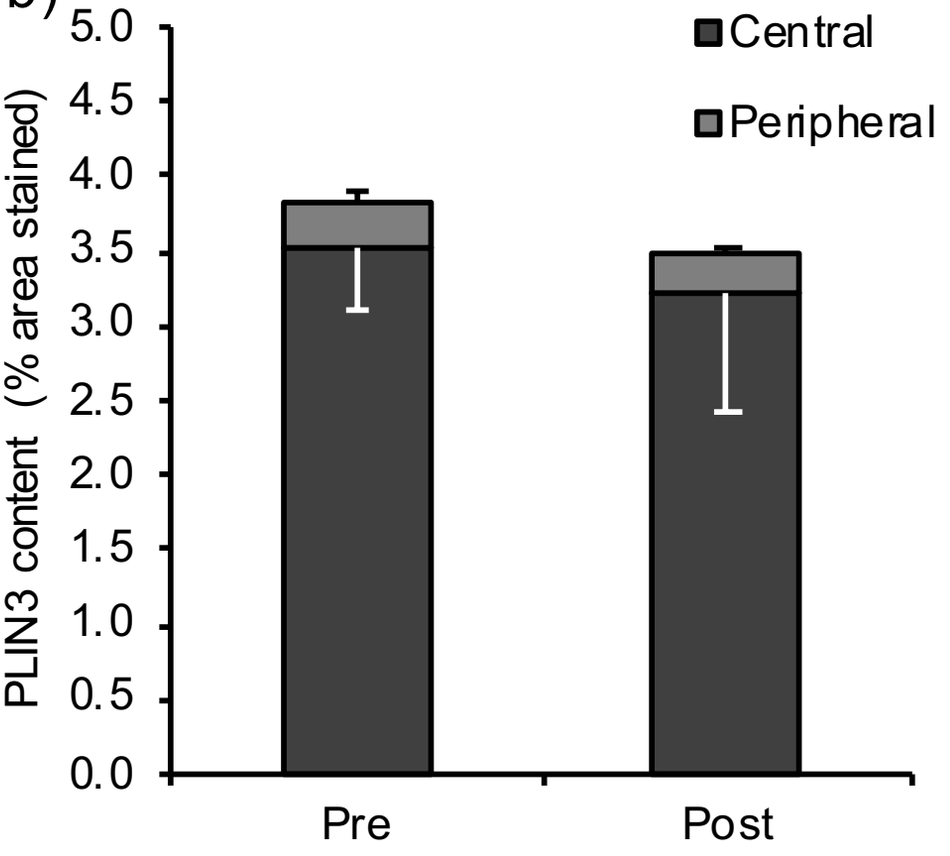
4)



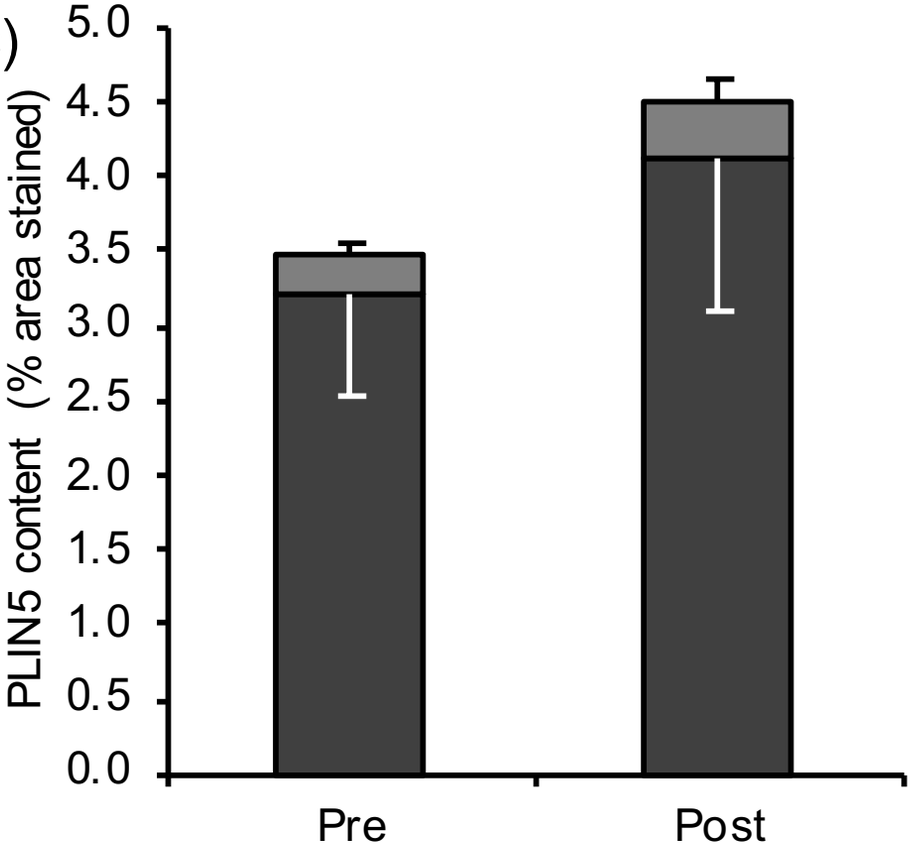
5) a)


