Exploring metabolic and molecular mechanisms regulating age-related declines in human skeletal muscle regenerative capacity

Miss Colleen Siobhan Deane

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Copyright statement

Abstract

The underlying mechanisms regulating the ability of skeletal muscle to regenerate after acute "damaging" eccentric or "non-damaging" concentric exercise in young human skeletal muscle is poorly defined. Age-related impairments in the regenerative mechanisms may contribute to the age-related loss of muscle mass and function, which has negative consequences for overall health and disease. Thus, the first aim of this thesis was to initially investigate multiple targeted mechanisms previously implicated in the regeneration process, over a comprehensive time-course following eccentric versus concentric exercise in young adults. Within this study it was found that post-exercise, in general, increased anabolic and repressed catabolic signalling preceded functional decline, whereas inflammation and ubiquitin proteasome system-related breakdown increased once functional recovery was initiated/achieved. Eccentric exercise led to greater anabolic signalling and inflammatory signalling response. As such, this study has provided a benchmark of muscle regeneration in young skeletal muscle, which implicates early anabolic and catabolic regulation in the rapid adaptation of muscle, whereas inflammation and ubiquitin proteasome system-related breakdown likely mediate longer term remodelling/adaptations, which may be greater following eccentric exercise. Using this benchmark, the aim of the second study was to identify age-related changes in targeted regenerative mechanisms. Concentric exercise did not cause a molecular regenerative response, whilst eccentric exercise induced anabolic signalling and satellite cell activation, prior to and at the nadir of force, respectively. Compared to the younger adults, ageing *per se* was associated with increased inflammation, whilst anabolic and catabolic signalling post-eccentric and concentric exercise was blunted. Interestingly, satellite cell activity was induced in the old only following eccentric

exercise. These data suggest that eccentric exercise is potentially more advantageous for promoting muscle growth versus concentric exercise in older adults. Whilst, compared to the young, the old displayed blunted molecular responses which might underlie blunted muscle growth during ageing. Furthermore, the activation of satellite cells in the old might be the result of the impaired molecular mechanisms being suboptimal for repair thus, requiring additional regenerative means. In order to further characterise ageing muscle and the mechanisms of muscle regeneration, RNA sequencing was performed at the time of peak anabolic signalling to highlight more global and novel molecular networks. Ageing *per se* revealed genes involved in blood vessel development, plasma membrane and cell-cell junction expression were down-regulated, thus implicating these processes in age-related muscle loss. Following concentric exercise in older adults, there was an up-regulation of structural transcripts whilst there was a general down-regulation of genes related to metabolism, which might suggest impaired metabolism post-concentric exercise. Perhaps the blunted transcript responses contribute to the often observed age-related blunting of muscle mass adaptations in response to exercise training. Collectively, the data from this thesis has important implications for developing interventions for maximising hypertrophic responses and for counteracting the suboptimal regenerative responses observed in older adults.

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overlap exists between up-regulated (top left Venn) and down-regulated (top right Venn) genes, 5 h after CON and ECC exercise in young participants. Up-regulated (bottom left Venn) and down-regulated (bottom right Venn) genes in older participants after CON and ECC exercise also display predominant overlap.

Figure 5.4. Transcriptomic profile of older participants 5 h post-CON exercise. (A) increased expression of GO term networks associated with cytoskeletal, cell adhesion and extra-cellular matrix (bottom left red insert) and blood vessel development (top right) (B) reduced expression of GO term network associated with mitochondrial metabolism (blue insert). Each node in the network represents a GO term with the size of each node corresponding to the number of genes associated with that term. Significantly enriched terms are coloured yellow with more significant terms a deeper shade.

Figure 5.5. Unique age-related transcriptomic response to CON and ECC exercise. Overlap of age-associated differentially expressed genes within contraction modes. Both young and older participants display unique signatures of differential gene expression 5 h post-CON (up-regulated, top left Venn; down-regulated, top right Venn) and post-ECC (up-regulated, bottom left Venn; down-regulated, bottom right Venn) exercise.

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Authors Declaration

I declare that the research within this thesis is the original work of the author, unless otherwise indicated within the text. This thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Abbreviations

1-RM	One repetition maximum
3MeH	3-methylistidine
4E-BP1	4E binding protein-1
20S	Ubiquitin 20S proteasome
AA	Amino acid
APE	Atom percent excess
BMI	Body mass index
CD68	Cluster of differentiation 68
CHO	Carbohydrate
CK	Creatine kinase
CON	Concentric
D_2O	Deuterium oxide
DOMS	Delayed onset muscle soreness
DNA	Deoxyribonucleic acid
E2	Ubiquitin conjugate enzyme
EAA	Essential amino acid
ECG	Electrocardiogram
ECM	Extracellular matrix
ECC	Eccentric
EDL	Extensor digitorum longus

eEF2	Eukaryotic elongation factor 2
EIMD	Exercise induced muscle damage
ES	Electrical stimulation
FAK	Focal adhesion kinase
FSR	Fractional synthesis rate
GC	Gas chromatography
HRP	Horseradish peroxidase
IL-1β	Interleukin 1 beta
IL-1ra	Interleukin 1 receptor antagonist
IL-6	Interleukin 6
IL-8	Interleukin 8
IRMS	Isotope ratio mass spectrometer
LEP	Leg extensor power
MAPK	Mitogen-activated protein kinase
MEB	Mitochondrial extraction buffer
MLP	Modified leg press
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger ribose nucleic acid
mTOR	Mechanistic/ mammalian target of rapamycin
MuRF1	Muscle RING finger protein-1

MVC	Maximal voluntary contraction
NCME	*N*-methoxycarbonyl methyl esters
NF-κβ	Nuclear factor-kappaB
NPB	Net protein balance
NSAIDS	Nonsteroidal anti-inflammatory drugs
OCT	Optimal cutting temperature
p70SK1	Ribosomal protein s6 kinase
PA	Phosphatidic acid
Pax7	Paired box protein 7
PBS	Phosphate buffer saline
PLD	Phospholipase D
PPT	Pain pressure threshold
RBE	Repeated bout effect
RE	Resistance exercise
RET	Resistance exercise training
RPE	Rate of perceived exertion
rps6	ribosomal protein s6
SC	Satellite cell
SPPBT	Short physical performance battery test
TA	Tibialis anterior
TC/EA	High temperature conversion elemental analyser

TNF-α	Tumour necrosis factor-α
tRNA	Transfer ribonucleic acid
UBI	Ubiquitin
UPS	Ubiquitin proteasome system
VAS	Visual analogue scale
VSMOW	Vienna standard mean ocean water

1 Literature Review

1.1 Skeletal Muscle: importance and plasticity

1.1.1 The physiological role of muscle

Skeletal muscle is the largest organ in the human body accounting for the majority of lean body mass and ~40-50% of whole body mass (188, 334). In occupying such mass, skeletal muscle performs key mechanical and biological functions essential to human life. One of the primary functions is to produce force and movement and maintain posture via the conversion of chemical into mechanical energy such that muscles pull on the bony lever system allowing joint movement (111, 167). This system permits the performance of necessary tasks for daily living such as rising from a chair and the more complex activities such as resistance exercise (RE).

Another fundamental role of skeletal muscle is its contribution to regulating whole body protein, carbohydrate (CHO), and fat metabolism (99, 134, 318, 340). Skeletal muscle is the principal store of amino acids (AA), which are called upon by other tissues in times of need (i.e. starvation, burn injury) to synthesise organ-specific proteins (111, 354). Skeletal muscles provide the largest site for glucose uptake (74, 318), which if disrupted has pathological consequences, such as the development of type II diabetes mellitus (75). Furthermore, skeletal muscle is a store for triglycerides, which are metabolised for the liberation of energy during exercise, however pathological increases in myocellular lipid content, as a result of altered metabolism, are observed in obesity (162).

Thus, skeletal muscle is critical for locomotory and metabolic functions in humans. However, during ageing there is the loss of muscle mass and function, termed

sarcopenia and dynapenia, respectively (223, 286). The loss of muscle strength (~ 2-4% per year) greatly exceeds the loss of muscle mass (~ 1% per year) (223). This was demonstrated in a five year follow up study period in 1678 older males, which found muscle torque declined by 16.1% where as the loss of thigh muscle mass equaled 5% (78). Reduced muscle strength is associated with poor physical function (330), increased falls (355) and is a predictor of all cause mortality (189). Moreover, the loss of muscle mass, mainly due to the loss of contractile protein, is associated with higher all-cause mortality (315), whereas greater muscle mass is associated with reduced all-cause mortality (308). In addition to ageing, detrimental declines in muscle mass and strength are observed in cases of muscle disuse and disease (295, 336). As such, the maintenance of skeletal muscle mass is essential for the preservation of functional and metabolic health throughout one's lifespan.

1.1.2 Muscle plasticity

Skeletal muscle is a highly plastic tissue capable of structural (i.e. increased muscle mass) and functional (i.e. increased muscle strength) remodelling in response to physiological demands such as exercise. For example, increases in skeletal muscle mass (hypertrophy) and strength are observed following 3 weeks of chronic resistance exercise training (RET) (38). Moreover, such plasticity means that muscle mass and function also rapidly deteriorate in response to pathophysiological cues such as disease and inactivity. To demonstrate, as little as 5 days of muscle disuse lead to decreases in skeletal muscle mass and strength (336).

Unaccustomed acute RE can induce disorganisation of the muscle contractile proteins leading to acute muscle dysfunction (i.e. reductions in force), which is typically restored within ~7 days owing to the intrinsic capability of muscles to regenerate (270). It is this process of rapid muscular regeneration that underpins its remarkable plasticity in response to exercise stimuli, whereby progressive accumulation of repeated regenerative responses to individual exercise bouts ultimately translate into muscle mass and functional gains. Muscle also rapidly adapts to initial unaccustomed RE by being less susceptible to muscle functional decline and sensations of muscle soreness during subsequent bouts of exercise, termed the repeated bout effect (RBE) (149). An impaired muscle regenerative capacity is associated with loss of muscle mass maintenance and health complications. For example, impaired muscle regeneration observed in the muscular dystrophies results in progressive muscle weakness and atrophy, culminating in early death (139). Understanding the mechanisms underpinning the processes of muscular regeneration from injurious events, such as unaccustomed RE, will therefore have important implications not only for optimising the hypertrophic adaptations to chronic RET but also for developing targeted countermeasures against pathological conditions of muscle wasting characterised by poor muscle regenerative capacity.

Within the human literature the terms muscle regeneration, muscle repair and muscle remodelling/adaptation are often interchangeably used and there is yet to be a specific and widely accepted definition for each of these terms. As such, it is imperative that these are defined within the context of this thesis from the outset. Herein, muscle regeneration is defined as; any metabolic or molecular mechanism which is exercise and is regulated (i.e. up/down regulated) between the onset of

functional decline up to the cessation of adaptive responses (i.e. mechanisms which are still regulated in response to acute exercise even once force has returned to baseline). As such, the term muscle regeneration within this thesis encompasses muscle repair which is defined as; mechanisms that are regulated between the nadir of force and until basal force is restored, and muscle remodeling/ adaptation which is defined as; mechanisms that are regulated beyond the restoration of muscle function.

1.2 Regulation of skeletal muscle plasticity by muscle protein turnover in response to acute exercise

The balance between the synthesis and the breakdown of muscular proteins (i.e. net protein balance (NPB)) determines skeletal muscle mass. On a day-to-day basis, during the post-absorptive period muscle protein breakdown (MPB) (0.08-0.11%/h (260)) exceeds muscle protein synthesis (MPS) (0.03-0.07%/h (71, 172, 341)) i.e. MPB>MPS, creating a net negative protein balance and loss of muscular proteins (39). With the provision of nutrients, particularly AA, there is a significant (~3 fold) but transient (~1.5-2 h) increase in MPS (12) and depression in MPB (~50%) (345) i.e. MPB<MPS, leading to a positive net balance and gain of muscle proteins. This dynamic equilibrium exists such that on a day-to-day basis net balance is neutral and mass is maintained (MPB=MPS). Cumulative periods of positive NPB results in muscle mass gains (38), whereas cumulative periods of negative NPB results in the loss of muscle mass (atrophy) (260).

Muscle protein turnover is critical for effective muscle regeneration following acute exercise and for muscle hypertrophy following chronic RET, since it governs the removal of damaged proteins (MPB) (45, 131) and deposits new functional proteins

essential for the restoration of muscle structure and function (MPS). The deposition and removal of proteins during the regenerative response to acute exercise may be a rapidly up-regulated acute response regulating the restoration of muscle function (23). The regulation of muscle protein turnover may also persist once function has been recovered to allow the muscle to remodel and adapt. Perturbations in either the synthesis and/or breakdown response following acute RE may attenuate acute structural and functional repair of skeletal muscles. Such perturbations could prolong functional deficits and structural damage, which may culminate in blunted muscle hypertrophy in response to chronic RET. However, the precise temporal interplay between MPS and MPB and how this relates to function following acute exercise is poorly defined.

1.2.1 Muscle protein synthesis and anabolic signals

1.2.1.1 Overview

The nucleus of skeletal muscle fibres contain deoxyribonucleic acid (DNA), which contains the genetic material used for the synthesis of proteins. Two key processes; transcription and translation, are needed to generate proteins from DNA. Transcription is the process of transcripting genetic information from DNA into messenger ribonucleic acid (mRNA). The process of translating mRNA into protein is translation and is synchronized by three distinct stages: initiation, elongation and termination, of which initiation and elongation are principally regulated in response to exercise stimuli.

Anabolic stimuli regulating muscle protein synthesis include exercise, nutrients and

hormones, which typically converge at the protein complex, mammalian target of rapamycin complex 1 (mTORC1) (Figure 1.1) (165). The phosphorylation of mTORC1 leads to the phosphorylation of the downstream protein substrate, ribosomal protein s6 kinase (p70S6K1), thereby phosphorylating eIF4B and eEF2, in turn enhancing protein translation (268). Additionally, phosphorylated p70S6K1 can enhance the activity of ribosomal protein s6 (rps6), which is increased in situations of enhanced protein synthesis (220). mTORC1 can also phosphorylate eukaryotic initiation factor 4E- binding protein (4EBP1), which is normally bound to eIF4E preventing the binding to eIF4G and thus the formation of the initiation complex (267, 285) inhibiting protein synthesis. When phosphorylated, eIF4E is released such that the initiation complex can be formed. As such, the regulation of mTORC1 activity can enhance translation initiation and elongation thus enhancing muscle protein synthesis. Although much regulation of exercise/nutrient/hormone-induced protein synthesis is regulated via the mTORC1 signalling pathway, other pathways such as the protein kinase B (PKB) (PKB/Akt) and mitogen-activated protein kinase (MAPK) pathways can affect protein translation (168, 350).

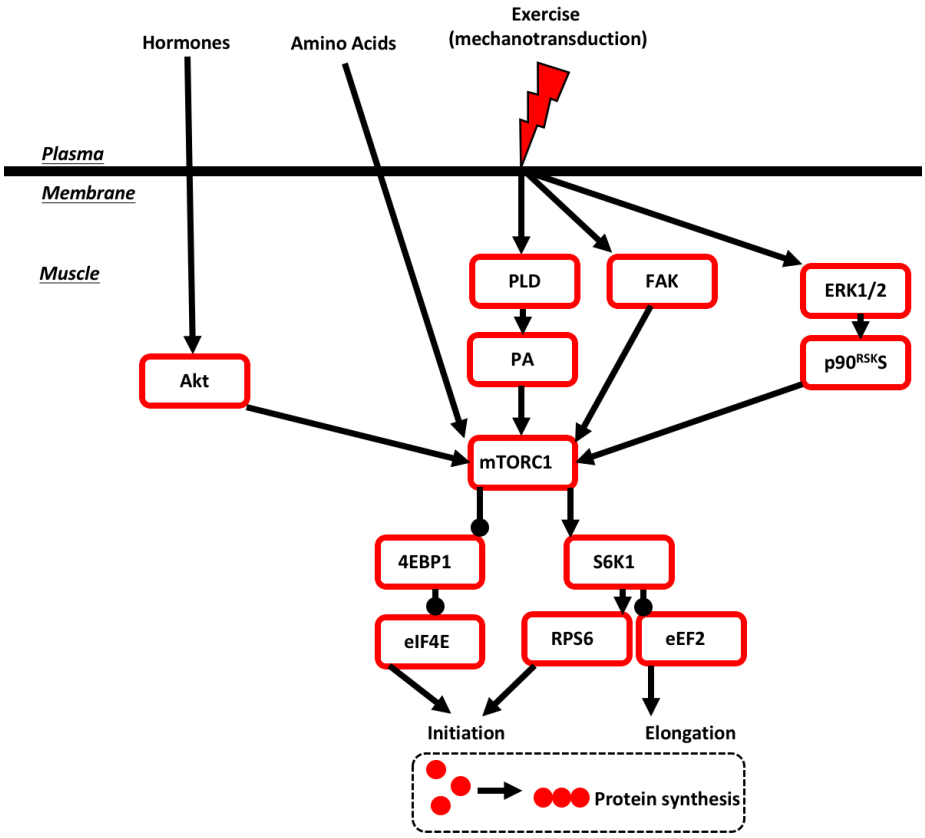


Figure 1.1. Intracellular signalling pathways in skeletal muscle known to be involved in the regulation of muscle protein synthesis. 4EBP1 4E binding protein-1; eEF2 eukaryotic elongation factor 2; eIF4E eukaryotic initiation factor 4E; *ERK1/2 extracellular signal-regulated kinase 1/2; FAK Focal adhesion kinase; PKB/Akt protein kinase B; mTORC1 mammalian target of rapamycin complex 1; P90^{RSK}S 90 kDa ribosomal S6 kinase; PA phosphatidic acid; PLD phospholipase D;* RPS6 ribosomal protein S6 and S6K1 S6 kinase 1.

1.2.1.2 Effects of acute RE

In response to an acute bout of RE, MPS is stimulated two-to-three fold (171, 260). In the fasted state myofibrillar MPS is elevated for ~4 h (173) and mixed muscle MPS is elevated for up to 48 h (260), although in the absence of nutrition the muscle

remains in a negative net protein balance due to the concomitant increase in MPB (i.e. MPB>MPS) (260, 261). The duration of RE-induced increases in myofibrillar MPS can be extended to at least 24-72 h when combined with AA consumption administered post-exercise and on a daily basis (72, 222), potentiating the anabolic effects of RE. The stimulatory effect of RE beyond 24-48 h, up until functional recovery (i.e. ~7 days) after exercise is poorly defined, at least partially due to limitations with traditional tracer techniques only providing a snapshot of MPS within a limited time frame (347). However, the recent validation and application of the first stable isotope tracer in metabolic research, deuterium oxide (D_2O) (294) provides an alternative to investigate MPS over longer time frames in humans (346, 347). Therefore, future studies utilising the D_2O tracer method can broaden current understanding of the cumulative and temporal MPS response to exercise, e.g. during muscle regeneration following acute exercise.

While the precise mechanisms regulating the RE-induced increases in MPS remain unclear, a wealth of human studies have reported increased phosphorylation of mTOR and downstream substrates (p70S6K1, rps6 and 4EBP1) are fundamental to the MPS response (14, 85, 91, 239, 273). This is demonstrated via the up-regulation of mTORC1 signalling in the hours following acute RE (72, 173). The mechanically-induced up-stream regulation of mTORC1 (i.e. mechanotransduction) is poorly defined. However, recent evidence has highlighted adhesion-related proteins such as focal adhesion kinase (FAK) and muscle intrinsic signalling via the production of the lipid second messenger, phosphatidic acid (PA)/ phospholipase D (PLD) are potential exercise-induced activators of mTORC1 (Figure 1.1). The accumulation of transient increases in MPS driven via increased growth signalling following acute

exercise can accumulate over a number of sequential exercise bouts leading to muscle growth (333).

1.2.2 Muscle protein breakdown and catabolic signals

1.2.2.1 Overview

MPB is a critical process for the maintenance of healthy muscle since it removes damaged organelles, proteins, protein aggregates and toxic products, which lead to cell death and thereby improper functioning of the contractile units of muscle (131). As such, removal of damaged proteins is likely critical to the successful structural and functional adaptations in response to RE/RET. Compared to current understanding of MPS and the underlying regulators, a lot less is understood in regards to MPB and the molecular regulations since fewer studies exist and also due to the fact that there are multiple proteolytic systems involved in regulating MPB (13). The four main proteolytic systems are; ubiquitin-proteosome system (UPS) (232), autophagy-lysosomal (238), calpain's (230) and caspase's (351) (Figure 1.2).

The UPS system is thought of as the main system for breakdown of the actomyosin proteins, which is independent of exercise mode (i.e. RE versus endurance exercise) (13, 245). Proteins degraded by the UPS are recognised by the 26S proteasome once at least four ubiquitin (Ub) molecules have been attached to the target protein. This is an ATP-requiring process regulated through the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase enzymes (E3) (232). Studies in MuRF1 or MAFbx (E3 ligases) knock-out models have shown muscle atrophy

was prevented (31), thus highlighting the importance of these two ligases in UPS-mediated muscle breakdown.