PLIN2 content (% area stained)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Pre
Post

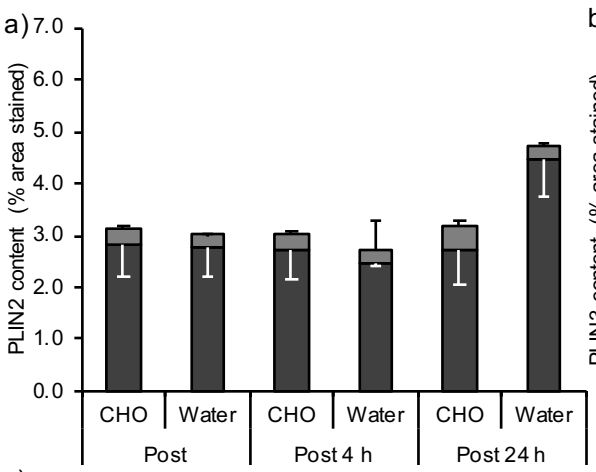
b)

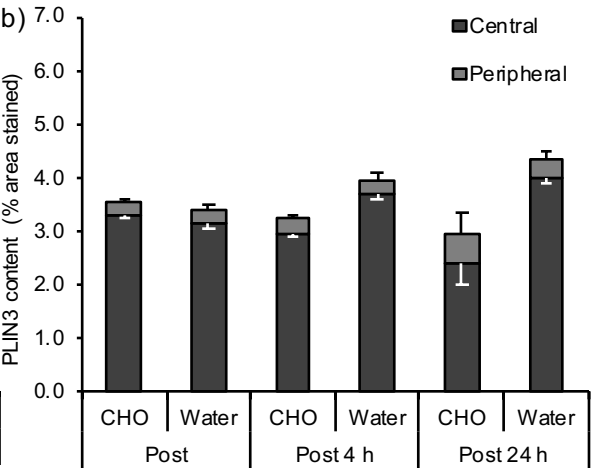

Central
Peripheral
PLIN3 content (% area stained)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Pre
Post

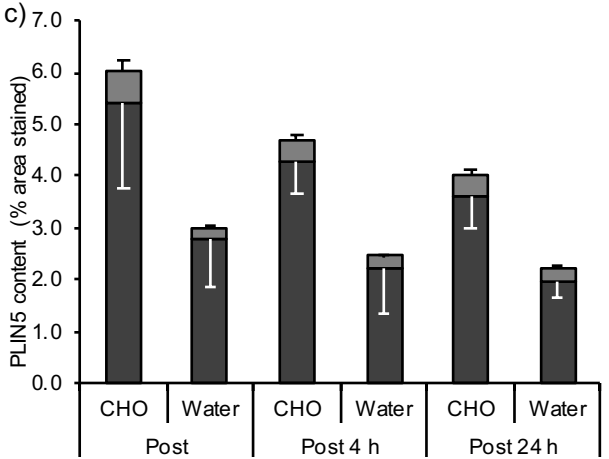
c)