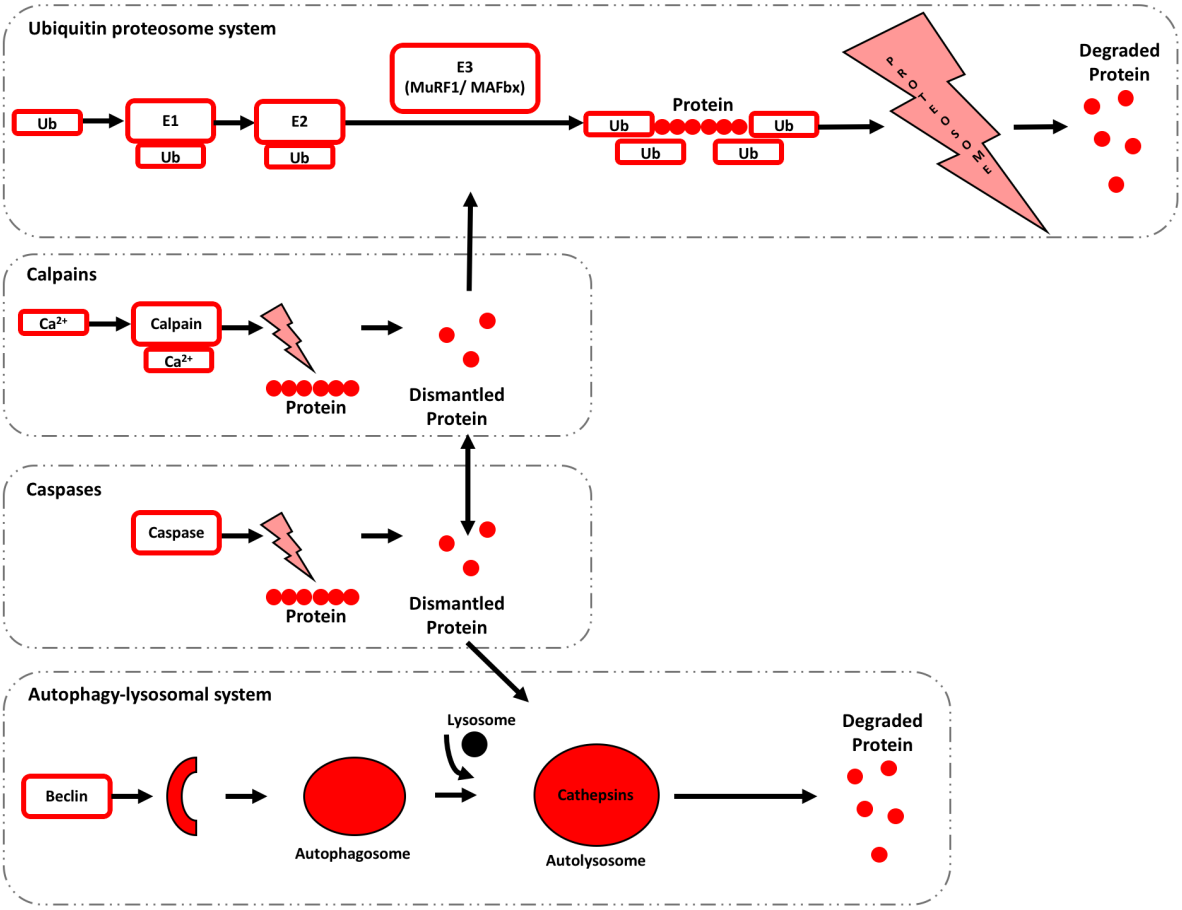


Figure 1.2. An overview of the four main proteolytic systems in skeletal muscle

Autophagy-mediated breakdown is also capable of degrading myofibrillar proteins (206) via the fusion of the protein containing-autophagosome with the lysosome to create an autolysome in which sequestered components are degraded (macroautophagy) (26). The lysosomal proteases, cathepsin B and L, are present in low levels within adult skeletal muscle and determine the proteolytic activity of the lysosomes (26, 235).

The calpains are activated in response to increased calcium levels and are capable of cleaving myofibrillar and structural proteins, which are then released into the cytoplasm and broken down or utilised for remodelling (20, 124). Similarly, caspases do not degrade proteins themselves but dismantle actomyosin structures to produce fragments for subsequent degradation (87). These cleaved fragments are proposed to be degraded by the UPS and autophagy pathways (123, 245).

1.2.2.2 Effects of acute RE

Coupled with RE-induced increases in MPS are concomitant increases in MPB (30, 361), which can increase by ~30% and persist for up to 24 h post-exercise (260). Despite the smaller magnitude and shorter increase in MPB versus MPS (260), the muscle remains in a negative net protein balance when fasted. In some cases no changes in MPB after exercise have been observed, although this may simply be due to the single 24 h time point measurement perhaps missing any earlier or later detectable changes in MPB (113). Despite the major role of MPB in muscle protein turnover particularly following acute exercise, the temporal MPB response is less well defined than the MPS response since direct measurements of MPB in humans are challenging and particularly invasive. As such, the use of molecular surrogates as indicators of MPB provides useful information regarding the exercise-induced regulation of MPB. Although, disassociation between molecular changes and MPB have been found (126), highlighting the limitation in measuring proteolytic surrogates.

The mechanisms by which MPB is up-regulated during the post-exercise regeneration period are currently unknown but all of the four main muscle proteolytic signalling pathways are likely implicated. To demonstrate, increased mRNA of the E3 ligases, MuRF1 and MAfbx, were observed immediately after acute RE, which peaked ~1-2 h post-exercise and returned to pre-exercise levels ~8 h after exercise (192). Thus, the UPS pathway is implicated in the immediate and rapid regulation of muscle recovery from exercise. One study reported reduced autophagy induction in healthy young males up to 24 h post-RE (113). This further provides support for the UPS system being the main proteolytic system during the post-exercise recovery period, and perhaps suggesting autophagy is not necessary. However, it could be speculated that autophagy is up-regulated at later stages of muscle regeneration.

1.3 Muscle protein turnover response to acute eccentric versus concentric exercise

RET has been identified as the safest and most effective method to improve muscle mass and function, even when compared to pharmacological interventions such as testosterone and growth hormone replacement (36). Conventional RE contractions can be segmented into two separate contractions, the eccentric (ECC) phase whereby the muscle lengthens whilst contracting and the concentric (CON) phase whereby the muscle shortens whilst contracting. During traditional RE exercise, the ECC phase i.e. the lowering phase, is under-loaded since contracting eccentrically produces greater force versus CON contractions (343), although the mechanisms which explain the differences in force production remain elusive (140). When performing isolated ECC exercise, this translates in to greater mass and functional gains when

compared to CON exercise training (283). However, the underlying protein turnover responses following ECC versus CON exercise are less well defined compared to conventional RE responses.

1.3.1 Muscle protein synthesis and anabolic signals

The investigation into contraction-specific differences of MPS and anabolic signalling during the regenerative period is limited to only a handful of studies. In fasted humans, mixed MPS was elevated above baseline at 3 (112%), 24 (65%) and 48 (34%) h post-ECC and -CON knee flexion/extension exercise (8 x 8 sets at 80% CON 1-RM), although no differences in MPS were observed between contraction types (260). Similarly, Gibala et al. (2000) found no differences in MPS 24 h post ECC versus CON exercise (117). However, there was no basal MPS rate determined by Gibala et al. (2000), thus it is impossible for exercise-mediated changes in MPS to be established; in fact, the data would suggest that MPS is not elevated since they reported MPS of ~0.04-0.07%/$h^{-1,}$ which is within previously reported basal ranges (39). Eight habitually active males who engaged in step up with one leg (i.e. CON exercise) and step down with the contralateral leg (i.e. ECC exercise) for a total exercise time of 12 minutes displayed elevated myofibrillar MPS in the fed state 6 h post exercise, which was still heightened at 24 h (72), but this was independent of contraction type. Furthermore, no MPS differences were observed between acute bouts of ECC or CON knee extensions (6x10 maximal repetitions) between 1-3 or 3-5 h post exercise (272).

Conversely, recreationally active males who performed 10 x 6 maximal ECC repetitions on one leg and the CON equivalent matched for work on the contralateral leg, found a more rapid rise in MPS at 4.5 h post-ECC exercise but MPS was similar between contraction-types at 8.5 h post-exercise (224). This would suggest (assuming breakdown is equal) that cumulative MPS following ECC exercise is greater compared to CON exercise. In addition to greater protein accretion, greater disruption to the myofibrillar contractile proteins (known as Z-disk streaming) was observed 1.5 h post-ECC versus CON exercise (224). Although, some authors suggest that Z-disk streaming represents remodelling as opposed to muscle damage since areas of Z-disk streaming contained increased myofibrillar-associated proteins such as desmin (356). It is hypothesised that greater disruption of the muscle proteins post-exercise requires a greater protein synthetic response to restore the structural integrity of the muscle contractile units, ultimately restoring muscle function. Furthermore, the greater protein accretion after acute ECC versus CON exercise (224) may explain the greater gains in mass and strength observed with chronic ECC training (283).

The results by Moore et al (2005), which show a contraction-specific difference in MPS, are in contrast to previous reports (72, 117, 260, 273), which may be explained by key methodological differences. Phillips et al. (1997) and Gibala et al. (2000) employed ECC exercise that was performed at 80% of the CON 1-RM, thus under-loading the ECC phase. Therefore, sub-maximal MPS stimulation might have occurred for the ECC phase. Since MPS progressively increases with increased CON load from 20% 1-RM to 60-90% 1-RM in young healthy individuals (173), it can be expected that the same happens in response to ECC exercise. Moreover, the muscle

fraction analysed differed between studies. For example, Phillips et al. (1997) and Gibala et al. (2000) measured mixed MPS, meaning that the sample incorporated myofibrillar, sarcoplasmic, collagen and mitochondrial proteins, where as Moore et al. (2005) measured myofibrillar MPS. Since RE has been shown to promote myofibrillar protein synthesis, at least in the trained state (349), it could be hypothesised that measuring mixed MPS may conceal the true changes that occur in the muscle myofibrillar fraction.

Additionally, the nutritional status of the participants differs between studies, since those in the Moore et al (2005) study were fed whilst Phillips et al (1997) investigated participants in the fasted state, thus suboptimal post-absorptive MPS may have masked any contraction-mediated differences. Furthermore, it is well acknowledged that the damaging effects of an unaccustomed bout of exercise i.e. muscle soreness and reduced muscle function, are attenuated following a sequential bout of exercise (i.e. RBE) (215). In the study by Rahbek et al (2015), participants were subjected to an exercise habituation protocol prior to the acute exercise bout which, although speculative, may have attenuated the MPS response due to the phenomenon of the RBE, masking any potential contraction mode differences since the exercise is no longer unaccustomed. However, no investigations have assessed MPS responses in tandem with the RBE, thus this is speculative.

In summary, the majority of the literature thus far suggests that the MPS responses up to 48 h post-ECC versus CON exercise are similar. However, as muscle regeneration is a process that extends beyond 48 h we are limited in our

understanding of the full temporal and cumulative MPS time-course following ECC versus CON exercise during the regenerative period, which continues for at least 7 days post-unaccustomed exercise (44, 63). Understanding MPS throughout muscle regeneration will aid the development of interventions intended to potentiate hypertrophic adaptations and counteract situations characterised by reduced regenerative capacity. Considering ECC exercise is capable of inducing greater functional deficits and ultrastructure changes, it must be the case that either: i) ECC exercise induces a greater and/or more prolonged MPS response to replace the greater volume of damaged and degraded proteins in order to restore muscle function or, ii) if there is no difference between ECC and CON exercise for MPS, improved efficiency of repair/ remodelling of existing proteins, which would not be detected with tracer-based measures of protein turnover, may account for enhanced chronic functional adaptation to ECC training. It is also possible that past research has missed vital early (i.e. 5 h) time points of investigation where muscle dysfunction may peak and thus warrants investigation, since this may be where contraction-induced differences are the most divergent. As such, the contraction-specific MPS responses remain unclear and warrant further investigation.

1.3.1.1 Anabolic signalling

Increased post-exercise MPS is mediated by contraction-induced increases in mTOR signalling (85), which is necessary for functional recovery post-exercise in rodents (23). However, the temporal relationship between anabolic signalling and functional decline and recovery in humans remains to be investigated. Since chronic adaptations to ECC versus CON exercise training differ (283), it is logical to postulate that anabolic signalling may also differ between the contraction types, at

least at some stage throughout the training programme. As for contraction-specific regulation of MPS, only a handful of studies currently exist which compare the anabolic signalling response to ECC versus CON exercise in humans. In fasted untrained human's, 4 x 6 unilateral maximal ECC contractions performed on a leg press caused significant increases in the phosphorylation of p70S6K^{Thr389} and rps6$^{Ser235/236}$ immediately after exercise which remained elevated 2 h post-exercise (91). Interestingly, performing 4 x 6 maximal CON or submaximal ECC contractions (same as maximal CON force), did not induce anabolic signalling. This might be due to the low number of contractions (91), since CON exercise has been shown to stimulate anabolic signalling elsewhere (72, 272). However, these data suggest that i) ECC exercise is a more potent stimulator of mTORC1 signalling than CON exercise, at least 2 h post-exercise, however no further measures were obtained beyond 2 h post-exercise in this study, and ii) when under-loaded (i.e. submaximal), ECC contractions have less potent anabolic effects compared to maximal ECC contractions. Further understanding of the temporal time course came from Rahbek and colleagues (2014) who found increases in the phosphorylation of mTOR^{Ser2448}, p70S6K^{Thr389} and rps6$^{Ser235/236}$ 1 h post ECC and CON exercise, which was maintained 3 and 5 h following ECC exercise only (272). This further supports the notion that ECC exercise results in a more prolonged increase in mTOR signalling but precludes understanding beyond 5 h post-exercise since no additional measurements were made (272). Conversely, Cuthbertson et al (2006) reported substantial increases in p70S6K1^{Thr389} within 3 h post-exercise, which remained increased up to 24 h post exercise, but no differences were observed between ECC versus CON contraction types (72). Interestingly, the stimulation of anabolic signalling was still detectable 24 h post-exercise regardless of exercise mode, which

is longer than reported by Rahbek et al (2014) following CON exercise. Although speculative, the familiarisation protocol employed by Rahbek and colleagues prior to the performance of an acute bout of exercise may have potentially diminished the longevity of anabolic signals shown following CON exercise (i.e. demonstrating a RBE) (55).

Thus, both ECC and CON exercise stimulate mTOR signalling, which in some cases is more persistent following ECC exercise although the duration of contraction-induced differences is unknown due to limited sampling time-points in previous studies investigating the early responses. Superior anabolic signalling post-ECC exercise may contribute to the greater acute contraction-induced increases in MPS (224) and chronic increases in muscle mass and function (283). However, investigations of contraction-specific temporal anabolic responses in tandem with MPS throughout the complete post-exercise muscle functional regenerative period are currently lacking. Such studies are required in order to understand the concordance between the two anabolic processes, which will highlight areas for intervention to potentiate anabolic responses, ultimately enhancing the regeneration process.

1.3.2 Muscle protein breakdown and catabolic signals

The invasive and technically challenging nature of measuring MPB directly in humans means that the literature regarding MPB during the muscle regenerative period is relatively sparse, nonetheless, studies do exist. Post-translational methylation of the actin and myosin histidine residues produces 3-methylistidine

(3MeH), which is not re-incorporated into protein thus the appearance (when assayed in muscle biopsy tissue) is often used as an indirect marker of MPB (95, 102, 171). This method has produced varied results, for example ECC (~40% of VO_{2Max}) or CON (~70% VO_{2Max}) cycling for 60 minutes had negligible effects on proteolysis over a seven day period (262). When investigated over a longer time-frame, untrained males demonstrated elevated 3MeH at 10-12 days post ECC cycle ergometry (250 W for 45 mins), indicative of enhanced myofibrillar MPB (95). Interestingly, following electrical stimulation (ES) in humans 3MeH was increased immediately post-ES, but no changes were observed following voluntary ECC contractions (138). The differences between the exercise mode may be explained by recruitment patterns inducing different levels of muscle damage. The Henneman's size principle is applied during voluntary contractions whereby there is progressive recruitment of small slow fibres at low contraction forces leading to larger faster fibre recruitment at higher contractile forces. This is reversed during ES, where larger faster fibres are recruited first, perhaps increasing the potential for muscle damage since type II fibres have been suggested to be more susceptible to damage (63, 127). However, the lack of change in 3MeH following voluntary exercise may also be due to the reliability and sensitivity of using 3MeH as an indicator of MPB, which has been questioned (171).

The application of 3MeH has been compared to the direct "tracee release method" developed by Zhang et al. (1996). This breakdown method sees the infusion of a tracer until isotopic equilibrium, at which point tracer infusion is ceased and tracer decay is monitored in the arterial and muscle intracellular pool (AA from arterial blood and intracellular protein breakdown) (Zhang et al. 1996). Mixed MPB

measured by the "trace release method" in fasted individuals found MPB was elevated 3 and 24 h post-ECC and post-CON exercise but had returned to baseline within 48 h (260). Conversely, there were no detectable changes when using the 3MeH method (260). These data support the idea that 3MeH may be an inadequate method to accurately measure day-to-day changes in MPB. This data also demonstrated that rates of MPB do not differ between ECC versus CON exercise when measured directly. However, the protocol employed ECC exercise at 80% of the CON 1-RM. As such, whether or not there are changes in MPB when comparing ECC versus CON exercise matched for contraction-specific relative intensity is unknown. It might be logical to hypothesise that greater MPB would be expected after ECC versus CON exercise due to greater muscle damage and potentially a greater volume of exercise-induced damaged proteins which need replaced. However, this remains poorly defined.

1.3.2.1 Catabolic signalling

Due to the difficulties with directly measuring MPB, many studies have investigated surrogate targets as indirect markers of MPB. Current evidence implicates a role for all of the major muscle proteolytic signals in the post-ECC and -CON exercise recovery period. For example, the day after a bout of ECC-bias downhill running mRNA levels of the muscle specific calpain 3 and calpain 1 were decreased, whilst calpain 2 was increased (98). Interestingly, calpain 2 peaked 1 day post-exercise in tandem with the most observed ultrastructure damage in participants, and thus implicates calpain 2 in the cleavage of damaged myofibrillar proteins (98). In addition to regulating myofibrillar turnover, calpains are also appreciated to have roles in membrane repair (218), the assembly and maintenance of attachment

complexes (92) and sarcomere remodelling (170), all processes implicated in the remodelling of muscle after damaging exercise and/ or for mediating longer term muscle adaptation. Perhaps, the differential regulation of different calpain isoforms implicates them in different remodelling roles throughout muscle regeneration. However, this is speculative and would need to be investigated further in tandem with functional recovery. Similarly, calpain 2 expression was up-regulated 24 h following ECC but not CON exercise in young healthy males (332). This shows that calpain 2 is regulated by contraction-type, which might be related to the levels of muscle damage (117). Perhaps greater myofibrillar disruption requires a greater calpain response for the cleavage of more damaged muscle proteins. Following acute ECC exercise on an isokinetic dynamometer (10 x 30 reps, 30°s, 1 min), calpain 3 autolysis (i.e. activation) was significantly increased 24 h post-exercise (230), by which point force had returned to normal. This would suggest to some extent that calpain 3 activation was not necessary for functional recovery, but might mediate sarcomeric repair and adaptation. Calpain 3 is recognised in sarcomere remodelling (170) and it is well acknowledged that the performance of a single bout of ECC exercise induces adaptation so that a subsequent bout of exercise is not as damaging (i.e. RBE). The authors speculate that perhaps calpain 3 mediates part of this remodelling process by adding extra sarcomeres in series (230). Raastad and colleagues (2010) investigated a more comprehensive time course of total calpain activity in response to 300 maximal ECC contractions and found calpain activity peaked 30 minutes after exercise (270). This rapid calpain response was not correlated to the number of fibers with myofibrillar disruptions, suggesting that calpain may regulate other processes in addition to/ rather than myofibrillar cleavage, such as the rebuilding of sarcomeres for adaptation (270). Compared to

ECC exercise, the calpain response to isolated CON exercise is poorly defined. It is postulated that isolated CON exercise might not necessitate as much of a calpain response compared to ECC exercise since less myofibrillar disruptions in need of cleavage or remodelling would be expected.

Similarly to calpains, caspase-3 dismantles the actomyosin structures which are subsequently degraded by the UPS (87). Following acute ECC exercise caspase-3 activity was increased 6, 24 and 48 h post-ECC-exercise (163, 351), likely due to ECC exercise-induced compromised muscle membranes allowing the influx of Ca^{2+}. The reported activation of caspase-3 overlaps to some extent with the calpain response, thus suggesting that both may initiate early protein cleavage of exercise-induced damaged proteins for subsequent degradation in response to ECC exercise. To the authors knowledge, no research to date has investigated the effects of isolated CON exercise on caspase-3.

Following calpain and caspase-induced dismantling, cleaved proteins are targeted for UPS-mediated degradation. No early changes were detected in the protein levels of the E3 ligase, MuRF1, as early as 30 minutes following acute ECC or CON exercise in young males (107). However, components of the UPS pathways have been reported to increase 6 h post-ECC exercise (352) and are still increased 48 h post-ECC exercise (351). The early up-regulation (i.e. 6 h post-exercise) may suggest rapid degradation in response to muscle damaged proteins, which might contribute to rapid adaptation necessary for functional recovery. Furthermore, the persistence at 48 h may suggest that degradation is also implicated in longer term muscle

adaptation. The effects of isolated CON exercise on UPS activity are poorly understood, although many studies have shown increases in the E3 ligases immediately after exercise (77) up to 24 h post-exercise (192) following conventional RE. UPS has also been implicated in muscle remodelling since 30 minutes downhill running increased proteasome enzyme activity at 14 days post-exercise (98). One report found no increase in ubiquitin-conjugated proteins, although this may be due to the single sampling time point at 24 h, therefore missing earlier or later changes (312).

Non-calcium activated breakdown pathways are also purported to be activated in response to ECC exercise. Thirty minutes of downhill running did not change cathepsin B or L post exercise or the day after exercise, however both cathepsins increased by ~50% at 14 days post-exercise (98). These data suggest that the cathepsins may be implicated in the longer-term skeletal muscle remodelling following acute ECC exercise, since declines in power had returned by this point and calpains, caspase-3 and the UPS system have been implicated in earlier time-points. As such, further research is needed to understand the effect of resistance based ECC versus CON exercise on the temporality of the cathepsin response during muscle regeneration.

In summary, despite the pivotal role of MPB for muscle regeneration and remodelling, limited research exists with regards to the temporality of the proteolytic pathways in response to acute ECC versus CON exercise. Such work is necessary since contraction-specific differences in the MPB and underlying molecular

proteolytic systems are plausible. This is because ECC exercise elicits greater muscle ultrastructural damage than CON exercise (117), thus theoretically this might represent greater volumes of exercise-induced damaged proteins. As such, following ECC exercise there may need to be a more extensive breakdown response in order to degrade these proteins. Furthermore, proteolytic systems may also be implicated in longer term remodelling demonstrated by the continued up-regulation once function has been restored.

1.4 Transcriptomic responses to acute eccentric and concentric exercise

Despite the great utility of measuring single or few target proteins/mRNA for characterising rapid physiological responses, such as early responses in muscle regeneration, the reality is that the biology is much more complex. In order to better understand the molecular networks regulating the rapid muscle regeneration response, researchers have started to implement the use of transcriptomics. Transcriptomics permits the detection of up to all of the mRNA (depending on the method used) at once within a biological sample, which can be analysed with advanced software to identify biological process/ cellular components/ molecular function networks that are regulated by the muscle damage and repair process. The most popular transcriptomic method for investigating gene expression is currently microarrays since it's affordable for most laboratory's and tens of thousands of transcripts can be detected at once (362). Although, more recently RNA sequencing has started to enter the field, likely due to the fact that all RNA transcripts can be detected and because the detection of transcripts is unbiased (i.e. no need for transcript specific probes) (329).

The utilisation of transcriptomics to broaden current understanding of the molecular networks regulating muscle regeneration has only been implemented in a handful of studies. Unique to ECC (versus CON) exercise was the up-regulation of cell growth, stress response, DNA damage, inflammation, ECM remodelling and signalling in young males (50). Since ECC exercise is associated with more muscle damage, this may explain the increase in damage and repair related transcripts. However, biopsies were taken at any time between 4-8 h post-exercise, thus there is a need for future studies to precisely define the temporal response, as this will be important for designing interventions to maximise growth responses and/ or speed up repair. Such work was done by Kostek and colleagues (2006) who found divergent transcriptional responses as early as 3 h following ECC versus CON exercise in healthy young males. Interestingly, it was shown that pathways and processes related to early growth and sarcomerogenesis were induced by ECC versus CON exercise. Such data may indicate the initiation of sarcomere repair and may also suggest the synthesis of new sarcomeres for the addition in series, part of muscle remodeling/adaptation. (169). However, this was using a muscle specific microarray and therefore may have missed out other potentially important players of muscle regeneration. Furthermore, no functional measures were made by Kostek et al., (2006), and thus investigations which detail the transcriptomic response at key functional time points throughout regeneration will provide valuable knowledge to the underlying molecular regulators.

1.5 Unaccustomed eccentric/ concentric exercise and its effect on skeletal muscle ultrastructure and function

1.5.1 Manifestations of unaccustomed exercise

Unaccustomed exercise, particularly ECC exercise, induces immediate declines in muscle force/function, which can typically recover within ~7 days (57, 148, 233). The extent of functional decline depends on the exercise mode, intensity and novelty (148). Whereby ECC exercise induces greater functional impairments compared to CON exercise (118), and those who are unaccustomed to exercise experience greater functional impairments compared to trained individuals (55, 57). Many theories exist to explain the mechanisms regulating force loss, although most are based on evidence from animal models and still today the precise mechanisms are unknown (148). The most prominent theory to date is an early theory put forward by Morgan (1990) referred to as the 'popping sarcomere theory'. This theory proposes that repeated lengthening contractions force weak sarcomeres to a point of no actomyosin overlap, which results in disrupted sarcomeres when the myofilaments are unable to re-interdigitate (226). This inability to re-interdigitate manifests as Z-line streaming (110), which disrupts the mechanotransduction networks responsible for transferring force from the sarcomere to the ECM via the Z-lines (145). Ultimately, the reduced number of functional contractile units impairs muscle function. Since this is generally applicable to lengthening ECC contractions, the popping sarcomere theory is thought to explain the greater damage observed in ECC versus CON exercise. Another theory attributes force loss to perturbations in excitation-contraction coupling (E-C coupling). To demonstrate, Warren et al (1993) proposed that damage to E-C components prior to Ca^{2+} release might lead to an inability to activate the contractile apparatus resulting in attenuated force following exercise. Mouse soleus

muscle exposed to electrically stimulated ECC contractions resulted in post-exercise declines in tension which were recovered by 50mM caffeine, which promotes contraction by stimulating the diffusion of Ca^{2+} from the sarcoplasmic reticulum (266, 337). However, heightening Ca^{2+} release has not always been shown to mitigate force loss (225). Some studies have suggested that 'popping' of sarcomeres leads to greater forces impinging on E-C apparatus such as the t-tubule, leading to E-C dysfunction and ultimately force loss, implicating both theories in the exercise-induced loss of force (226).

Initial evidence of actomyosin disruptions came from Friden et al. (1983) after ECC exercise induced Z-disk streaming. Many reports have since shown exercise induces Z-disk disruptions (224, 234), which have been reported to get progressively worse over time (234), peaking in disruption between 1-4 days post-exercise (248). Indeed, Z-disk disruption correlates with reduced force-generating capacity (270), making Z-disk streaming the most accurate indicator of functional decline, thus weighing support for the role of impaired contractile unit function and/or force transmission as the cause of post-ECC functional decline.

However, CON-induced Z-disk disruption has been shown, albeit to a much lesser magnitude than that induced by ECC exercise, denoted by significantly fewer disrupted fibres (118). The common and well acknowledged interpretation of Z-disk streaming is that it represents mechanically-induced damage of the sarcomeric proteins (109). However, this has been challenged with the notion that Z-disk streaming represents muscle remodelling since areas of Z-disk streaming contain

actin and desmin proteins possibly contributing to sarcomerogenesis (356). Perhaps, greater Z-disk streaming observed after ECC exercise represents a greater remodelling response needed to bring about muscle adaptation. Such remodelling may contribute to the attenuated muscle damage observed after a second damaging bout of exercise (i.e. RBE), although this is speculation. ECC exercise also induces the immediate separation of the ECM from the myofibres (310). ECC-induced ECM disruption, as indicated by increases in the ECM adhesion-modulating protein tenascin C, may contribute to force loss since the ECM is responsible for transferring ~70% of lateral force produced by myofibres (63).

After unaccustomed exercise is the delayed presentation of muscular soreness, termed delayed onset muscle soreness (DOMS), which is characterised by the tenderness to palpate the muscle (2, 7, 142). Soreness typically increases within the first 24 hours after exercise, peaks at ~24-48 h and subsides between 5-7 days after exercise (57). Many theories have been put forward with regards to the mechanisms of DOMS. One theory suggests DOMS is the result of an accumulation of lactic acid, although this has been refuted since CON exercise caused greater levels of lactate but lower levels of soreness (11, 52). Another theory implicated increased muscle spasms as a cause of DOMS, although the lack of reproducibility has also lead to this theory being refuted (2, 52). The contribution of muscle inflammation to DOMS has been appreciated. Increased infiltration of inflammatory cells and muscle swelling observed following exercise are implicated in the sensitisation of the nerve endings stimulating sensations of muscle soreness (108, 148). However, discordance between soreness and infiltrating inflammatory cells has been noted (158), as such inflammation does not fully explain DOMS. The mechanically induced disruption to

the contractile apparatus, which is associated with force loss, is not expected to induce significant muscle soreness since there are no pain receptors intramuscularly. Furthermore, it has been observed that the level of muscle soreness did not correlate with well known indirect markers of muscle damage (237), therefore providing evidence that mechanical damage of the sarcomeres is not necessarily a major contributor to DOMS. However, the mechanical disruption of the plasma membrane is thought to allow an infiltration of calcium into the cell, which can activate proteases such as calpains. The proteolytic activity at the Z-lines is thought to increase the chemical stimulation of nociceptors increasing muscle soreness, this is known as the enzyme efflux theory (52, 133). Additionally, mechanically-induced tears to the ECM are postulated to contribute to DOMS (2, 7, 142). This is postulated to be the result of ECM damage increasing inflammatory mediators, which in turn stimulate the nociceptors generating sensations of muscle soreness (63). In support of this theory, markers of ECM synthesis and inflammatory cells within the ECM increased in response to damaging exercise (63).

Collectively, many theories have been hypothesised to contribute to DOMS, although no one theory appears to fully explain the onset of DOMS (148). It has thus been suggested that an amalgamation of all of the theories likely contribute to DOMS (148). Further insight into the regulation of DOMS may be achieved by investigating all of the plausible theories in tandem with muscle soreness measures in response to unaccustomed exercise.

Another manifestation of unaccustomed exercise is the increase in membrane permeability and subsequent leakage of the muscle specific protein, creatine kinase, into the blood, which is used as an indirect marker of exercise-induced muscle damage (EIMD) (236). Mechanisms regulating the permeability of the membrane are unknown, although theories have been put forward. Initial hypothesis suggested that the mechanical stress associated with exercise induces increased membrane permeability (217), although this is losing credibility since histological analysis in humans show no evidence of significant sarcolemma disruption despite elevated plasma CK (185).

1.6 Skeletal muscle regeneration from unaccustomed exercise

Following exercise-induced disruptions i.e. DOMS, force declines, ultrastructural and ECM damage, a myriad of intrinsic metabolic and molecular mechanisms appear to co-ordinate the regenerative response restoring the structural and functional phenotype of the muscle. Effective regeneration is necessary for the normal maintenance of healthy muscle in response to habitual activities of daily living, to continue with daily tasks following strenuous exercise and for muscle growth during RET. The precise mechanisms and the temporality of such processes regulating regeneration are poorly defined in humans. Traditional models of muscle regeneration suggest that four time-dependent and inter-related phases regulate regeneration; 1) degeneration 2) inflammation 3) regeneration and 4) remodelling/repair (48, 65) (Figure 1.3).

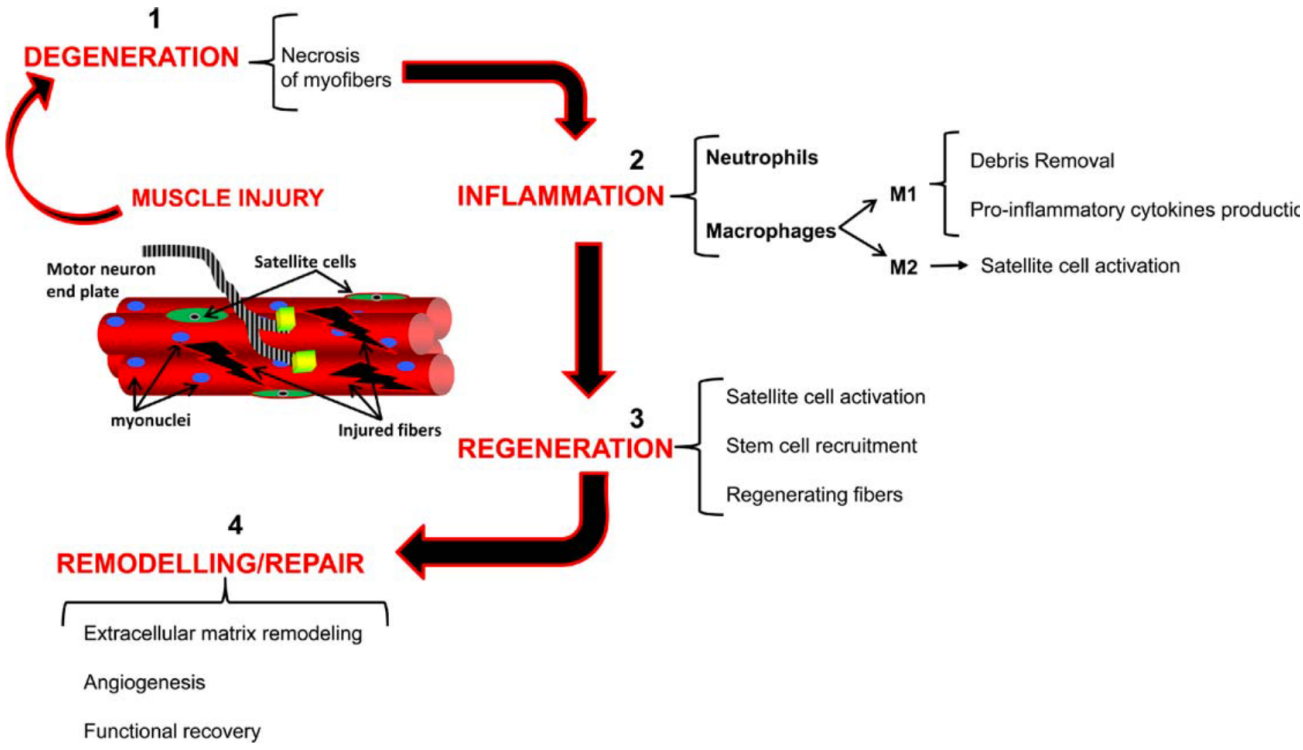


Figure 1.3. Traditional model of muscle regeneration (taken from (48)***).***

1.6.1 Degeneration

In response to injury, damaged muscle fibres become ruptured and spontaneously contract creating gaps which divide the fibre into two half's (159). This gap becomes occupied with hematoma from damaged blood vessels. Within hours of sarcolemma disruption a new membrane is formed, known as the contraction band, to isolate the area of damage preventing widespread necrosis (146). This is based on evidence found in rats using contusion and toxic based methods of injury (159, 275), thus it is unknown whether these event occurs in humans following voluntary contraction. Considering the lack of evidence of support membrane damage in humans (185), it is plausible that these sequence of events do not occur in humans. The necrosis of

muscle fibres is a consequence of infiltrating calcium, activating calcium-mediated proteolytic pathways to breakdown disrupted contractile proteins at least in rodents, denoting the degeneration phase (240). These degenerative/ necrotic events have been consistently shown in rodent models however, in healthy humans the presence of fibre necrosis following voluntary exercise is debated (63, 357). This would indicate that damage in rodents requires a re-establishment of muscle fibres, whereas humans require only sections of fibres or individual sarcomeres to be repaired (157).

1.6.2 Inflammation

Enhanced systemic and local inflammatory cells stimulate the post-exercise inflammatory response, which co-ordinates the phagocytosis of cellular debris and stimulates satellite cells (SC) for subsequent regeneration/ repair (320). The importance of efficient inflammation for effective muscle regeneration has been evidenced by rodent studies which show that the depletion or inhibition of inflammatory cells leads to slower and blunted muscle regeneration, which may be attributable to slower debris removal or reduced macrophages-related factor release, which stimulate SC needed for regeneration (4, 324). Such definitive work is yet to be done in humans, although the presence of an inflammatory response post-exercise in humans is well characterised.

The release growth factors and cytokines such as tumor necrosis factor-alpha (TNF-α) from the blood vessels stimulate the migration of inflammatory cells to the damaged fibres (202). The first inflammatory cell population to invade the damage site are neutrophils, which further stimulate inflammation via the secretion of pro-

inflammatory cytokines (48). Eosinophils later invade the site contributing to cellular debris removal and SC activation (296). Soon after, blood derived monocytes are released and differentiate into pro-inflammatory M1 macrophages (8, 202, 296). Macrophages are the predominant inflammatory cell throughout the inflammatory process, accounting for 46-100% of infiltrating inflammatory cells (291), which are responsible for phagocytising cell debris, secreting pro-inflammatory cytokines such as TNF-α and release reactive oxygen species, which can further damage muscle tissue (48, 296, 322). M1 macrophages later convert into M2 macrophages to initiate the regeneration process via the promotion of SC and angiogenesis, via the release of factors and cytokines/ growth factors, respectively (48, 296).

This sequence of events in post-exercise/ injury in human muscle is well acknowledged although the precise role in regulating functional decline and repair needs better characterisation (248). The systemic inflammatory response to unaccustomed exercise within humans has been extensively reviewed previously (248, 314). Most studies show increases in plasma interleukins (IL-6, IL-8, IL-1ra), and in some cases soluble TNF-α receptor 1, immediately after exercise which can remain increased for more than 24 h post exercise (66, 250–252, 255, 325), although results are variable likely due to the different exercise protocols used eliciting different levels of damage. Whether these increases in cytokines are a cause or a by product of muscle damage is questioned since comparisons between ECC versus CON exercise are less common. In one study, ECC cycling elicited greater increases in plasma IL-6 and CK compared to CON cycling (43), demonstrating ECC evokes a greater systemic cytokine response. Interestingly, other studies have shown no

difference in downhill versus level running (252). Thus, the origin of plasma cytokines remains elusive.

Thus far, the local muscular inflammatory response in humans has received less attention than the systemic inflammatory response. Local inflammatory responses have been shown to be mediated after exercise and moreover, are contraction-type sensitive. For example, mRNA of TNF-α, IL6, IL-1β increased 4 h post ECC but not CON exercise and remained elevated for up to 24 h post-exercise (50, 143). Increased TNF-α has been implicated in promoting MPB (183), mediated by NF-κB signalling (130), therefore exercise-induced inflammation may promote local inflammatory cytokine up-regulation (TNF-α), stimulating muscle degradation. The greater inflammatory response after ECC versus CON exercise is likely due to the presence of greater muscle damage, necessitating inflammation to clear cellular debris and MPB to degrade exercise induced damaged proteins, paving the way for successful remodelling. The transcription factor, NF-κB, has been shown to increase in skeletal muscle following ECC exercise (151), and controls the transcription of >150 target genes (244), some of are implicated in growth responses. For example, transcription of c-Myc via NF-κβ subsequently activates genes promoting muscle hypertrophy (344) ribosomal biogenesis and protein synthesis (280). Moreover, c-Myc gene expression increased following RE which strongly correlated to protein synthesis (342). As such, the activation of inflammatory pathways may also regulate anabolic and catabolic mechanisms. Greater inflammatory responses observed post-ECC exercise may be one mechanism which facilitates the greater adaptations of skeletal muscle mass and function.

Cytokines also regulate the invasion of leucocytes to the area of muscle damage. The local inflammatory cell response shows early neutrophil invasion, where neutrophils were increased 45 minutes post downhill running, and remained elevated for 5 days, which was positively correlated with Z-disk damage (101). Thus neutrophils are thought to facilitate the removal of cell debris i.e. damaged Z-disk related proteins (321). Macrophages later infiltrate the muscle to the site of damage. Following ECC exercise, no changes in macrophages within muscle fibres were observed, however there were increases following ES (63). Instead, macrophages were found to increase within the perimysium and endomysium 24 and 96 h following voluntary exercise, which was also observed 96 and 192 h following ES (63). The difference in macrophage findings between voluntary and ES studies is likely due to the extent of muscle damage and necrosis, where by the greater the damage, the greater the macrophage response and infiltration into the muscle in order to remove damaged cells and initiate remodelling. Perhaps, voluntary-contraction induces greater muscle damage to the ECM (compared to the myofibrilar structures), which is critical to the proper functioning of muscle and muscle metabolism. Thus, macrophages are recruited to the ECM to restore ECM homeostasis. Similarly, following acute unaccustomed ECC exercise, macrophages increased in the periymysial and endomysial tissue 2 days following exercise and remained elevated 4 and 8 days post-exercise (64). This prolonged elevation in macrophages may represent both the up-regulation of the early M1 macrophages for the debris clearance, followed by the later up-regulation of M2 macrophages to initiate adaptive processes. Deyhle et al (2016) reported no changes in muscle infiltrating macrophages 2 and 27 days following 300 maximal ECC contractions (82). This may not be all that surprising since voluntary exercise has previously been shown to increase macrophages in the

ECM only, and not within muscle fibres (63). Furthermore, total (CD68) macrophages were quantified as opposed to either subpopulation of pro M1 (CD11b) or anti M2 (CD163) inflammatory macrophages, which potentially could have masked any changes in either subpopulation.

Differences in exercise protocols and the lack of sequential muscle biopsies throughout the literature means that current understanding of the temporal local cytokine and leucocyte cell response during muscle regeneration following voluntary exercise is poorly defined (248). Virtually no evidence exists which compares the inflammatory response post-ECC versus CON exercise over a comprehensive time course, particularly in the context of how inflammation temporally relates to other essential aspects of muscle regeneration such as MPS, anabolic signalling and proteolysis. Such research is warranted to ordain a fundamental understanding skeletal muscle regeneration.

1.6.3 Regeneration

Current evidence suggests that the regenerative phase is initiated by the activation of quiescent SC, which migrate away from the basal lamina to the site of injury and enter the cell cycle for several rounds of proliferation (88, 89). Committed SC i.e. myoblasts, exit the cell cycle to differentiate into myocytes which fuse to form *de novo* muscle fibres or fuse into pre-existing damaged myofibrils increasing the capacity for protein synthesis by the addition of a nucleus (89). Rodent studies suggest SC are indispensable for muscle regeneration following injury (180, 210). Five days post cardiotoxin-induced injury caused small fibres (indicative of *de novo*

fibres, the result of SC fusion (211)) with centralized nuclei, indicative of muscle regeneration in wild-type animals. However, in $Pax7^{+}$ eliminated mice there was no regenerative responses denoted by no fibres with centralised nuclei, demonstrating that SC are an essential requirement for muscle regeneration following injury (180). Similarly, mice subjected to mechanical overload by synergist ablation had an ~8 fold increase in small regenerating fibres, which was significantly blunted in mice subjected to >90% SC ablation (210). SC are also purported to be important for functional recovery since mice subjected to γ-irradiation in order to prevent SC proliferation, displayed delayed functional recovery compared to the non-irradiated group (276). However, these responses are following supraphysiological 'damage' (i.e. cardiotoxin-induced injury) eliciting supraphysiological regenerative responses, and therefore do not necessarily mean SC's are essential to human muscle regeneration in a physiological context.

In humans, SC activation following acute ECC exercise occurs as early as 24 h post exercise, which typically peaks at 72 h, falling back towards baseline thereafter, at least in mixed muscle (i.e. both type I and II fibres) (302). The SC response has been shown to be contraction-specific. To demonstrate, Hyldahl et al (2014) reported exercise-induced muscle damage, evidenced by histological and functional measures, and mixed muscle SC activation 24 h following acute ECC exercise but not following a work and intensity matched bout of CON exercise. This suggests that the SC response may be a result of either the presence of muscle damage or due to the unique lengthening nature of ECC exercise. Although only speculative, increased SC proliferation may suggest a greater number of nuclei within pre-existing muscle fibres in order to repair the damage. Increased nuclei will provide greater DNA and

thus increase the potential for muscle growth. Whereas, following CON exercise the lack of SC response may suggest that the DNA capacity is great enough to bring about the required remodelling responses. Perhaps, greater SC responses following ECC versus CON exercise contribute to greater chronic adaptations. However, the work by Hyldahl et al (2014) only included the one post-exercise time point and thus may have potentially missed any other contraction-specific regulation at earlier or later time points. Although SC are purported to be activated in response to unaccustomed exercise in order to mediate regeneration, they have been shown to not be essential for hypertrophy. This is since muscle hypertrophy occurs in the absence of SC activity, probably because pre-existing myonuclei control a larger proportion of the cytoplasm when stimulated (210). As such, many questions remain around the role of SC in human skeletal muscle regeneration.

The regenerative phase is also purported to be characterised by increased protein synthesis, essential for the deposition of functional proteins for muscle repair, adaptation and growth (221). However, as previously mentioned there is a lack of studies which have investigated the muscle protein synthetic response beyond the 24-48 h post-exercise time frame, due to the limiting nature of traditional tracer methods. Traditional tracer methods are only capable of measuring MPS over short time-frames (i.e. 8-12 h), thus more advanced methods are required in order to ordain the MPS response during muscle regeneration following exercise. Advances in mass spectrometry has led to the oldest stable isotope tracer, deuterium oxide (D_2O), being validated and successfully implicated in measuring cumulative MPS in humans over longer-time frames compared to traditional tracers (38, 346, 347). Therefore, there is a need for further studies in humans which utilise D_2O tracers for

the measurement of MPS throughout the complete post-exercise functional recovery period. Such studies would provide a more accurate representation of the MPS response over the regenerative time-frame, and how this relates to other indices of muscular regeneration, as opposed to snap shots provided by stable-isotope infusion based tracers.