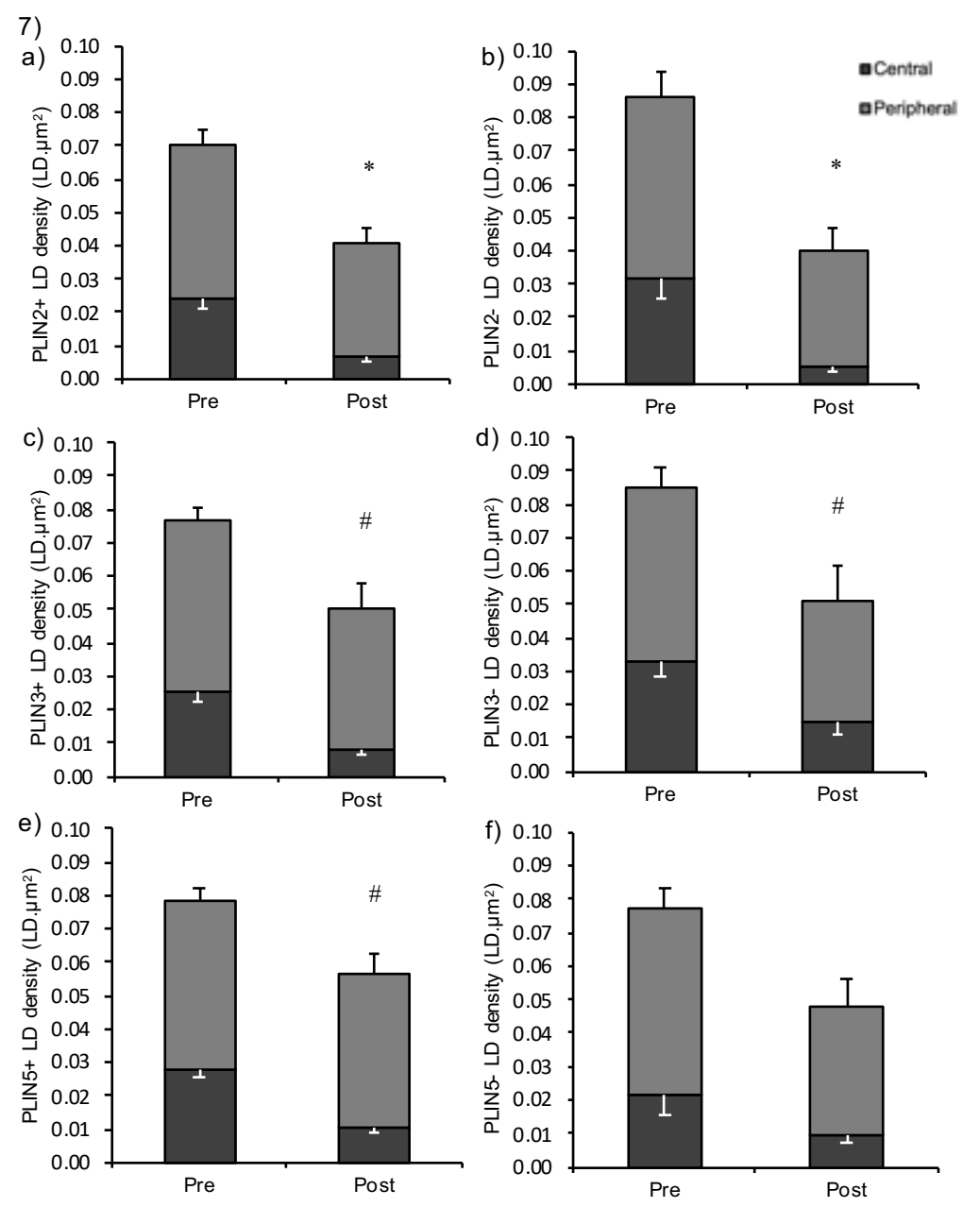

PLIN5 content (% area stained)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Pre
Post