1.6.4 Remodelling/repair

The final phase of the muscle regeneration process is referred to as remodelling-repair and is characterised by connective tissue remodelling, angiogenesis and the recovery of muscle function (48). In regards to connective tissue repair, in order to connect the broken edges of the damaged myofibre observed in rodents, fibrin and fibronectin derived from the blood combine to form a matrix to which fibroblasts can attach and produce collagens I and III (159). This results in the formation of scar tissue (116). The production of excessive scar tissue can lead to incomplete muscle repair and subsequently attenuated muscle function, reduced muscle elasticity and an increased risk of future susceptibility to EIMD. Repeated bouts of unsuccessful repair characterised by an accumulation of scar tissue are hypothesised to contribute to muscle loss (54).

In summary, our current understanding of the temporality of the mechanisms contributing to muscle regeneration from both supra-physiologic (i.e. ES) and physiologic (i.e. exercise) mediated muscle injury is largely based upon rodent studies and correlative human studies of limited temporality. There exists no such study that has compared the contraction-specific regulation of multiple key

regenerative mechanisms over a comprehensive time-course simultaneously. Such investigations are necessary in order to enhance current fundamental understanding of the mechanisms regulating muscle remodelling process in humans following exercise. This will have significant implications for developing interventions which optimise hypertrophic adaptations to chronic resistance exercise training and for developing countermeasures against conditions of muscle wasting characterised by suboptimal muscle regenerative capacity.

1.7 Ageing skeletal muscle

The UK population is ageing: demographics show more than 10 million people within the UK are aged ≥ 65 years, which is set to double by the year 2050 (62). A key healthcare concern associated with ageing is the progressive loss of muscle mass (sarcopenia) (286), and function (dynapenia) (69, 227). Following peaks in muscle mass between the ages of 20-30 (353), the decline in muscle mass begins around the fourth decade of life (155) with losses of ~ 1% per year, equating to around 8% per decade, until ~ 70 years of age (223). Therefore, older individuals between the ages of 70-80 will only retain 60-80% of the muscle mass they had at ~ 30 years old (353). Thereafter, the rate of muscle loss increases to ~15% per decade in those beyond 80 years of age (78). The loss of muscle mass is primarily characterised by the loss of contractile proteins, observed by fewer and smaller muscle fibres, particularly of the type II muscle fibres (181). In addition to the loss of mass, there is an age-related loss of strength, which declines at a faster rate equating to 2-4% per year in men (125). Such losses in muscle strength are primarily a result of increased fat and connective tissue infiltrating between muscle fibres (70, 79).

Sarcopenia is associated with multiple physiological impairments such as reduced physical function, frailty and poor physical performance and can increase the risk of falls leading to a loss of independence (25). Additionally, since skeletal muscle is the largest tissue for glucose disposal, sarcopenia is associated with diabetes (306, 307) and an increased risk of mortality (175). Combined, the public healthcare bill associated with sarcopenia totalled $18.5 billion in the US alone in year 2000, equating to ~1.5% of the total expenditure on health (156). No European study to date has assessed the economic burden of sarcopenia (25), although it was estimated to be £5.7 billion annually in the UK (MRC, 2012).

Several putative mechanisms underpinning the onset and progression of sarcopenia have been reported. These include blunted responses to anabolic stimuli i.e. exercise and nutrition (71, 128, 173), reduced SC functionality (160), chronic inflammation (293), hormone imbalances, neurodegeneration, genetic factors (258), inadequate protein intake (243), ectopic fat deposition and physical inactivity (35). Therefore, the etiology of sarcopenia is multifactorial. However, despite evidence for a role of all these processes in sarcopenia, the precise causes are still not fully delineated. As such, further work is needed to demarcate the mechanisms regulating sarcopenia in order to develop therapeutic interventions, which offset sarcopenic onset and progression.

1.8 Muscle protein turnover in ageing at rest and during muscle regeneration after acute exercise

Studies have investigated the possibility that deficits in the basal rate of MPS during ageing contribute to chronic muscle loss, however the majority of data shows that

there are no age-related differences in basal MPS (112, 173). The acute MPS response following exercise is however, blunted in the elderly compared to the young (112, 173) (i.e. anabolic resistance). Increased MPS is essential during regeneration to replenish the exercise-induced degraded and damaged proteins, thus restoring muscle structure and function and facilitating hypertrophy. Considering the age-related blunting of MPS in response to traditional RE paradigms (173), it is logical to hypothesise that the blunted MPS response during acute muscle regeneration from unaccustomed exercise may lead to a slower recovery of muscle function due to the slower replacement of damaged proteins with newer functional proteins. The relevance of investigating such acute responses is that impaired regenerative responses to acute exercise (whether MPS mediated or otherwise) may accumulate over repeated bouts of post-activity regenerative cycles leading to incremental muscle loss with age.

In contrast to reports of anabolic blunting, limited evidence suggests that older humans are able to stimulate MPS during the recovery period following an acute bout of exercise to the same extent as younger counterparts. For example, in older untrained fasted individuals following an acute bout of RE (6 sets of 8 reps 80% 1-RM), mixed MPS was increased 10 minutes post-exercise but not 60 or 180 minutes post-exercise, whereas the young did not increase MPS until 180 minutes post-exercise (298). This demonstrates a rapid but transient MPS response in the old which, the authors hypothesise may be due to an increased MPB in the old, freeing AA for MPS in the fasted state, or the exercise stimulus may not have been strenuous enough for the young thus delaying MPS (298). Others have shown a similar temporal response but a blunting of MPS in older adults. To demonstrate, an

acute bout of RE at 60-90% of 1-RM led to increases in myofibrillar MPS 1-2 h post-exercise in young fasted recreationally active males, which also increased in older adults but was suppressed compared to the young (173). Fry et al (2011) also reported no increase in mixed MPS in fasted physically active old males following 8 sets of 10 repetitions at 70% 1-RM, whereas MPS increased 3 hours' post-exercise and remained elevated 24 h after RE in the young males. The study by Fry et al (2011) reports no changes in MPS in the old where as Kumar et al (2009) do report an increase, albeit blunted compared to the younger males. The difference in findings may be due to the time point investigated as Fry et al (2011) measured MPS rates 3 h post-exercise where as Kumar et al (2009) report differences in MPS rates at 1-2 h post-exercise, thus Fry and colleagues may have missed any potential increases in MPS. In support of this idea, Kumar et al (2009) report no increase in MPS 2-4 h post-exercise suggesting that the age-related MPS response during recovery may be transient, more so than young adults. Furthermore, inconsistencies may be due to the muscle fractions analysed i.e. myofibrillar vs. mixed MPS.

Thus, the current consensus is that the MPS response within older humans following an acute bout of RE is blunted compared to young humans (112). However, several limitations exist within the current literature. Currently, the time frame of investigation often has not extended beyond 24 h, thus the MPS response during later regenerative time points when other processes such as inflammation are thought to peak and function returns remains unknown. This may be explained by the limited applicability of traditional tracers for measuring longer-term responses thus the utilisation of D_2O as a protein tracer in future research will help to delineate the temporal MPS response during regenerative periods in the old. Nonetheless, such

temporal understanding of the MPS response in relation to other aspects of regeneration in ageing muscle will provide insight into mechanisms regulating the MPS response, which could then be targeted with anabolic interventions aimed at maximising growth responses. Based on the literature reported, it may be suggested that MPS will not change beyond 24 h in older males as studies have reported it returning to baseline within 60 minutes - 4 h post-exercise or not changing at all, however these studies have employed conventional RE. The MPS response may be more extensive following 'damaging' ECC contractions compared to 'non-damaging' CON contractions for two reasons; i) more ultrastructual damage may occur indicating the need for greater repair and ii) greater mechanical input (i.e. greater force lifted) following ECC may induce greater anabolic signals to make muscle proteins. If this were the case (i.e. that older people had enhanced MPS following ECC versus CON exercise), this may have implications on designing chronic exercise training programmes to maximise adaptations in mass and strength. Furthermore, such acute comparisons might be able to predict chronic adaptability.

1.8.1 Anabolic signalling

Age-related blunting of the anabolic signalling response to acute exercise appears to underlie the diminished MPS response. For example, in tandem with blunted MPS, suppressed phosphorylation of p70S6K1^{Thr389} and 4EBP1$^{Thr37/46}$ was observed 10 minutes, 1, 2 and 4 hours after acute RE in older versus younger counterparts (173). Thus, the blunted rise in MPS may due to suppressed increases in anabolic signalling. Extending current understanding of the anabolic signalling time course, Fry et al (2011) reported blunted MPS in tandem with attenuated increases in mTOR, p70S6K1, 4EBP1 signalling at 3, 6 and 24 hours after acute RE in older

compared to younger humans. Others have demonstrated no attenuation in translational signalling in older compared to younger untrained fasted humans 24 h following an acute bout of RE, despite blunted MPS (208). Hypothetically, blunting of anabolic signalling may not be as extensive following ECC exercise, since the greater mechanical strain may require greater anabolic signalling to repair greater structural damage and functional deficits. However, comparisons between exercise modes in older adults remains to be performed. Therefore, age-related blunting of mTOR signalling pathway following acute exercise may contribute to a reduced regenerative capacity compared younger counterparts, although age-related comparisons have not yet been investigated.

1.9 Age-related transcriptomic profile; baseline and in response to acute RE

Since the age-related loss of muscle mass and function is multi-factorial, the utilisation of transcriptomics provides an innovative tool to identify multiple potential gene regulators of sarcopenia. As RNA sequencing is still a relatively expensive and novel tool, not a large amount of data exists, however microarray have been implemented in a number of studies (259, 304, 339). At the transcriptomic level, ageing is associated with reduced expression of genes related to mitochondrial function (313) and energy metabolism (i.e. mitochondrial protein synthesis, tricarboxylic acid cycle activity) and an up-regulation of genes encoding proteasome components (339). Such metabolic dysregulation may contribute to muscle dysregulation during ageing.

In response to conventional RE, which induced muscle damage in old and younger humans (denoted by plasma CK), stress and cellular compromise, inflammation and immune responses, necrosis, and protein degradation transcripts increased in the older adults (317). Thus, muscle damage seems to induce a greater response at the transcriptional level, which is related to catabolic events. Perhaps this could explain the less successful adaptations of older muscle to RET. If this is the case, perhaps ECC exercise in older adults would exacerbate this response, since ECC exercise induces more muscle damage. No studies have investigated the effects of contraction type on the transcriptomic response in older adults.

1.10 Ageing and muscle regeneration to acute exercise

Muscle regeneration is necessary for day-to-day muscle maintenance and co-ordinates muscle adaptation in response to exercise. Impairments in the metabolic and molecular mechanisms regulating muscle regeneration is expected to accumulate and lead to perturbations in muscle mass and function over time. It has been hypothesised that the susceptibility to muscle damage is heightened in older muscle, and that recovery of muscle function is pro-longed. If so, these two functional responses must be underpinned by altered regenerative mechanisms, which may contribute to sarcopenia.

1.10.1 Susceptibility to exercise-induced muscle damage

Studies in rodents have shown that in response to a bout of ECC exercise, older muscles are more susceptible to a greater level of muscle damage evident through greater declines in muscle function compared to younger muscles (271).

Additionally, older muscle display prolonged or incomplete muscle regeneration evident by sustained force declines and the loss of muscle mass (271) compared to younger rodents. Therefore, it has been hypothesised that greater muscle dysfunction and impaired regeneration following acute exercise in older muscle may culminate over successive exercise bouts and thus contribute to the development and progression of sarcopenia and dynapenia (97). For example, greater force deficits were observed in older (26-27 months) versus younger (2-3 months) mice 10 minutes (43 vs. 64% of the control value) and 3 days (44 vs. 58% of the control value) following 75 ECC contractions (359). Similarly, Brooks & Faulkner (1996) found the force deficit in old single fibre preparations to be greater that in the young (42). Radner and Faulkner (2006) found force to return to baseline 2 months after 225 ECC contractions in the young, whereas the old still had a 32% reduction in force of the plantar flexor muscles. Others have found similar force decrements in young and old rodents but a faster recovery in the young (271). For example, 225 lengthening contractions of the EDL in young and old mice resulted in similar decrements of maximum isometric tetanic force 3 days post, which returned to baseline by 28 days in the young but not completely in the old (41). In rats, 24 lengthening contractions of the TA resulted in similar functional decrements 1 and 2 days post-contraction in young and old, although recovery of force took significantly longer in the old compared to the young (14 vs. 5 days, respectively) (209).

Whilst evidence within rodents is suggestive of a heightened damage and prolonged recovery following unaccustomed exercise in older muscle, evidence within humans is equivocal. Some have reported no age-related differences in regards to the extent of muscle functional decline (3, 56, 289). For example in active young and older

women matched for strength, 24 contractions of the forearm flexors at 115% isometric strength led to increases in CK and pain and a reduced ability to flex the arm yet no differences between the ages were observed (56). Some have shown no age-related delay in regeneration (3). A recent study found no difference in force declines or regenerative capacity in young versus older humans, concluding that exercise or co-morbidities may lead to impaired muscle regeneration rather than ageing *per se* (44). Conversely, other studies have shown age-related differences in the extent of muscle damage (201, 263, 288). At the histological level, immediately after ECC exercise in the form of resisting the backward motion of ergometer pedals (3 x 15 minutes, 80% VO_{2max}) >90% of fibres in the old had damage compared to only 5-50% in the young, which may be attributable to reduced muscle mass and VO_{2Max} in the old (201). However, the same biopsy incision site was used (different fascia incision), which may have overestimated the damage due to damage induced by the previous biopsy sample. Ultrastructure damage was also observed to be greater in older compared to younger women (288). Interestingly, Ploutz-Snyder et al., (2001) found greater declines in strength in sedentary older compared to sedentary younger women following an acute ECC exercise protocol, which were non-existent when older women were RE trained (263), demonstrating the protective effect of RE in ageing. Other studies demonstrate similar strength declines between young and old humans, however strength was recovered in the young by 3 days post-exercise, but remained lower than baseline at 5 days post-exercise in the old (73). This might be due to reduced MPS in ageing, meaning not enough proteins essential to functional muscle recovery were synthesised (16). Contrary to most research, one study reported greater damage in younger compared to older males, denoted by larger decrements in force and larger increases in CK and soreness (177). The region

of interest within this study was the arm, which is less affected by the sarcopenic process compared to the legs which might explain why damage was greater in the young.

Due to the association of ECC exercise being detrimental to muscle health in ageing humans (i.e. heightened sensitivity to muscle damage and pro-longed repair), the safety and use of ECC exercise in older populations has been questioned (194). However, more recent evidence has shown a similar time course in the decline of muscle function and the recovery following ECC exercise, in older versus younger adults (44). As such, the responses of older muscle to ECC exercise are still unclear. Potentially, ECC exercise offers greater utility in maximising anabolic responses compared to CON exercise in older adults, like it has been shown to in the young (224). Uncovering the true metabolic and molecular responses to ECC versus CON exercise has important implications for understanding the safety and effectiveness of such exercise interventions in the older population.

1.10.2 Inflammation

Chronic low-grade inflammation is associated with ageing and has been purported to contribute to sarcopenic progression (29). However, most research thus far is in non-human models i.e. cells and rodents, whilst limited research has actually assessed local basal inflammation in older humans (249). Whether or not the release of cytokines is actually increased in ageing adults at rest compared to younger counterparts remains inconclusive since conflicting reports have been published. For example TNF-α mRNA has been reported to be similar (137) and also higher (179)

in older compared to younger humans at rest. Furthermore, most of these studies have assessed the mRNA expression rather than the protein levels, and therefore cannot inform on protein changes. Further research is required to delineate the basal local inflammatory state between the ages. It is intuitive to hypothesise that 'inflammaging', where there is an age-related low-grade chronic inflammation, may lead to excessive macrophage responsiveness in turn increasing the number of fibrotic factors recruited. This response will result in excessive formation of ECM and infiltration of adipocytes at the same time reducing the SC responsiveness (202). Repeated cycles of inadequate repair in the ageing muscle in response to successive bouts of damaging exercise may culminate in excessive ECM as opposed to muscular proteins leading to reductions in muscle mass, function and quality. Thus, age-related perturbations in the basal inflammatory state may lead to perturbed muscle regeneration (76).

The cytokine response following acute exercise in older compared to younger humans has produced varied findings. Reports have shown TNF-α concentrations to either increase similarly (137) or remain unchanged (277) in response to exercise. Although difference may be attributable to the different genders recruited for the study. In regards to local inflammatory responses, macrophage infiltration has been shown to be lower in older compared to younger men following unaccustomed exercise. Sedentary young and older males who completed acute RE displayed no changes in total macrophage (CD68) count 72 h post-exercise, and no basal significant difference in macrophages was reported between the ages (269). Pro-inflammatory macrophages (CD11b) were higher at rest in young compared to old, and 72 h following exercise both pro and anti (CD163) inflammatory macrophages

increased in the young only (269). The lower number of macrophages following exercise in the elderly may perturb muscle regeneration since macrophages later change phenotype in order to recruit SC's for regeneration. Overall, there is a paucity of data which has directly assessed the temporal local inflammatory response to unaccustomed exercise in older compared to younger humans following EIMD. Furthermore, how the inflammatory response following acute exercise relates to regenerative processes (i.e. MPS etc.) in older humans remains to be detailed.

1.10.3 Regeneration/remodelling/repair

Age-related perturbations in the SC response following acute-exercise may compromise muscle regeneration. However, reports regarding the basal SC status remain controversial, and some authors have found SC number to be reduced (292, 326) whilst others have reported no change (84, 287) in older compared to younger muscle. Several differences between studies may explain discrepant finding such as the choice of muscle biopsied, the number of fibres analysed, the antibodies used for detection and the training history of participants (327). Studies have also shown that the age-related reduction in SC is also fibre-type specific. Indeed, Verdijk et al. (2007) demonstrated type II fibre atrophy to be associated with fewer SC compared to type I fibres (327). Whether this age-related fibre-type specific decline is a cause or consequence of age-related muscle loss is unknown. In addition to an age-related reduction in SC content, the functionality (i.e. proliferation/differentiation) of SC in response to acute exercise is thought to be compromised in ageing and may contribute to impaired muscle regeneration following damaging exercise. To demonstrate, 92 maximal unilateral ECC contractions increased SC proliferation in both the young and old 24 h post-exercise but the magnitude of increased SC

proliferation was blunted in the older humans (141 vs 51%, respectively) (84). When assessing fibre type specific changes, McKay et al. (2012) reported type II muscle fibre SC to increase during the 48 h recovery period following exercise in the young but not in the older humans (216). A key factor regulating the blunted SC response in the old may be attributable to the higher level of myostatin observed in the type II SC, since myostatin has been implicated in the cell cycle inhibition of SC (216). A recent study described a more comprehensive time course, which found the exercise-induced increase in SC content to be delayed in the old compared to the young, increasing above baseline 72 h post-exercise rather than 48 h post, and a blunted SC activation response (303). These age-related perturbations may be due reductions in the activity of SC regulatory pathways, since MyoD expression increased to a lesser extent in the old. However, these findings were in response to conventional RE as opposed to either isolated ECC or CON, thus the response to different contraction types may be different. Blunted regenerative responses of SC during ageing may also be underpinned by diminished Notch signalling, which is critical for SC proliferation and differentiation (61). However, Buford et al. (2014) reported no age-related difference in SC at baseline or in response to ECC-induced muscle damage. A few methodological differences between studies may explain discrepant findings, for example type II specific changes in SC have been reported in several studies (216, 326, 327) however, Buford et al. (2014) sampled the gastrocnemius which typically has a higher proportion of type I fibres compared to the vastus lateralis and thus may explain the absence of age-related differences. Buford et al. (2014) also controlled for confounding factors including physical activity level, thus the nonexistence of an age-related dysregulation may suggest that other factors are more pertinent than ageing *per se*.

Overall, the literature would suggest that acute unaccustomed exercise elicits a SC response in older humans although whether it is blunted or similar to younger individuals requires further investigation. More studies which investigate a comprehensive temporal SC response following damaging vs. non damaging exercise in older humans are needed in order to delineate the role of SC in age-related skeletal muscle regeneration.

1.11 Summary

Skeletal muscle regeneration involves the timely co-ordination of multiple metabolic and molecular processes in order to successfully repair the form and function of skeletal muscle following acute exercise. As such, successful muscle regeneration is critical to the maintenance and augmentation of muscle mass and function across the lifespan. ECC versus CON exercise elicits greater functional decline of skeletal muscle following an acute bout of exercise which may evoke a greater regenerative response in order to repair the greater muscle damage. A contraction-specific difference in the regenerative response may therefore underlie differences in chronic adaptations (i.e. muscle mass and strength). Previous human studies have attempted to characterise the regenerative processes, however, a key limiting factor is the lack of temporal investigative time points. Furthermore, few attempts have been made to addresses the time-course of multiple regenerative processes within one single study. Research is thus warranted which aims to extensively delineate the time-course of multiple processes underlying muscle regeneration in healthy young muscle following acute ECC versus CON exercise. By comprehensively defining the normal muscle regenerative process, we can identify regenerative dysregulation, which may contribute to cases of muscle loss such as sarcopenia. Rodent data suggests muscle

regeneration following exercise in older rodents is impaired, which may contribute to the progression of sarcopenia. Few human studies have attempted to understand the mechanisms regulating age-related muscle regeneration, and those that have do not corroborate with rodent data. Discrepancies are most likely due to the time points investigated and the methods used to induce muscle damage. Thus, further research is warranted which aims to understand the temporal metabolic and molecular mechanisms regulating muscle regeneration in older humans, to identify whether there are age-related perturbations in the regenerative mechanisms, to which may contribute to the progression of sarcopenia. Although targeted markers of muscle regeneration (i.e. single or few target proteins/mRNA) provide key insight into the putative mechanisms regulating muscle regeneration, the biology is much more complex. The recent OMICS development, in particular transcriptomics, allows the detection of all mRNA at once within a sample using RNA sequencing techniques. As such, further work should implement novel RNA sequencing techniques to identify global molecular networks involved in regulating muscle regeneration in younger and older adults. In doing so, this will highlight key opportunities for interventions aimed at maximising muscle growth responses or rejuvenating impaired responses.

1.12 Aims of this programme of research

The overall aims of this programme of research are three-fold:

1) to define the temporal metabolic and molecular mechanisms regulating muscle regeneration in young healthy males,

2) to determine whether the mechanisms of muscle regeneration following exercise display altered responses in older adults, and

3) to investigate novel gene regulators of exercise-induced muscle regeneration between young and older adults.

In order to achieve these aims, objectives are as follows:

1) To investigate functional (force, power, soreness, pain, SPPBT), metabolic (myofibrillar MPS) and molecular (anabolic, catabolic and inflammatory signalling, histochemical analysis of inflammation and SC) markers of muscle regeneration in young healthy males following ECC ('damaging') vs. CON ('non-damaging') exercise

2) To investigate functional (force, power, soreness, pain, SPPBT), metabolic (myofibrillar MPS) and molecular (anabolic, catabolic and inflammatory signalling, histochemical analysis of inflammation and SC) markers of muscle regeneration in old healthy males following ECC ('damaging') vs. CON ('non-damaging') exercise and compare to the young healthy males

3) To investigate differential gene expression at baseline and 5 hours following ECC and CON exercise in young and older healthy sedentary males.

2 General Methods

This chapter outlines key general principles relating to the methods and sample analysis performed within this thesis.

2.1 Serial skeletal muscle biopsies

In order to characterise the temporality of the key regenerative mechanisms within human skeletal muscle it is vital to obtain serial biopsies. Previous reports have shown the biopsy procedure itself can induce ultrastructural (9), immunological (200), biochemical (319), protein signalling (9), and gene expression (132, 331) changes, some similar to those observed after ECC exercise (200). However, there are various factors which may account for the observed responses. For example, Aronson *et al.* (1998) reported biopsy-induced changes when the same incision site was used to take a serial biopsies, however when spaced ~5cm apart, no protein signalling changes where observed (9). Malm *et al.* (2000) only spaced serial biopsies by 2 cm, which is considered too close, thus by separating out the biopsy sites further it may reduce the chances of capturing biopsy-induced changes. Furthermore, the damage observed by RE is ~four fold greater than biopsies only (309), thus the exercise-induced regenerative responses can still be identified.

More recent studies have shown no effect of serial biopsies through the same incision site on inflammatory or protein signalling cascades, most likely due to the angling of the biopsy needle ~3 cm (132) or ~5 cm (85) away from the previous site. Utilising new incision sites placed 2.5 cm apart was shown not to effect the muscle transcriptome (231). In summary, by strategically placing serial biopsies at least 3 cm apart (as within this study), the capture of responses unrelated to the exercise i.e. due to the biopsy technique, can be avoided.

2.2 Muscle soreness

Measures of DOMS are highly subjective and there is yet to be a gold standard device or method to assess DOMS in humans (58). To overcome this, the 10 cm visual analogue scale which ranges from no soreness (0 cm) to very very sore (10 cm) (Figure 2.1) (34, 264) and an algometer (FPX Algometer, Wagner Instruments, Greenwich, Connecticut) (Figure 2.2), which has been previously established as a valid and reliable way to measure pressure pain threshold (PPT) threshold (103), were utilised in tandem.

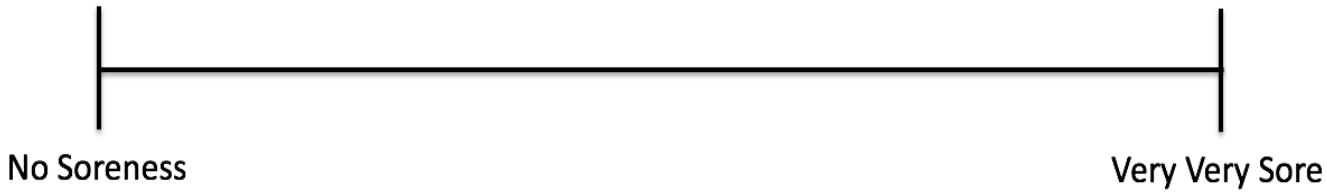


Figure 2.1. Visual analogue scale (VAS) used to measure reported soreness (not to scale)

Figure 2.2. Image of an algometer used to measure pain pressure threshold

2.3 Modified leg press

Participants performed an acute bout of unilateral ECC and unilateral CON exercise on a modified leg press (MLP) (Technogym, Gambettola, Italy) (Figure 2.3), specifically designed to be able to perform either type of isolated contraction, as described previously (106, 107). The MLP was chosen to conduct the exercise protocols as opposed to an isokinetic dynamometer (ID), which is frequently used in the literature (63, 138), as it elicited greater declines in force compared to the ID during pilot testing (data not shown), suggestive of greater skeletal muscle damage (57).

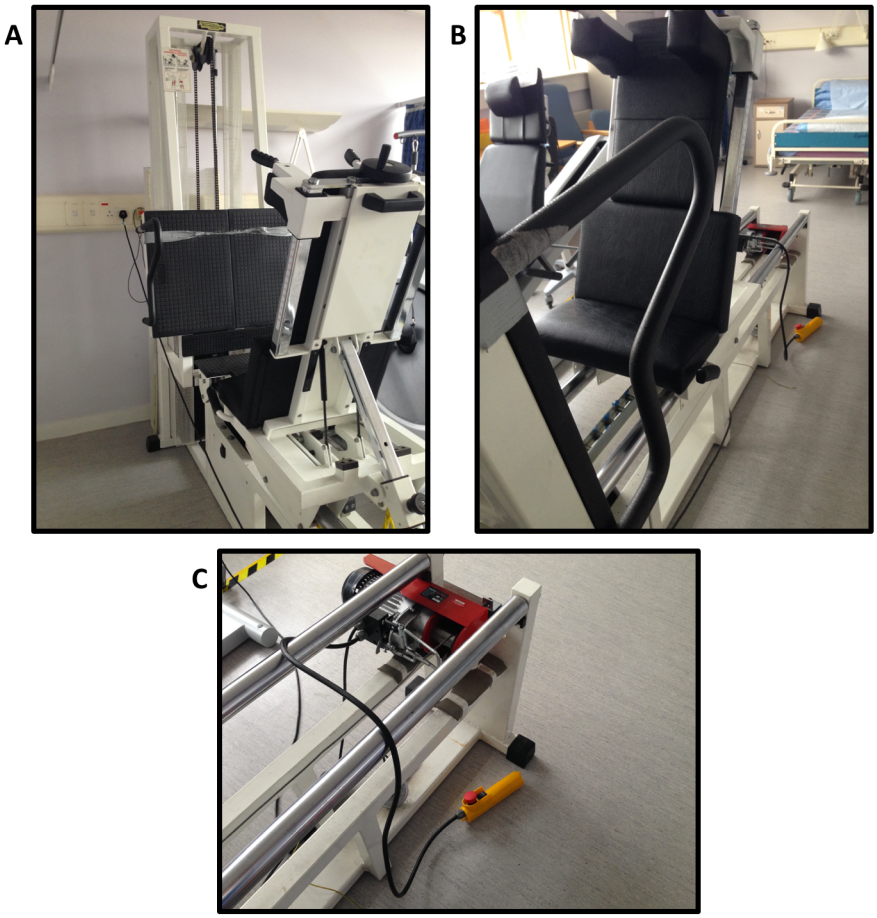


Figure 2.3. Leg press (A) modified with an electric winch and controller attached to the back of the machine (seen in B and C) which controls the movement of the chair by pulling or releasing a steel cable allowing the isolation of ECC or CON movements

2.4 Principles of sample analysis

2.4.1 Plasma creatine kinase

Creatine kinase is an enzyme located within skeletal muscle that is released into the blood stream following intense exercise and is thus considered a marker for exercise-induced muscle membrane disruption (233). The broad principles of this assay are; 1) creatine kinase within the sample will catalyse the transfer of a high energy

phosphate group to ADP from creatine phosphate, 2) the resultant ATP phosphorylates glucose to produce glucose-6-phosphate in the presence of hexokinase, 3) glucose-6-phosphate is then oxidized by glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucldotide phosphate (NADP) is reduced to nicotinamide adenine dinucldotide phosphate reduced (NADPH). It is the rate at which NADPH is formed which is monitored at 340 nm and is directly proportional to creatine kinase activity.

2.4.2 Using deuterium oxide as a tracer to measure muscle protein synthesis

Tracers have been used for many years within the area of skeletal muscle biology to determine reliable rates of muscle protein synthesis. The principle of common stable isotope tracers such as L-[*ring*- $^{13}C_6$]-phenylalanine is that a known amount of labelled (i.e. tracer) and unlabelled (i.e. tracee) amino acid mix with the endogenous pool and over time are incorporated into protein (114). Measuring protein enrichment (tracer/tracee) against precursor enrichment (precursor: product labelling ratios) can then used to determine the fractional synthesis rate of MPS (114). Despite great utility of this method, it is not without limitations. For example, such tracers require expensive sterile infusions, cannulations, multiple muscle biopsies performed by trained personnel and are limited to acute measurements of MPS (<8-12 h) in a well-controlled laboratory environment (347). As such, more advanced tracer methods are required in order to measure MPS over longer time frames in free-living situations, in order to unravel the MPS response during physiological processes such as skeletal muscle regeneration.

The re-introduction and validation of D_2O has led to the availability of a tracer which can permit longer-term, free-living measurements of MPS, whilst also negating the need for sterile infusions, controlled laboratory spaces for large amounts of time and requires reduced clinical needs. D_2O is a stable isotope that is ingested orally and rapidly equilibrates within the human body water pool within ~2 h (152), labelling all intracellular AA, in particular alanine (40, 114) (Figure 2.4). Rapid equilibration ensures that labelling gradient differences (i.e. body water versus alanine) are practically non-existent, as carbon-bound hydrogens on alanine are not subject to non-specific exchange (114). The assumption is made that alanine follows the enrichment of body water and plasma alanine closely reflects intracellular alanine and alanyl-transfer ribonucleic acid (alanyl-tRNA), the true protein synthesis precursor (40). Therefore, by taking non-invasive saliva samples it is thus possible to determine ^2H labelling of the body water, which is used as a suitable surrogate precursor for alanyl-tRNA (40). Muscle biopsies permit the detection of deuterium-labelled alanine into protein, which combined with the body water enrichment allow the calculation of the fractional synthesis rate (FSR). In humans, MPS measurements using D_2O can be detected over several hours (346), days (27) and weeks (38), yielding similar results to traditional L [ring-13C6]-phenylalanine tracers (346).

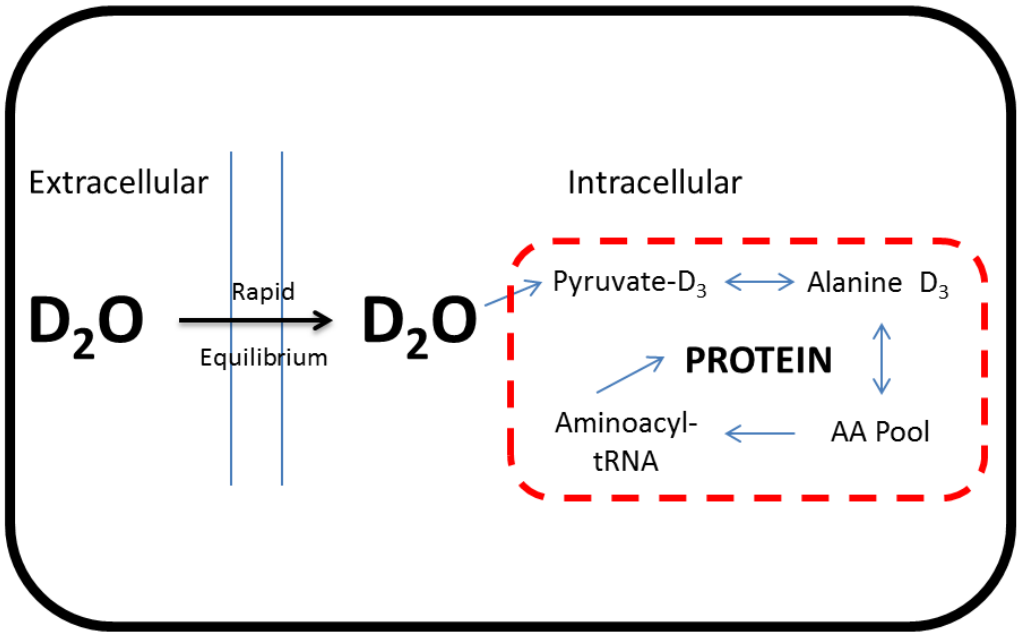


Figure 2.4. Illustration depicting D_2O equilibrating and incorporating into the protein pool (adapted from* (40, 114)*).

2.4.3 Immunoblotting

Immunoblotting (otherwise known as western blotting) allows the investigation of molecular pathways involved in the regulation of transcription and translation following exercise which underpin key physiological responses such as muscle regeneration. A few of the main applications of immunoblotting include measuring post-translation modifications (such as phosphorylation (an indication of activation status)), protein abundance and protein localisation in a sample (21). The principle of immunoblotting is to detect a specific protein by creating an antibody:protein complex which can then be detected (with specific detection methods) and is achieved with the following steps; 1) extract protein fraction of interest from sample, 2) quantify protein content, 3) separate the proteins (based on molecular weight) using electrophoresis, 4) transfer separated proteins to a membrane with a high

affinity for proteins, 5) reduce non-specific protein binding by 'blocking' the membrane using milk or bovine serum albumin, 6) incubate the membrane in the antibody specific for the protein of interest, 7) incubate the membrane in a secondary antibody that is linked to chemiluminescent (or another label) and 8) detection and quantification of the signal (21). The greater the density of the signal detected means greater levels of phosphorylation which is used as a proxy of greater activity of that protein (214).

2.4.4 Histochemistry and immunohistochemistry

Histochemistry and immunohistochemistry permit the investigation of specific molecular targets on frozen skeletal muscle tissue sections, which can be observed with light microscopy and fluorescent microscopy allowing the determination of the localisation of such targets. The principles of histochemistry and immunohistochemistry are similar to immunoblotting in that antibodies are utilised in order to detect the protein/s of interest in the sample and can be achieved with the following steps; 1) skeletal muscle must be cut into thin (~ 6-8 μm) sections using a cryostat set at ~ -22°C, 2) muscle sections are then mounted onto glass slides and left to air dry for at least 1.5 hours to improve section adherence, 3) blocking solution is added to reduce non-specific binding, 4) the section is incubated in the primary antibody of interest, 5) the muscle section in then incubated in a biotinylated secondary antibody with specificity for the first antibody, 6) an avidin-biotin complex (ABC) reagent is added to the section (30 minutes after being made to allow the ABC to form), this allows the secondary antibody to link the primary antibody to the ABC, 7) this complex can be visualised with the addition of diaminobenzidine (DAB HRP) horseradish peroxidase (HRP), which stains the

target protein brown. By utilising secondary antibodies which are visualized in different ways (i.e. fluorescent versus DAB HRP), double staining can be achieved such that more than one target protein can be visualised. This is particularly informative when wanting to look at the fibre-type specificity of a particular protein in skeletal muscle.

2.5 Statistical Strategy

To determine the correct statistical test all data were first checked for normal distribution. Data which failed normal distribution were subjected to non-parametric analysis. Friedman's non-parametric test was used for not normally distributed ordinal scale data (212) followed by Dunn's non-parametric multiple comparison test. Data which were normally distributed were subjected to parametric analysis. In the event of significance being detected, post-hoc multiple comparisons test were used to determine the point of significance. When comparing contraction types within age group (i.e. ECC versus CON exercise in young or older participants), a two-way (exercise type x time) repeated measures (time) within subject's ANOVA will be used since both contraction types were performed by the same participant. The post-hoc test used was the Bonferroni correction since it can account for running many statistical analysis on many different dependent variables. When comparing age for a specific contraction type (i.e. young versus older participants for ECC or CON exercise) a two-way mixed-model ANOVA design was employed since comparing young versus older participants is between subjects and the time points measured following ECC or CON exercise is within subjects (i.e. within: time, between: age).

3 Exploring the Mechanisms Underpinning Human Skeletal Muscle Regeneration Following Acute Eccentric versus Concentric Exercise

3.1 Abstract

Background: Skeletal muscle regeneration involves a myriad of coordinated metabolic and molecular processes essential for muscle repair, and the maintenance and augmentation of muscle mass and function. However, the time-course of such mechanisms remain poorly defined. The aim of this study was to investigate multiple metabolic and molecular mechanisms implicated in human skeletal muscle regeneration over a comprehensive time-course following ECC versus CON exercise in young participants. **Methods:** Eight young (22±1 y) healthy exercise naïve participants performed a single bout of unilateral ECC (7×10 repetitions at 80% of ECC one-repetition maximum) and unilateral CON exercise (7×10 repetitions at 80% of CON one-repetition maximum). Functional (muscle soreness, sensitivity to pain, peak torque, power and lower body function), biochemical (plasma creatine kinase), metabolic (MPS) and molecular responses (mTORC1 signalling, proteolytic activation, inflammatory signalling, macrophage infiltration, satellite cell proliferation) were measured at baseline (BL), immediately (0), 5, 24, 72 and 168 h after ECC and CON exercise. **Results:** Expectedly, both exercise modalities resulted in reduced peak torque, which was greater (0 & 5 h, $P<0.05$) and persisted for longer (72 h, $P<0.0001$) following ECC exercise. Increased mTOR (0 & 5 h, $P<0.05$), p70S6K1 (0 & 5 h, $P<0.05$) and rps6 (5 h, $P<0.05$) following ECC exercise, increased p70S6K1 (5 h, $P<0.05$) following CON exercise and repressed cathepsin L (37kDa, 0-24 h, $P<0.05$) following both exercise modalities preceded functional recovery. Increased TNF-α (ECC: 24-168 h, $P<0.05$; CON: 72-168 h, $P<0.05$) and NFκβ signalling (ECC: 72 h, $P<0.05$), macrophage infiltration (ECC: 72-168 h, $P<0.05$; CON: 72 h, $P<0.05$) and MuRF1 signalling (168 h, $P<0.05$) occurred alongside functional recovery after both ECC and CON exercise. No change in

satellite cell proliferation was observed. **Conclusion:** Overall, this study shows increased anabolic signalling and the repression of a lysosomal marker precede functional recovery and therefore may be implicated in rapid adaptation, whilst inflammatory signalling, macrophage infiltration and UPS activation occurred after functional recovery was initiated and thus might regulate chronic muscle adaptation. Despite similar temporality between contraction types, ECC exercise was associated with magnified anabolic signalling and inflammation. Combined, this may augment the post-exercise net anabolic environment which might explain the greater strength and mass adaptations observed following chronic ECC training.

Specific acknowledgements

I would like to acknowledge each clinician that performed muscle biopsies for this study: Miss Catherine Boereboom, Dr. Haitham Abdulla and Dr. Syed S I Bukhari. I would like to acknowledge the research technicians Amanda Gates and Margaret Baker for obtaining blood samples via the venepuncture technique. Finally, I wish to acknowledge Exeter Clinical Laboratory for performing plasma creatine kinase analysis.

3.2 Introduction

Skeletal muscle is a highly plastic tissue, capable of intrinsically repairing structural abnormalities and restoring muscle dysfunction induced by electrical stimulation (ES) or unaccustomed, particularly eccentric (ECC), voluntary exercise (63). Pathological perturbations in muscle regeneration, for example as observed in muscular dystrophies, causes progressive muscle weakness and wasting (60). Normal functioning of the regenerative process also assures contraction-induced adaptation, such that muscle is more resistant to subsequent 'damaging' contractions (i.e. repeated bout effect (RBE)) (215). Successful regeneration/ adaptation over several exercise bouts such as RET, culminates in increased muscle mass and strength (141). Therefore, successful regeneration is critical for the maintenance, post-exercise adaptation and growth of skeletal muscle (15). As such, understanding the regulatory mechanisms of muscle regeneration is a fundamental, yet poorly understood aspect of skeletal muscle biology. Delineating such mechanisms will provide insight for optimising the muscular response to chronic exercise training and will also provide a healthy benchmark against which to identify perturbed regenerative responses in clinical populations, such as the ageing (97).

Much of the current understanding of the metabolic and molecular processes regulating contraction-induced muscle damage and regeneration comes from animal models. Such models display immediate and persistent declines in peak torque (254), cytoskeletal disruption denoted by the progressive loss of desmin (186) and dystrophin (360) as early as 5 minutes post-exercise, myofibrillar disruption denoted by Z-disk streaming (241), marked inflammatory infiltration within the muscle fibres ~24 h after exercise (195), a later onset (48 h post-exercise) and prolonged (120 h

post-exercise) increase in muscle protein synthesis and breakdown (195) and satellite cell proliferation (276). This sequence of events conforms to the traditional, well established view that regeneration occurs in four sequential and time-dependant stages: degeneration, inflammation, regeneration and repair/remodelling (48, 65). More recently, data has implicated additional molecular mechanisms such as mTOR signalling for functional recovery (23), Ca^{2+} activated calpains for the cleavage of myofibrillar proteins (124, 229), activation of NFκB to promote the transcription of survival genes (228, 279) and up-regulated autophagic and UPS systems during the recovery period (164). These data highlight more novel mechanisms implicated in the regulation of exercise induced muscle damage (EIMD) and muscle regeneration, at least in rodents.

However, data generated in rodents may not accurately represent human processes of regeneration due to the supraphysiological methods used (i.e. ES) (63). Disparities between rodent and human muscle regeneration is evident since the metabolic and molecular regenerative events and the time-course of these events differs. For example, in humans following voluntary ECC exercise the presence of fibre necrosis, loss of desmin and plasma membrane permeability is questioned (357), there is the early onset of increased anabolic signalling and MPS (~3–48 h post-exercise) (72, 260), MPB is a rapid transient event (~3–24 h post-exercise) (260) and inflammatory cells infiltrate the ECM as opposed to the muscle fibre (63). Thus, in order to understand physiological regeneration in humans, voluntary contractions are more suitable. Furthermore, several critical processes involved in muscle regeneration have been over looked in human models. A key process being the central role of muscle protein turnover responses to acute exercise in orchestrating

the degradation of exercise-induced 'damaged' muscle proteins, and subsequent *de novo* protein synthesis to replace degraded proteins. No studies to date have directly measured muscle protein synthesis and/ or breakdown responses > 48 h post-exercise, or in temporal correlation to other putative regulators of muscle regeneration (72, 172, 173, 224, 260). Additionally, the precise role of more novel regulators such as proteolytic markers; calpains, cathepsins and local inflammatory signalling markers; TNF-α and NFκβ in regulating either muscle functional recovery and/or muscular adaptation following acute exercise are poorly defined.