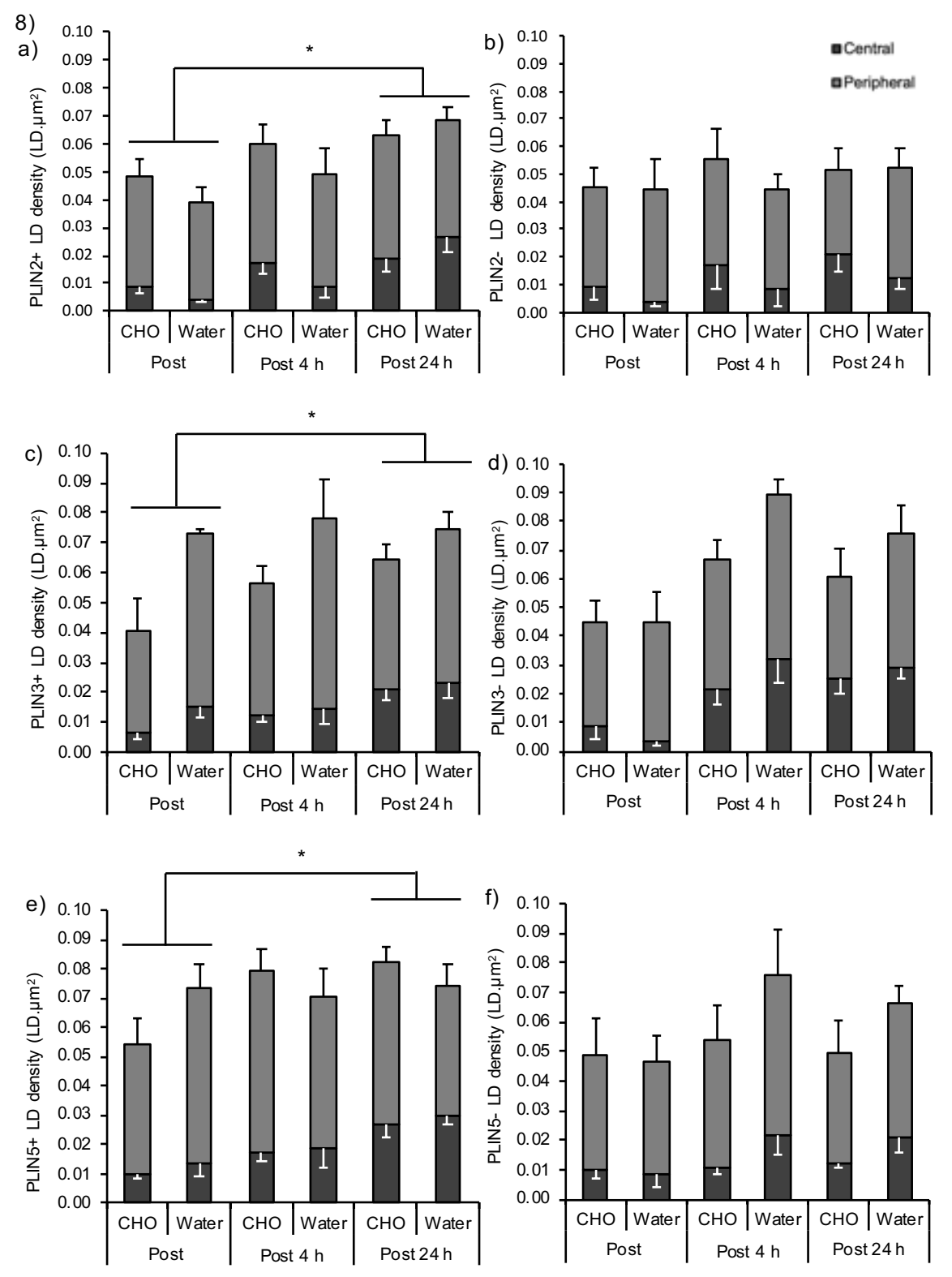
6)


a)
PLIN2 content (% area stained)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
CHO
Water
CHO
Water
CHO
Water
Post
Post 4 h
Post 24 h


b)
PLIN3 content (% area stained)
Central
Peripheral
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
CHO
Water
CHO
Water
CHO
Water
Post
Post 4 h
Post 24 h


c)
PLIN5 content (% area stained)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
CHO
Water
CHO
Water
CHO
Water
Post
Post 4 h
Post 24 h


7)
a)
b)
c)
d)
e)
f)
PLIN2+ LD density (LD.µm²)
PLIN2- LD density (LD.µm²)
PLIN3+ LD density (LD.µm²)
PLIN3- LD density (LD.µm²)
PLIN5+ LD density (LD.µm²)
PLIN5- LD density (LD.µm²)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
Pre
Post
Central
Peripheral
*
#


8)
a)
b)
c)
d)
e)
f)
PLIN2+ LD density (LD.µm²)
PLIN2- LD density (LD.µm²)
PLIN3+ LD density (LD.µm²)
PLIN3- LD density (LD.µm²)
PLIN5+ LD density (LD.µm²)
PLIN5- LD density (LD.µm²)
Central
Peripheral
*
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
CHO
Water
Post
Post 4 h
Post 24 h