Unaccustomed voluntary ECC contractions, where the muscle lengthens under tension, are commonly used as a method for inducing skeletal muscle damage and thus regeneration in humans compared to CON contractions, where the muscle shortens under tension (196). This is because markers of damage and regeneration (i.e. muscle soreness, dysfunction, inflammation) are exacerbated following ECC exercise (57). In addition to greater muscle damage, ECC training leads to greater chronic muscle adaptation demonstrated through greater gains in muscle mass and function, compared to CON exercise (283). Despite greater muscle damage, and greater chronic adaptations following ECC exercise, CON exercise training does induce chronic adaptations (i.e. increased muscle mass and function), therefore there will be a exercise-induced remodelling response. However, the extent of the regenerative response will likely differ between the two contraction types (highlighted by different chronic adaptations), although this concept is poorly defined. Thus, examining putative regenerative mechanisms between contraction modes will give greater insight into predictors of the remodelling process, rather than investigating either mode in isolation.

Understanding the temporality of key mechanisms regulating skeletal muscle regeneration (i.e. anabolic, catabolic, inflammatory and SC responses) following voluntary exercise is key since early metabolic and/ or molecular changes that precede functional recovery will provide insight into the putative mechanism regulating recovery of muscle function. Furthermore, metabolic and/ or molecular changes that occur once functional recovery has been initiated/ restored may underlie longer-term adaptations i.e. RBE and muscle growth in response to RET. This will have important implications for the development of training programmes aimed at maximising growth responses. Furthermore, this will produce a benchmark of healthy regenerative responses, which will be useful in identifying perturbed regenerative responses in pathological situations.

Therefore, the aim of this study was to investigate multiple metabolic and molecular mechanisms implicated in human muscle regeneration over a comprehensive time-course following ECC versus CON exercise in healthy young adults.

3.3 Methods

All testing took place in the Clinical, Metabolic and Molecular Physiology laboratories, part of the MRC-ARUK Centre of Excellence for Musculoskeletal Ageing Research based in the School of Medicine, University of Nottingham, Royal Derby Hospital, Derby.

3.3.1 Ethical considerations

All procedures and amendments within the study were approved by the University of Nottingham Research Ethics Committee and conformed to the Declaration of Helsinki. Invasive muscle biopsies were performed by clinical research fellows and intravenous cannulation and blood sampling was performed by trained phlebotomists. All procedures were conducted using aseptic techniques and were performed in designated clinical or exercise laboratories with at least two people present.

3.3.2 Participant recruitment and screening

Participants recruited for the study were physically inactive young healthy males (22±1y, 23±2kg·m-^{2} body mass index, 265±18kg ECC 1-RM, 152±14kg CON 1-RM) and were targeted by mailshots, local advertising in magazines, posters, flyer distribution, word of mouth and social media. Initial contact with participants included the provision of the participant information sheet and a health questionnaire. Interested participants returned the health questionnaire and were allocated a time and date for a screening session, providing no health contradictions were pre-disclosed.

Participants attended the health screening session at ~9 am, fasted from 10 pm the previous evening. During the screening all of the risks and procedures were explained verbally as well as in writing and informed consent was obtained prior to performing a medical examination which included; recording past medical history, height, weight, body mass index (BMI), heart rate, blood pressure, a blood test (urea

and electrolytes, liver function test, thyroid function test, coagulation, full blood count, fasting glucose and lipid profile), peak flow and an electrocardiogram (ECG). Participants were excluded if they were physically active and/ or had a history of exercise training within the previous 12 months since exercise elicits muscular adaptation and can therefore attenuate the regenerative response (215). Participants were also excluded if they were taking any nutritional supplements or had the following: BMI >30 kg/m^2, a history or symptoms of cardiovascular/respiratory disease, thyroid disease, anaemia, diabetes, gastrointestinal disorders, liver disease, vertigo and malignancy or taking chronic medication known to affect muscle metabolism such as nonsteroidal anti-inflammatory drugs (NSAIDS), statins, paracetamol and/or aspirin.

Following medical examination, participants performed a battery of functional tests which were used as the participant's baseline functional measures if they were enrolled in the study. These tests were; the short physical performance battery test (SPPBT), peak torque of the quadriceps measured by maximal voluntary contraction (MVC) performed on an isokinetic dynamometer and quadriceps power.

3.3.2.1 SPPBT

The SPPBT is a measure of lower extremity function, which is predictive of mortality (135). The SPPBT is composed of three tests completed in the following order; balance, gait speed and chair rise. The balance test consisted of three balances 1) a side by side 2) semi-tandem and 3) tandem stand. After a visual demonstration of each balance, participants were required to complete each balance unaided without moving their foot position. When completed or if the participant was unable

to hold a balance for 10 seconds the rest of the balance tests were terminated and a score was given (Table 3.1). The gait speed test was a 2.44 m short walking course where participants were instructed to walk from the beginning of the course to the end at their normal walking speed. To ensure participants would walk at their normal speed, a visual demonstration was provided whist verbally saying phrases such as 'walk at your normal walking speed, as if you were walking to the shops or out on a walk'. At the end of the course, participants were asked to return to the start and repeat the gait test twice. The average of all three tests was taken and an overall score was given according to Table 3.1.

Table 3.1. Scoring categories for the SPPBT

Balance Test		**Gait Speed**		**Chair Rise**	
Time (Sec)	**Score**	**Time (sec)**	**Score**	**Time (sec)**	**Score**
Side-by-side 0-9, or unable	0	Could not do any	0	Could not do any	0
Side-by-side 10, semi-tandem <10	1	> 6.52	1	> 16.7	1
Semi-tandem 10, tandem 0-2	2	4.66-6.52	2	16.6-13.7	2
Semi-tandem 10, tandem 3-9	3	3.62-4.65	3	13.6-11.2	3
Tandem 10	4	<3.62	4	<11.1	4

Chair rise was tested by placing a chair against the wall and asking the participants to sit in the chair with their arms across their chest and rise to a full stand and then fully sit down. This description was to ensure the chair was in a secure place and the

participant understood the movement, a visual demonstration was also provided beforehand. Participants were verbally instructed to start in the seated position and rise up and repeat this action an additional four times equaling five times in total, as quickly as they could. Participants were scored according to Table 3.1. Following the completion of all three tests, all test scores were tallied and participants were scored out of a total of 12.

3.3.2.2 Peak torque

Peak isometric torque of the quadriceps was measured by isometric MVC using an Isokinetic Dynamometer (ID) (Humac Norm, CSMI, Stoughton, USA). Participants were seated and secured into the chair by strapping the lower leg of interest onto a pad on the ID lever arm, the upper leg was strapped down with a Velcro pad and a seatbelt was worn across the torso. Adjustments were made to the position of the chair and the ID unit in order to line up the dynamometer fulcrum with the knee center of rotation at 90°, with full extension being 0°. Participants were instructed to push against the level arm as hard as they could for 5 seconds whilst being given verbal encouragement. Participants were asked to complete 3 x 5 second contractions at a 90° angle, each attempt was separated by 60 seconds.

3.3.2.3 Power

Unilateral quadriceps power was measured using the leg extensor power rig (University of Nottingham, Nottingham, UK), which has been deemed safe across all ages (22). The LEP consists of a seat for the participants to sit in and two foot plates which are connected a chain and a flywheel with a lever on the side to break the

wheel (Figure 3.1) (22). Participants were instructed to place one foot on the foot pedal and on the count of three to push the footplate as hard and as fast as they could until the leg was extended (see Figure 3.1 B). Participants returned the foot to the starting position and were asked to perform an additional four attempts before switching to the contralateral leg and performing five contractions.

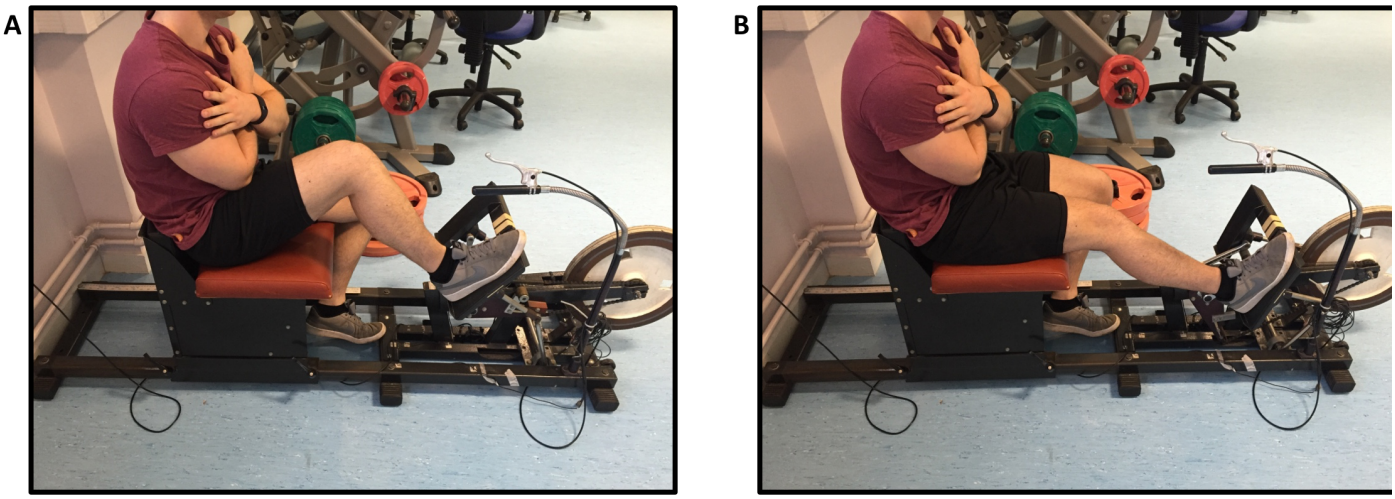


Figure 3.1. Participant on the Nottingham leg extensor power rig at rest (A) and at the end of an attempt at producing peak leg power (B).

Participants were free to withdraw from the study at any time without reason. All personal details and test results were kept confidential, anonymous and in a lockable filing cabinet and/or a password protected computer file. At least 72 h before commencing the study and throughout the whole duration of the study, participants were asked to refrain from exercise, nutritional supplements and NSAIDS.

3.3.3 Study design and procedures

Participants were required to visit the laboratory on five separate occasions over twelve days (Figure 3.2). On the first visit (baseline), participants arrived fasted at ~9 am and were asked to report the soreness in each leg measured using the VAS, as previously described (93, 178). This required participants to slowly rise from a chair and then sit back down, then immediately assess the pain in the quadriceps of each leg and mark correspondingly on the scale for each leg. PPT was measured by palpating the algometer parallel onto a region of interest of the quadriceps and graded pressure was applied until the pressure became uncomfortable, before the point of pain, for the participant as described previously (93). The approximate point of the origin, insertion and mid-belly of the *m. vastus lateralis* and the mid-belly of the *rectus femoris* and the *m. vastus medialis* were measured whilst the subject was in a seated position with the legs at a 90° angle. All points were marked out on the first day of the study and participants were asked to draw over the same point if it was fading to ensure consistency of measures throughout the study.

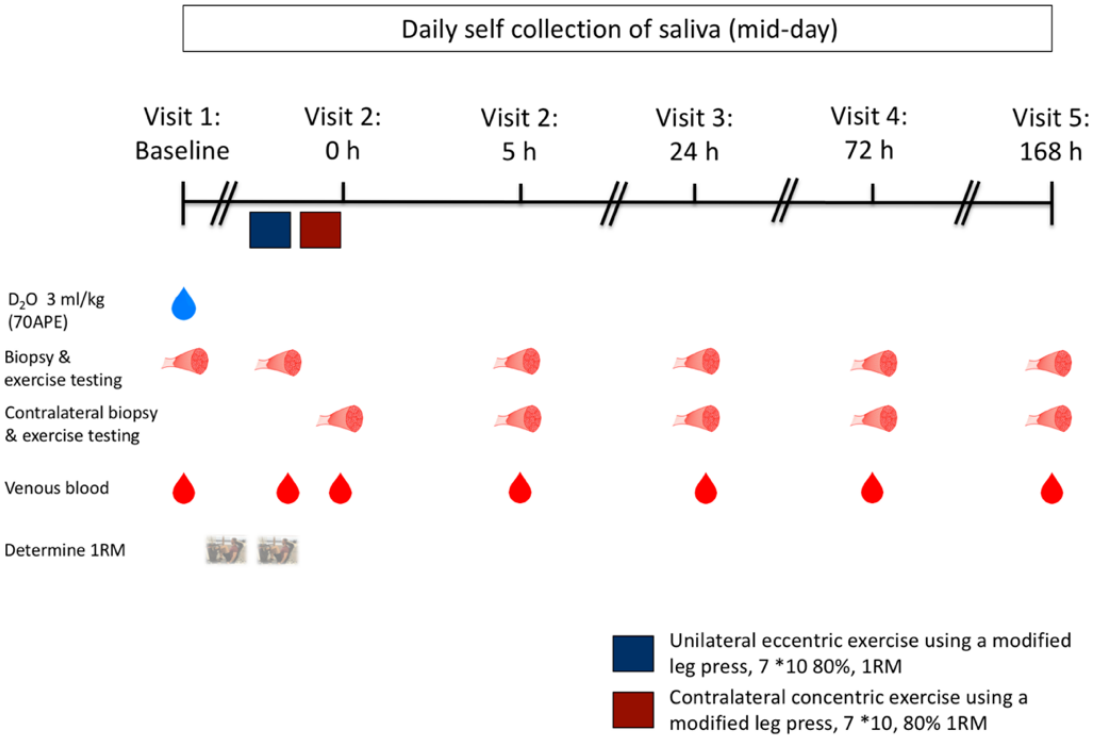


Figure 3.2. Study protocol.

Participants provided a baseline saliva sample prior to a baseline muscle biopsy. The biopsy sites were marked out on the first day of the study by a clinician. Initially the biopsy site was shaved (if needed), cleaned and anaesthetised with 1% Lidocaine and a small incision (~1 cm wide and ~2 cm deep) was made with a scalpel penetrating the skin and the fascia. Muscle biopsies were performed using the conchotome technique (83), yielding ~200 mg of tissue. Biopsy tissue was rapidly washed in ice-cold PBS, blotted on gauze to remove excess blood, and obvious fat or connective tissue was removed with a scalpel and the muscle was snap frozen in liquid nitrogen. Biopsy tissue for histological/immunofluorescence analysis was blotted on phosphate buffer saline (PBS) soaked gauze and placed on top of a small amount of optimal cutting temperature (OCT) compound on a piece of cork and submerged into

liquid nitrogen cooled isopentane. To avoid any freeze damage, the consistency of the isopentane was ensured to be almost frozen with a liquid pool in the middle and at the top before use. Samples were left in isopentane for ~1 min and then rapidly transferred into a liquid nitrogen frozen bag and placed in liquid nitrogen. All muscle tissue was subsequently stored at -80°C for further analysis.

Biopsy incision sites were closed with one stitch and covered with a large waterproof plaster. Participants were given verbal instructions on how to look after the biopsy sites and were provided with an information sheet with contact details in case of an emergency. Participants had the stitch removed within 5-7 days. As the use of NSAIDS were prohibited throughout this study, participants were prescribed with codeine for pain relief and were instructed to take it only if they needed it, not exceeding the maximum doses.

Following the biopsy, participants provided a blood sample drawn by venipuncture from the antecubital fossa vein collected in to 3 x 4 ml lithium heparin blood tubes. Participants then consumed 3ml/kg of D_2O (70 atom percent, Sigma-Aldrich, Poole, UK) (up to a maximum of 250 ml). The D_2O dose was consumed in 3 equal doses separated by ~45 minutes each to minimise any associated side effects of D_2O i.e. nausea and vertigo (114). Participants were required to collect daily saliva samples (at least 30 minutes after eating or drinking) at mid-day every day throughout the duration of the study, with the exception of the first day where two samples were collected; one before D_2O consumption, and one 3 hours post to determine peak body water enrichment. Saliva (~300 μl) was collected in sterile plastic tubes and

refrigerated until the next study visit, where it was handed to the investigator to determine body water enrichment.

Ninety-six hours later, participants arrived at ~8.30 am for visit 2 (0 h), having consumed a 250 ml liquid high energy nutritionally complete drink (Fortisip, Nutricia, Netherlands) at 07:00 am but remained fasted thereafter until visit 2 was complete at ~5.30 pm. The high energy nutritionally complete drink was included so that the participants had a standardised meal prior to the long study visit (~9 h). Participants performed isolated ECC exercise on one leg, and isolated CON exercise on the contralateral leg using the MLP. Legs were randomised to unilateral ECC and unilateral CON exercise. In order to perform an ECC contraction, the electronic motor attached at the back of the MLP was controlled to pull the chair back via a steel cable connecting the electric motor/ winch to the weight stack until the participant was in a starting position (exercise leg ~180°). When the chair was being pulled back, participants were instructed to not resist or facilitate the movement of the chair to ensure that no CON exercise component was being performed. Once the chair was released, participants lowered the weight stack in a controlled manner taking ~ 3 sec to complete the movement i.e. ECC contraction. To perform a CON contraction, participants started with the leg at 90° and lifted the weight until they reached ~180°, making the contraction last ~3 seconds. The electric motor then performed the lowering ECC phase of the contraction, participants were instructed to not lower the weight to ensure no ECC contraction was performed (Figure 3.3).

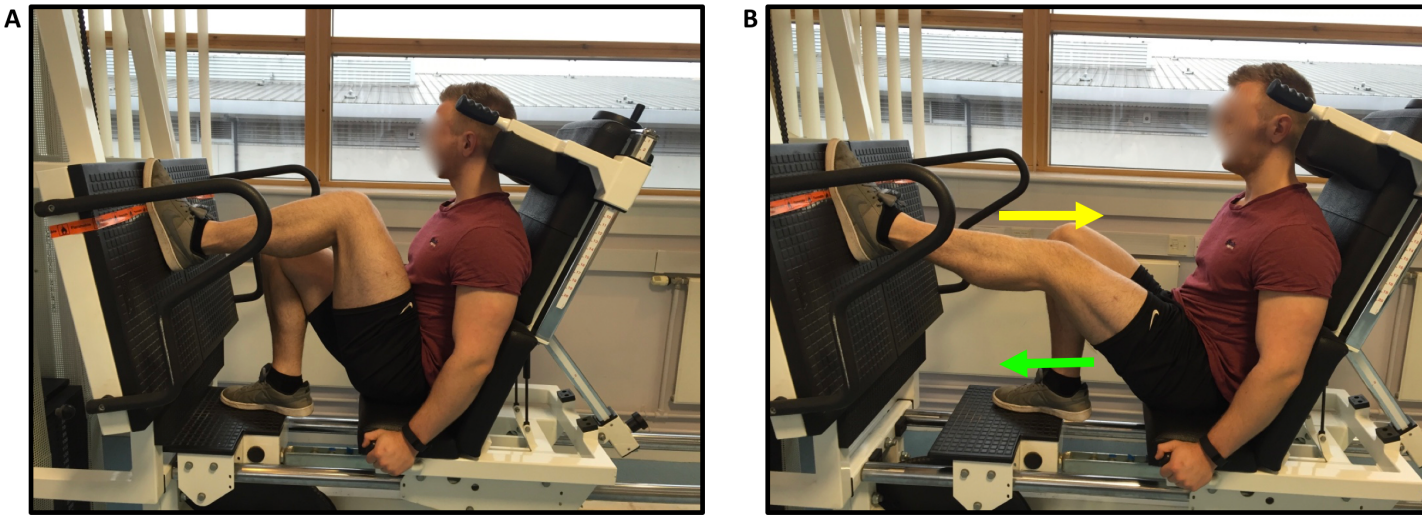


Figure 3.3. Participant on the MLP in the starting position with the leg ~90° (A). During an ECC contraction, participants lowered the weight depicted by the green arrow, during a CON contraction the participant lifted the weight stack depicted by the yellow arrow (B).

Initially, participants were given verbal instructions and a visual demonstration of how to perform an isolated ECC and an isolated CON contraction on the MLP. Participants then performed 4 repetitions at 20 kg to become familiar with the isolated contraction followed by a warm-up consisting of 2 sets of 6 reps set at 40 kg. Participants then had their ECC/CON one-repetition maximum (1-RM) determined. To begin, the first 1-RM attempt was an estimate of the participants 50% 1-RM, based on the participants age and perceived strength. Borg's scale of rate of perceived exertion was used as a gauge of how hard participants found the weight, aiding the determination of the subsequent weight to be tested. Subsequent attempts were increased in weight until the participant could no longer perform the contraction in a controlled manner, throughout the full range of motion and at a controlled speed (~ 3 seconds long). Each attempt was separated by 3 min.

Following 1-RM determination, participants performed the unilateral exercise protocol which consisted of 7 sets of 10 repetitions (3 second contractions) at 80% of ECC or CON 1-RM, with 2 minutes' rest between sets. Immediately following cessation of the exercise, participants reported their soreness (VAS) and PPT was tested followed by a blood sample and muscle biopsy. Immediately following the biopsy, peak torque was measured by MVC followed by measurements of power and SPPBT. After the initial exercise bout, participants followed the same regime on the contralateral leg doing the opposing randomised exercise.

Five hours following the cessation of the first exercise protocol (visit 2, 5 h) participants reported leg muscle soreness, PPT was measured, and a blood sample and muscle biopsy was taken from each leg. Immediately following the biopsy, peak torque, power and the SPPBT were measured. Participants arrived at the laboratory fasted 24 h post-exercise for visit 3, 72 h post exercise for visit 4 and 168 h post exercise for visit 5. During these visits, participants reported leg soreness, PPT was measured, a single blood sample was taken and a muscle biopsy was obtained from each leg and the same functional exercise tests were performed.

3.3.4 Data analysis

3.3.4.1 Plasma Creatine Kinase Analysis

Plasma was analysed for CK content as an indicator of muscle membrane damage. Blood samples were centrifugated at 3,200 rpm for 20 min at 4 °C after which, plasma was drawn, aliquoted and stored at -80°C until further analysis. Plasma CK was measured using a commercially available creatine kinase reagent for use on a

clinical chemistry analyser (Roche P800, Roche Diagnostics, Germany) by the Exeter Clinical Laboratory.

3.3.4.2 Body water enrichment

The measurement of body water enrichment has been described previously (38, 106, 346, 347). Upon collection, saliva was transferred into an Eppendorf and centrifuged at 13,300 rpm for 10 min at 4°C. The supernatant was aliquoted and stored at -20°C for later analysis. Prior to analysis, samples were defrosted and 50-100 µl of saliva was placed in the cap of an inverted auto-sampler vial. Samples were placed inverted on a heating block for 4 h at 95°C and then rapidly cooled on ice for 10 min. This allowed the collection of water distillate which was then transferred into a vial insert within a new auto-sampler vial.

To determine body water enrichment, saliva samples (0.1 µl) underwent direct liquid injection in to a high temperature conversion elemental analyser (TC/EA; Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK) and were immediately converted into H_2 gas. The sample was sent to a connected Isotope Ratio Mass Spectrometer (IRMS, Delta V Advantage, Thermo, UK) where the $^2H/^1H$ ratio was determined (347). Each sample was injected sequentially four times to minimise sample carryover. A standard curve of a known D_2O amount was run alongside to ensure accuracy of the machine.

δ^2H (deuterium isotopic enrichment) was converted into atom% using the following equation:

$$Atom\% = \frac{100 \times AR \times (\delta^2 H \times 0.001 + 1)}{1 + AR\ (\delta^2 H \times 0.001 + 1)}$$

Where *AR* is based on the Vienna Standard Mean Ocean Water (VSMOW), which is 0.00015595 and represents the absolute ratio constant for deuterium. Atom% was then converted into atom% excess (APE) by correcting to the baseline sample (347).

3.3.4.3 Muscle fractionation

To determine deuterium labelling of alanine in the myofibrillar muscle fraction, ~40-50 mg of frozen skeletal muscle tissue was rapidly homogenised with scissors in 10 μl mg^{-1} ice cold homogenisation buffer (containing 50mM Tris-HCL, 1mM EDTA, 1mM EGTA, 10mM β-glycerophosphate, 50mM NaF, 0.5mM Na_3VO_4 and a complete protease inhibitor cocktail tablet, pH 7.5) (Roche, West Sussex, UK). Homogenates were placed on a Vibrax shaker at a speed of 1,500 rpm for 10 min at room temperature followed by centrifugation at 11,000 g for 10 min at 4°C to pellet the myofibrillar, mitochondrial and collagen fraction. The supernatant containing the sarcoplasmic proteins was transferred into a new Eppendorf and frozen at -80°C for immunoblotting analysis. The remaining pellet was washed with 500 μl of homogenisation buffer and centrifuged at 11,000 g for 15 min. The supernatant was discarded and this step was repeated to ensure all sarcoplasmic proteins were removed. The pellet was transferred into a pre-cooled dounce homogeniser using 500 μl mitochondrial extraction buffer (MEB) containing 20mM MOPS, 110mM

KCl and 1mM EGTA, pH 7.5. The pellet was manually homogenised 15 times to ensure adequate extraction of the mitochondria fraction without causing them to burst. The sample was transferred back into an Eppendorf using a further 500 µl MEB and centrifugated at 1,000 g for 5 min at 4°C to pellet large organelles. The resultant myofibrillar and collagen pellet were kept on ice whilst the supernatant containing the mitochondria was removed to a fresh Eppendorf and centrifugated 11,000 g for 15 min at 4°C to pellet the mitochondria. The supernatant was discarded and 500 µl MEB was added and centrifugated at 11,000 g for 15 min at 4°C. The supernatant was removed and discarded and the mitochondrial pellet was stored at -80°C for later analysis. To solubilise the myofibrillar fraction 750 µl of 0.3 N NaOH was added, vortex mixed and incubated in a water bath at 37°C for 30 min, vortex mixing at 15 and 30 min. Samples were centrifuged at 13,000 rpm for 10 min at 4°C and the solubilised myofibrillar fraction was transferred to a new boiling tube whilst the insoluble collagen pellet was washed twice with 1.5 ml 70% ethanol centrifuged at 10,00rpm for 5 min at 4°C, supernatant discarded and the collagen pellet was stored at -80°C. A further 750 µl of 0.3 N NaOH was added to the myofibrillar supernatant and centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant was combined. To precipitate the myofibrillar proteins, 1 ml of 1 M perchloric acid was added to the supernatant, vortex mixed and cooled in the fridge (4°C) for 20 min after which samples were centrifuged at 3,200 rpm for 20 min at 4°C. The pellet was washed with 2 ml 70% ethanol and centrifuged at 3,000 rpm for 5 min at 4°C, this final step was repeated.

All muscle fraction pellets were added to 1 ml 0.1M HCl and 1 ml Dowex H^+ resin and hydrolysed in the oven overnight at 110°C. Liberated amino acids were purified on Dowex H^+ resin via cation exchange chromatography then eluted in NH_4OH, dried and derivitised to their *N*-methoxycarbonyl methyl esters as described previously (38, 147, 346, 347). Samples i.e. the AA, were re-suspended in 60 µl distilled water and 32 µl methanol, followed by the addition of 10 µl pyridine and 8 µl methylchloroformate and immediately vortex mixed for exactly 30 seconds. Samples were left at room temperature to react for 5 min followed by extraction in 100 µl chloroform and 100 µl of 0.001 M of $NaHCO_3$ to isolate the MCME AA. Molecular sieves were added to each sample and left at room temperature for 30 seconds to remove excess water before the sample was transferred into auto-sampler vials.

3.3.4.4 GC-pyrolysis-IRMS analysis

Gas chromatography-pyrolysis-isotope ratio mass spectrometry was used to analyse the incorporation of deuterium into protein bound alanine, as previously described, within the myofibrillar muscle fraction (38, 347). A standard curve of a known amount of L-Alanine-2,3,3,3-d4 enrichment was run alongside each set of samples to test the measurement accuracy of the machine.

3.3.4.5 Calculation of fractional synthesis rate

The incorporation of deuterium-labelled alanine into bound protein was calculated to determine myofibrillar FSR using the body water enrichment (corrected for the mean number of deuterium moieties incorporated per alanine i.e. 3.7 and dilution from the

total number of hydrogens in the derivative i.e. 11) as the surrogate precursor labelling between biopsies (38, 347).

Myofibrillar fractional synthesis rate (FSR) was calculated using the following equation:

$$FSR = -Ln\left\{\frac{-1\left[\frac{APEala}{APEp}\right]}{t}\right\}$$

Where APE_{Ala} is the deuterium enrichment of protein-bound alanine, APE_P is the mean precursor enrichment over the time period and t is the time between biopsies (38, 347).

3.3.4.6 Immunoblotting

The sarcoplasmic supernatant obtained through the fractionation process was measured on a NanoDrop Lite Spectrophotometer (Thermo Scientific, Hemel Hempstead, UK). Samples were standardised to 1mg/ml by diluting with homogenisation buffer and 3 x Laemmli loading buffer followed by vortexing and heating at 95°C for 5 min. Precisely 15 µg of sample was loaded into individual lanes on Criterion-XT Bis-Tris-12% SDS PAGEs (Bio-Rad, Hemel Hempstead, United Kingdom) for electrophoresis at 200 V for 55-60 min. Gels were placed in transfer buffer for ~10 min to equilibrate prior to cold transfer onto a methanol-soaked PVDF membrane at 100 V for 45 min. Membranes were subsequently blocked in 2.5% low-fat milk (diluted in tris-buffered saline and 0.1% Tween-20

(TBS-T)) for 1 h at room temperature. Following 2 brief washes in TBS-T, membranes were gently rocked overnight at 4°C whilst incubated with the primary antibody. Primary antibodies were used at a dilution of 1:2000 in 2.5% bovine serum albumin (BSA) in TBS-T against the phosphorylation of $mTOR^{Ser2448}$, $eEF2^{Thr56}$, $p70S6K1^{Thr389}$, $rps6^{Ser240/244}$, $4EBP1^{Thr37/46}$, NFκβ $p65^{Ser536}$ and for total content of Beclin 1, TNF-α (New England Biolabs, Hertfordshire, UK), MuRF1 (ECM Biosciences, Versailles, KY, USA), Cathepsin L and Calpain 1 (Abcam, Cambridge, UK). Following overnight incubation, membranes were washed in TBS-T (3x 5 min) and subsequently incubated in HRP-conjugated anti-rabbit or anti-mouse secondary antibody (1:2000 in 2.5% BSA in TBS-T; New England Biolabs, Hertfordshire, UK) for 1 h at room temperature whilst rocking. Membranes were washed in TBS-T (3 x 5 min) and subsequently submerged in Chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA, USA) for ~5 min and immediately imaged on a Chemidoc MP (Bio-Rad Laboratories, Hertforshire, UK). Protein bands were quantified by densitometry using Image Lab Version 5 (Bio-Rad Laboratories, Hertforshire, UK) ensuring no pixel saturation. Protein loading differences were corrected to total coomassie stained protein (338). Relative arbitrary units were normalised to coomassie stained proteins.

3.3.4.7 Histology and Immunohistochemistry

3.3.4.7.1 Muscle sectioning

Serial transverse sections were cut at 6 μm using a cryostat (Leica CM1850; Leica Microsystems, Kista, Sweden) set at -22°C. Sections were mounted on Superfrost Plus slides (Thermo Scientific, Hemel Hempstead, UK) and left at room temperature

to air dry for at least 1.5 hours to improve adherence to the slide, before being stored at -80°C for subsequent staining.

3.3.4.7.2 Muscle staining

Pre-cut muscle sections were removed from -80°C freezer and left to thaw for at least 15 minutes. Slides were dabbed free of any excess water with tissue, avoiding direct contact with the muscle sections. A large circle was drawn around all the sections on the slide with a pap pen to form a hydrophobic barrier preventing the solutions from running off the slides, reducing the risk of the sections drying out. Slides were placed in coplin jars and washed in PBS (1x) for 10 min after which they were gently tapped and shaken dry and blocked in normal goat serum (Vector Laboratories, S-1000, Burlingame, CA) for 20-30 min at room temperature. The blocking agent was removed by gentle tapping and shaking of the slide and the primary monoclonal antibody was added for targets outlined below and incubated at 37°C for 1 h. Following primary antibody removal, slides were washed in 1x PBS for 10 min, twice, and then incubated in biotinylated horse anti-mouse secondary antibody (Vector Laboratories, BA-2000, Burlingame, CA) at room temperature for 1 h. Slides were washed in PBS (x1) for 10 min, twice. Slides were incubated in VectaStain ABC reagent (made 30 min before use to allow the avidin-biotin complex to form) (Vector Laboratories, PK-6100, Burlingame, CA) for 1 h and left at room temperature. Slides were washed in PBS (x1) for 10 min, twice. To visualise the antibody-antigen binding, an ImmPACT diaminobenzidine (DAB) HRP substrate kit (Vector Laboratories, SK-4105, Burlingame, CA) was added for 45 seconds, staining the target brown. Slides were immediately placed in tap water to stop the DAB reaction and left under a running tap for 5 min. Sections were

dehydrated in 35, 70 and 100% ethanol for 2 min each and left to dry before mounting.

3.3.4.7.2.1 Total fibre number

Serial cross sections were stained for muscle fibre type and the cell membrane in order to count the total number of fibres. Primary antibodies against slow myosin (A4.951, Developmental Studies Hybridoma Bank, Iowa City, Iowa) and laminin for the cell membrane (2E8; Developmental Studies Hybridoma Bank, Iowa City, Iowa) were added. Total fibre number was counted using Image J software (National Institutes of Health, USA).

3.3.4.7.2.2 CD68

The macrophage staining protocol was similar to that previously described (203). The primary antibody CD68 (DAKO, M0718, Hamburg, Germany), considered a marker of total macrophages, was added. Sections were visualised under the microscope for counting with a Leica CM E light microscope (Leica Microsystems CMS, UK). All $CD68^{+}$ were counted at a 10x magnification throughout the whole muscle section.

3.3.4.7.2.3 Pax7

To assess fibre type specific $PAX7^{+}$ cells, marker of quiescent and activated SC, a two step staining process was used, similar to one that has been previously described (17). Initially, the section was treated as described above. In brief the section was thawed, washed, blocked, incubated with the primary monoclonal antibody against

Pax7 (Developmental Studies Hybridoma Bank, Iowa City, Iowa) followed by biotinylated horse anti-mouse secondary antibody. Vectastain ABC and DAB substrate were used to stain the Pax7+ cells brown and could be visualised using light microscopy. The same section was then incubated with slow myosin (A4.840; Developmental Studies Hybridoma Bank, Iowa City, Iowa) and laminin (2E8; Developmental Studies Hybridoma Bank, Iowa City, Iowa) at 37°C for 1 h, washed with PBS and subsequently incubated in Alexa Fluor 546 (Thermo Scientific, Hemel Hempsted, UK) in a dark staining tray at room temperature for 1 h. Sections were mounted with Molecular Probes ProLong Gold Antifade Mountant (ThermoFisher Scientific, Hemel Hempstead, UK) and visualized using fluorescent microscopy (Nikon Eclipse 50i, Badhoevedorp, The Netherlands) where the basal lamina and type I fibres were fluorescent and type II fibres were unstained. Images of the whole section were taken at 20x magnification; fibre-type specific SC were counted in the whole section.

3.3.5 Statistical Analysis

The distribution of all data was analysed for normality using the Kolmogorov-Smirnov test (accepted if $P > 0.05$). Data are presented as mean ± S.E.M. Paired t-tests were performed to determine differences between ECC vs. CON 1-RM. Ordinal data (visual analogue scale) and data which failed normality testing (plasma CK concentrations) were analysed using the non-parametric Friedman one-way ANOVA. In the event of significance being detected, Dunn's multiple comparisons test was used to determine the point of significance. The Dunns test was chosen as it is the appropriate multiple comparisons test for data that is not normally distributed. One person was removed for the determination of plasma CK concentrations, since

an outlier was detected (> 2 standard deviations from the mean). Differences were detected using repeated-measures (time) two-way (exercise type x time) ANOVA with a Bonferroni correction using GraphPad Prism 6 (La Jolla, CA, USA). A log transformation was performed for the phosphorylation of $mTOR^{Ser2448}$ to achieve normal distribution, followed by a two-way RM ANOVA. To draw temporal comparisons between functional, metabolic and molecular outputs, data sets were normalised over a range of 0-100% according to the data span (i.e. for each set of data, 0% represented the lowest whilst 100% represented the highest value). For metabolic (FSR) and molecular (immunoblotting, histochemical and immunohistochemical staining) an *n* of 7 was used since a 5 h post-ECC exercise biopsy could not be obtained for one participant. The α-level of significance was set at $P < 0.05$.

3.4 Results

3.4.1 Muscle function, soreness and plasma CK responses to ECC vs CON exercise

No effects of time or contraction type were observed for the SPPBT (data not shown). Compared to baseline, peak torque significantly declined immediately (0 h) ($P<0.05$) and remained significantly decreased 5 and 24 h post-CON exercise ($P<0.0001$) and there was a strong trend (p=0.05) for force decline 72 h post-CON exercise (Figure 3.4 A). Post-ECC exercise, peak torque also declined at 0 h ($P<0.001$) and remained decreased 72 h post-exercise ($P<0.001$), with a trend for decline 168 h post-ECC exercise ($P=0.08$); thus, ECC resulted in a prolonged peak force decrement versus CON exercise. When comparing the level of force decline between contraction modes, the reduction in peak torque was significantly more

pronounced following ECC versus CON exercise at 0 and 5 h post-exercise (P<0.05). Thus, unaccustomed ECC exercise cause greater and more sustained declines in peak torque versus CON exercise. Peak power was only impaired post-ECC exercise, with significant impairments observed at 0, 5 and 24 h post-exercise (Figure 3.4 B).

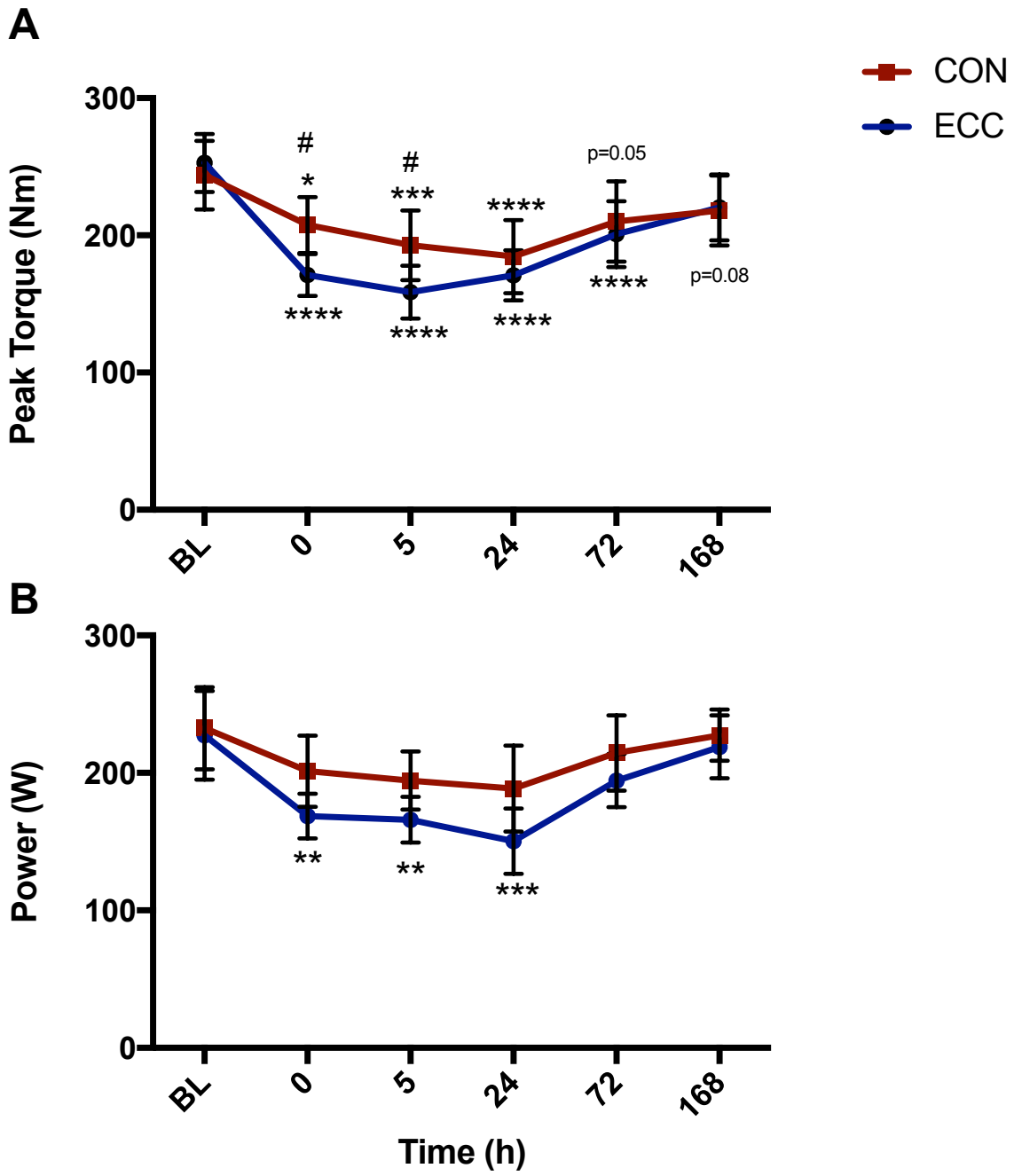


Figure 3.4. Declines in peak torque (A) and power (B) following ECC vs. CON exercise. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. *indicates significant difference at that time point compared to baseline (P<0.05), ** (P<0.01), *** (P<0.0005), **** (P<0.0001) and #indicates significant difference between contraction types at that time point (P<0.05).*

CON exercise resulted in significantly increased sensations of muscle soreness (Figure 3.5 A) 24 h post-exercise only (P<0.01), although there was a strong trend for reduced pressure tolerance 5 h post-CON exercise (P=0.06) (Figure 3.5 B). However, after ECC exercise muscle soreness was increased 0, 5, 24 and 72 h post, and pressure tolerance was reduced at 5, 24 and 72 h post-ECC exercise (Figure 3.5 A-B). Unaccustomed ECC contractions therefore induce an earlier onset and sustained presence of muscle soreness and impaired pressure tolerance versus CON exercise. No significant differences between the two contraction types were found.

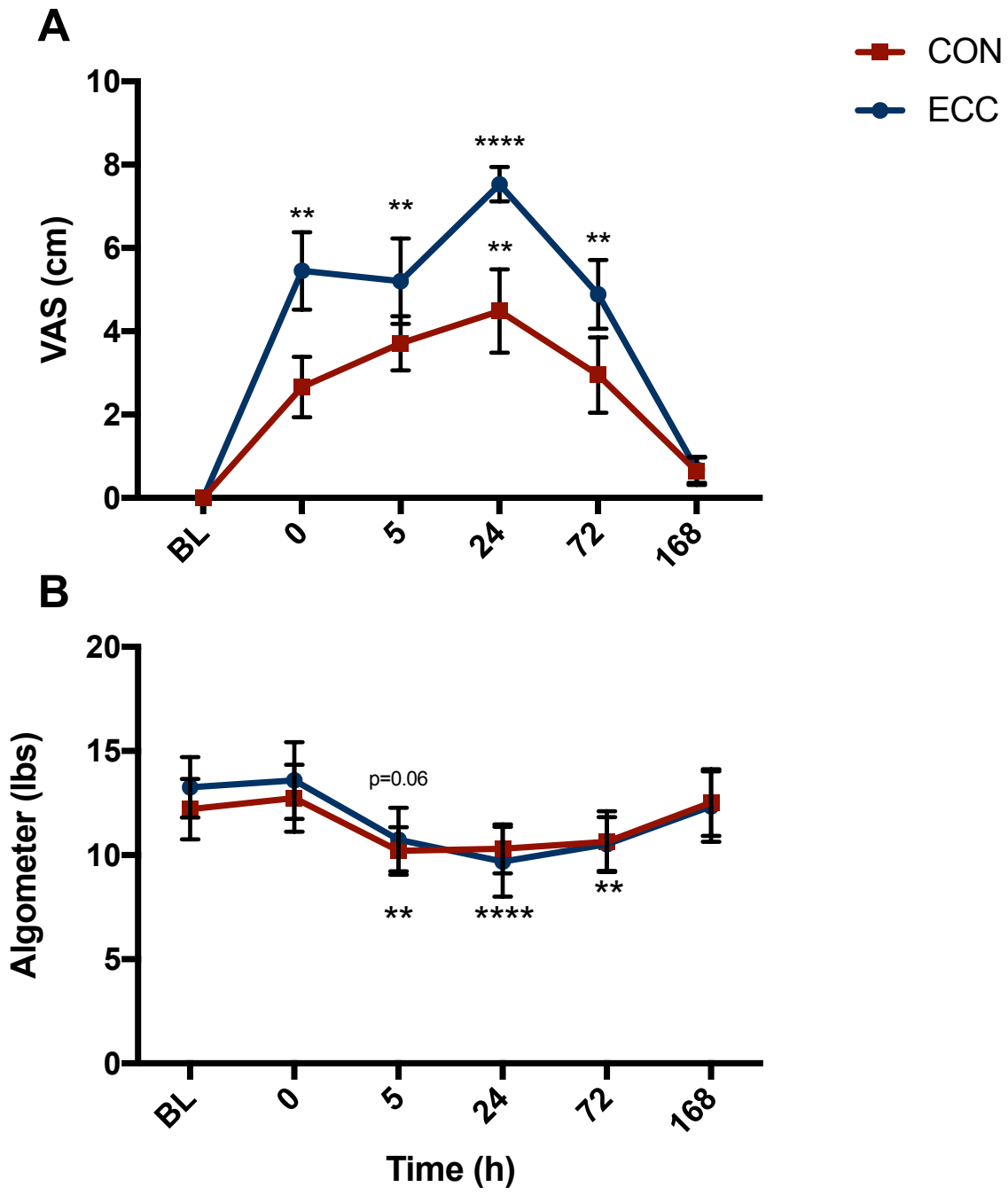


Figure 3.5. Perceived muscle soreness measured using the VAS (A) and changes in the PPT (B) pre and post ECC vs. CON exercise. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively.* ** indicates significant difference at that time point compared to baseline (P<0.01), **** (P<0.0001) and # indicates trend at that time point compared to baseline (P=0.06).

Indicative of post-exercise muscle membrane disruption, plasma CK concentration was elevated 24 (P<0.05) and 72 h (P<0.01) post exercise (Figure 3.6). However, contraction mode-specific effects could not be delineated due to CK content being measured in the plasma and not in the skeletal muscles.

This data show increases in soreness (VAS) and CK coupled with declines in PPT, peak torque and power indicating that both ECC and CON exercise are capable of inducing muscle disruption, which is exacerbated and prolonged following ECC versus CON exercise.

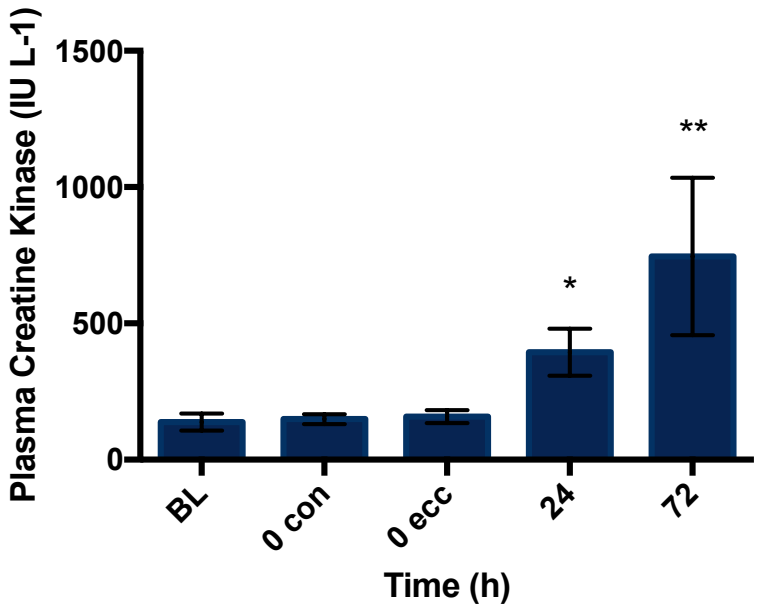


Figure 3.6. Plasma creatine kinase levels prior to and up to 72 h following exercise. *BL, 0 con, 0 ecc, 24 and 72 refer to baseline, 0 h post-CON exercise, 0 h post-ECC exercise, 24 and 72 h post-exercise, respectively. n=7. *indicates significant difference at that time point compared to baseline (P<0.05) and ** (P<0.01).*

3.4.2 Muscle protein synthetic response to ECC vs CON exercise

A single D_2O bolus (70 atom percent) of 3ml/kg led to a peak in body water enrichment of 0.314 ± 0.007% 3 h post consumption and an enrichment of 0.159 ± 0.005 on the final day (Figure 3.7 A). Body water enrichment followed an exponential decay pattern, decaying throughout the trial at ~0.014% per day (Figure 3.7 B). Myofibrillar MPS did not significantly change throughout the time course of the study following either ECC or CON exercise although data were highly variable between 0-5 h (Figure 3.8 A). When these data were collapsed i.e. combining analysis of post-exercise changes in MPS by using all ECC and CON exercise FSR values together, there was still no exercise-induced change in MPS (Figure 3.8 B).

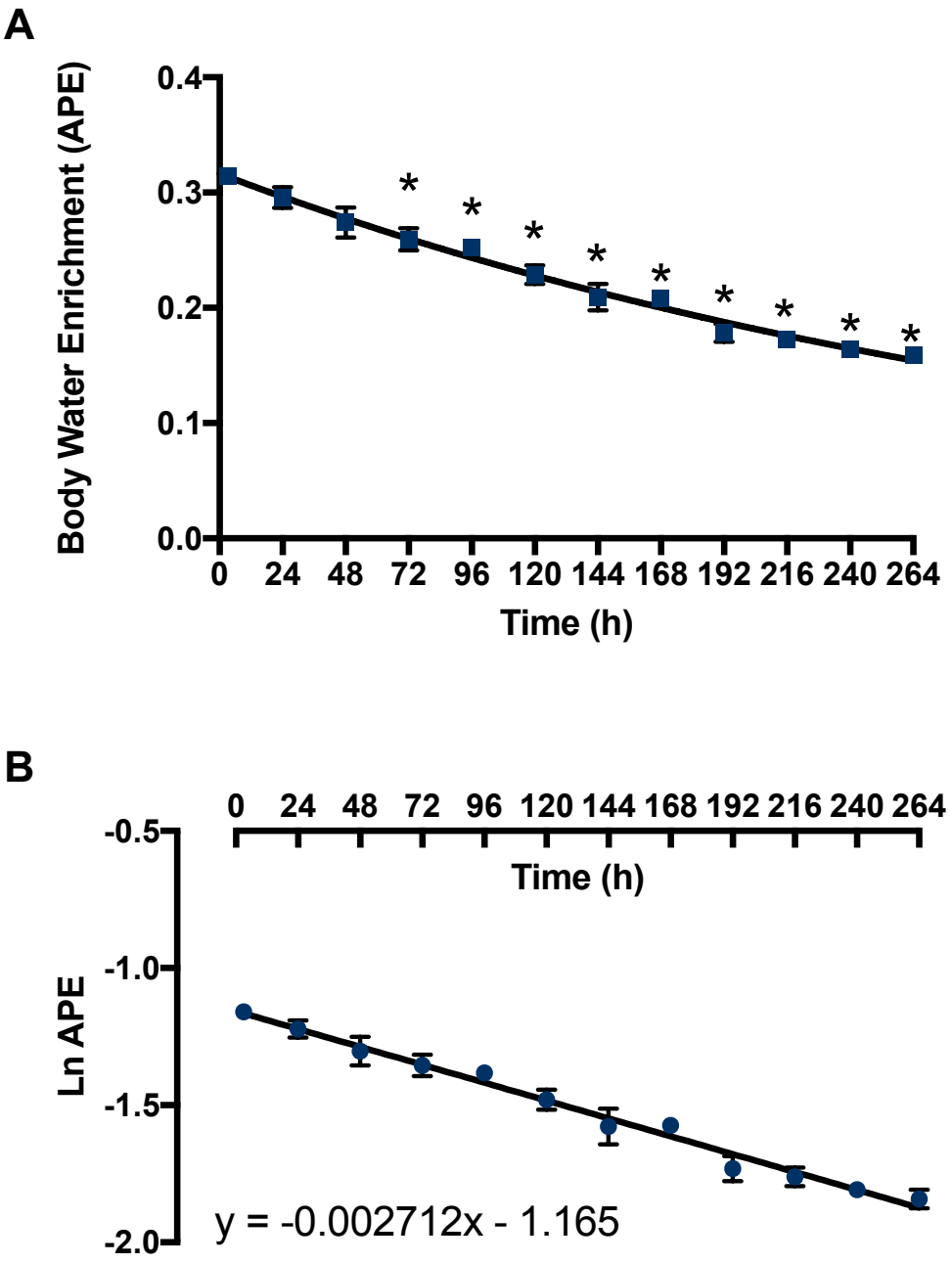


Figure 3.7. Deuterium body water enrichment and muscle protein incorporation A) time course of body water enrichment over 11 days following the oral consumption of D_2O and B) natural logarithm transformed body water enrichment to determine decay constant and half life. *0, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 refer to 0, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 h post-ingestion of D_2O, respectively. * indicates significant difference from initial day 0 body water enrichment ($P<0.005$).*

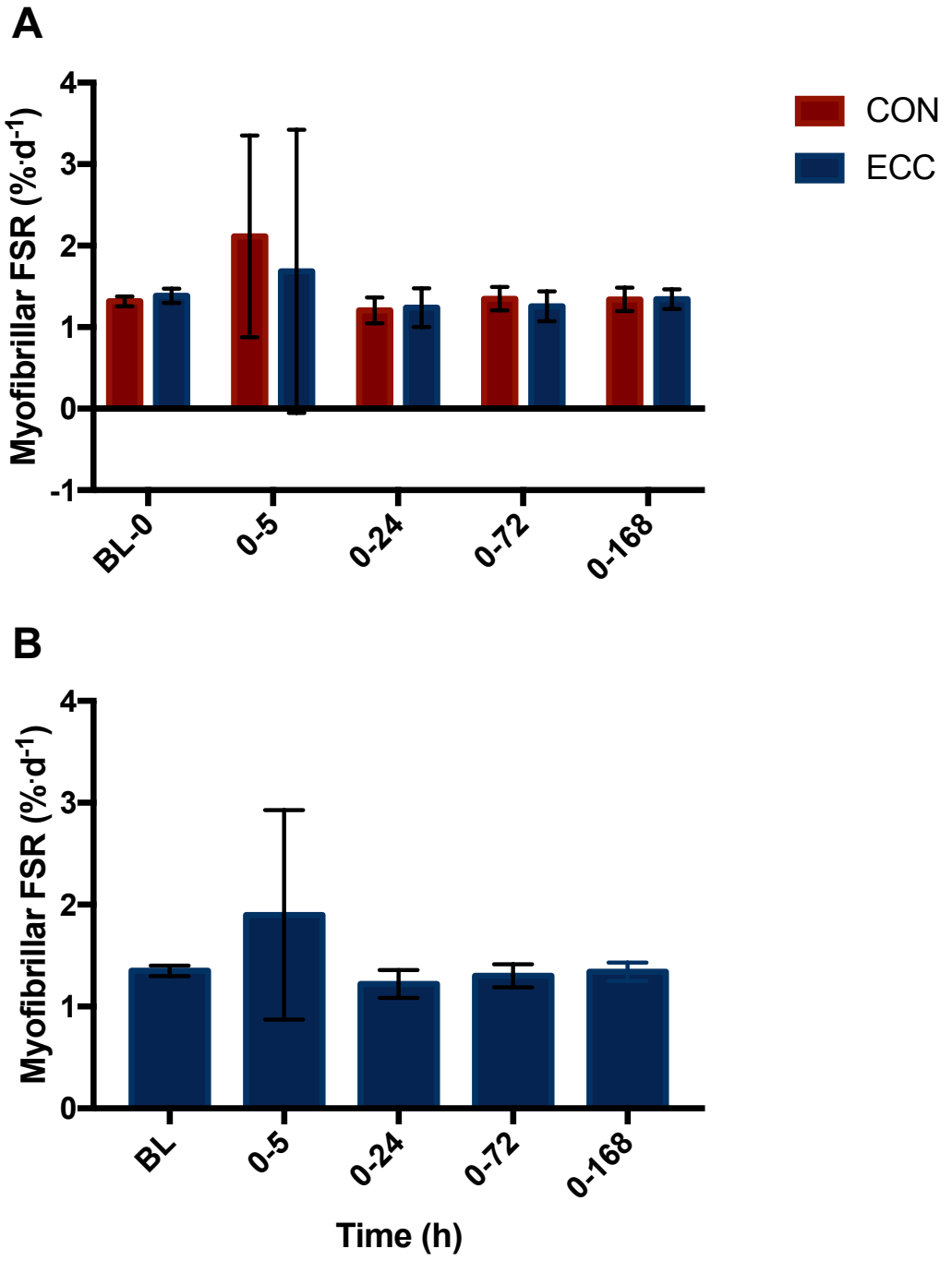


Figure 3.8. Baseline and temporal response of myofibrillar FSR up to 168 h post-exercise A) following ECC versus CON exercise and B) when the data was collapsed i.e. independent of contraction-type. *BL-0, 0-5, 0-24, 0-72, 0-168 refers to BL-0, 0-5, 0-24, 0-72, 0-168 post-exercise, respectively. ** indicates significant difference from BL (P<0.01).*

3.4.3 Anabolic and catabolic signalling responses to ECC versus CON exercise

ECC exercise induced increased phosphorylation of anabolic signalling protein mTOR^{Ser2448} and its downstream effector p70S6K1^{Thr389} immediately (P<0.05) and 5 h (P<0.005) post-ECC exercise (Figure 3.9 A-B). This early activation precedes the CON-induced phosphorylation of p70S6K1^{Thr389} at 5 h post-CON exercise (P<0.05). In comparison to CON, ECC exercise induced significantly greater phosphorylation of p70S6K1^{Thr389} immediately post-exercise. As with mTOR^{Ser2448}, only ECC exercise significantly up-regulated rps6$^{Ser240/244}$ at 5 h (P <0.01) with a strong trend for phosphorylation 24 h post-ECC exercise (P=0.05) (Figure 3.9 C). No significant changes in the phosphorylation of 4EBP1$^{Thr37/46}$ or eEF2^{Thr56} were observed at any time point following either ECC or CON exercise (data not shown).

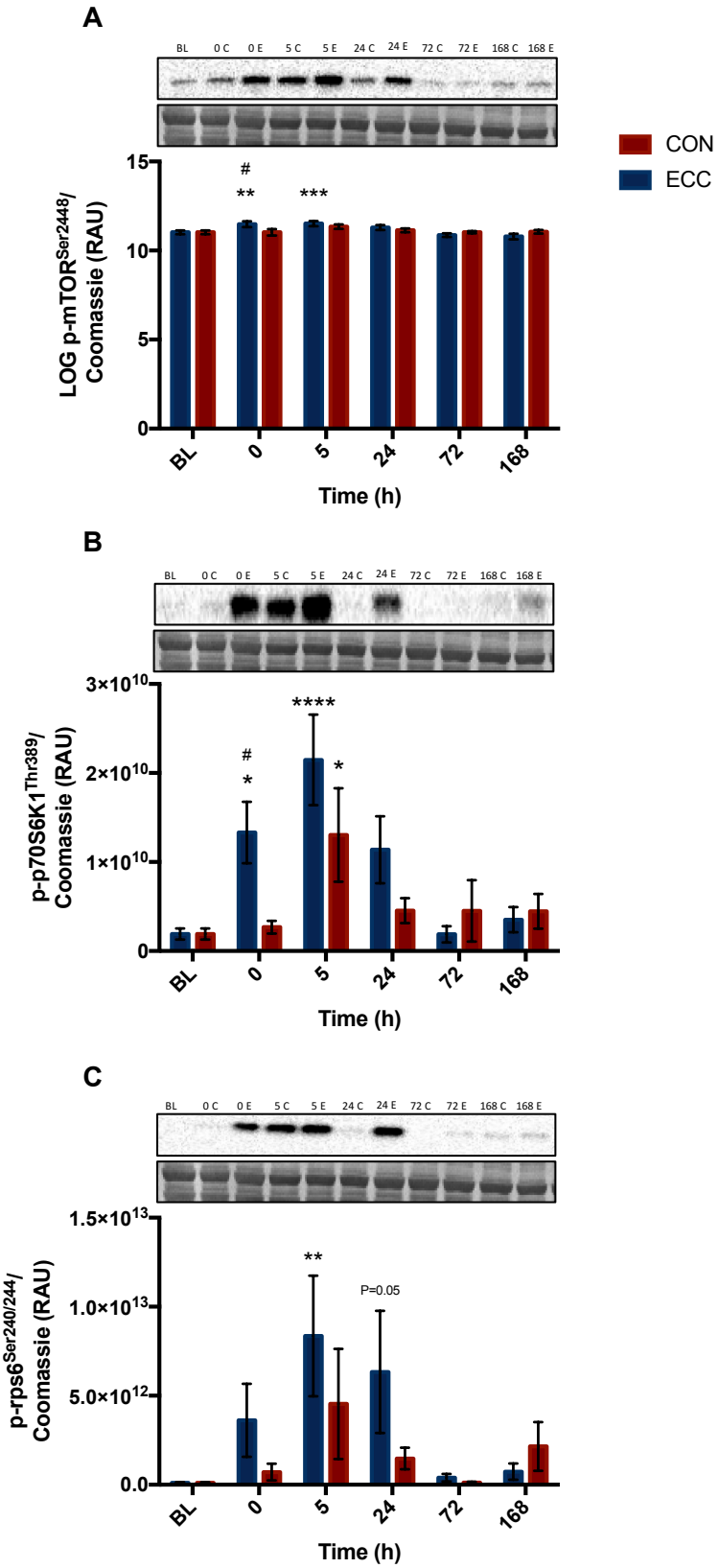


Figure 3.9. Temporal response of anabolic mTOR pathway signalling markers; mTOR^{Ser2448} (A), p70S6K1^{Thr389} (B) and rps6$^{Ser240/244}$ (C) following ECC versus

CON exercise. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. RAU relative arbitrary units. * indicates significant difference at that time point compared to baseline (P<0.05), ** (P<0.01), *** (P<0.005), **** (P<0.001) and # denotes significant difference between groups at that time point (P<0.05).*

Muscle proteolytic regulation following ECC versus CON exercise revealed the lysosomal protease Cathepsin L (37 kDa) was down-regulated following both contraction types 0 to 24 h post-exercise (P<0.05) (Figure 3.10 A). The ubiquitin ligase MuRF1 (UPS marker) increased 168 h post ECC and CON exercise (Figure 3.8 B), when function was regained. No changes in pro Cathepsin L (42 kDa), Cathepsin L (25 kDa), Beclin 1 (autophagy) or Calpain 1 (calcium-dependent cysteine protease) were observed at any time point following either contraction mode (data not shown).

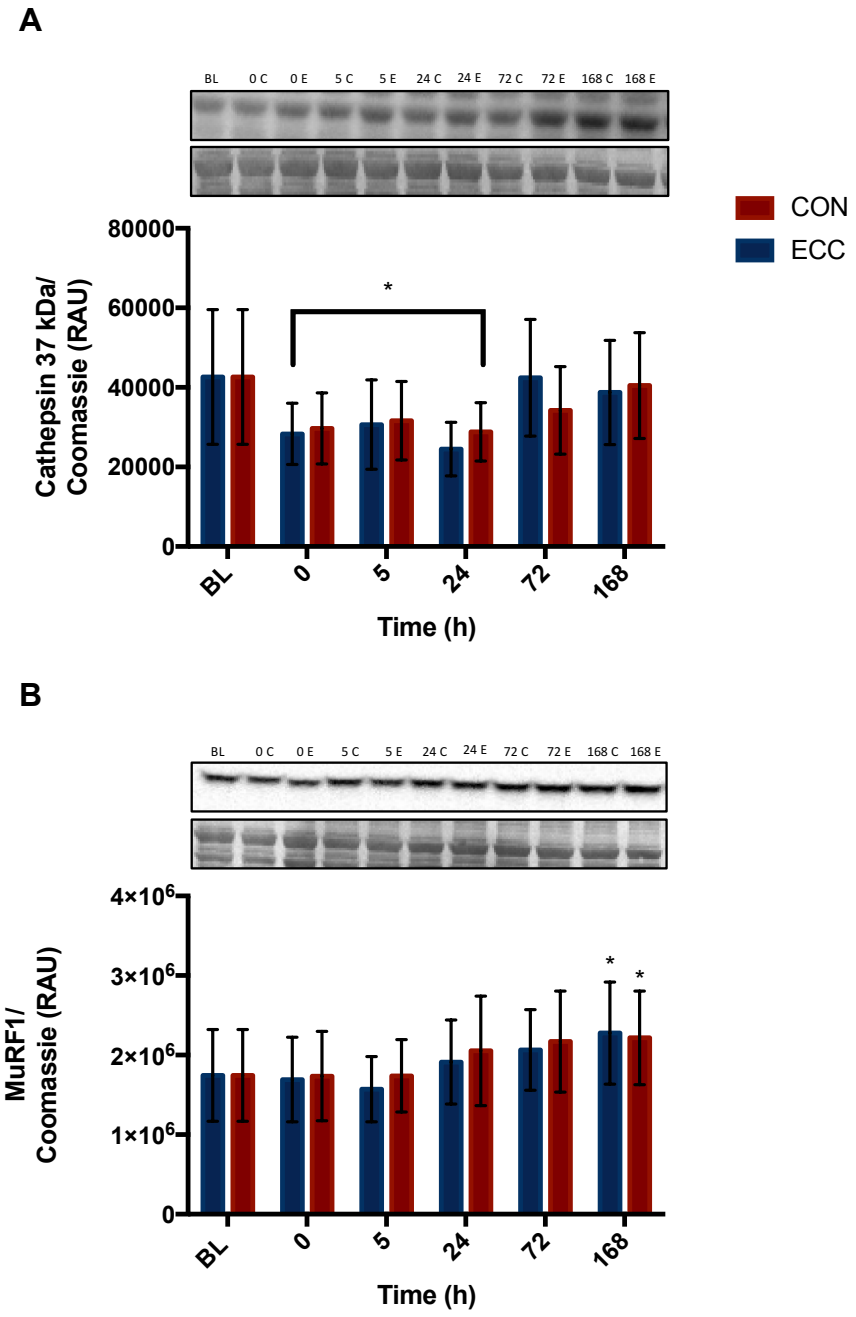


Figure 3.10. Temporal response of proteolytic markers; active Cathepsin L (A) and MuRF1 (B) following ECC versus CON exercise. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. RAU relative arbitrary units. * indicates significant difference at that time point compared to baseline (P<0.05).*

3.4.4 Inflammatory response

Immunoblotting against the inflammatory protein TNF-α revealed increased TNF-α 24, 72 and 168 h post-ECC exercise (P<0.05). This rise in TNF-α post-ECC exercise presented earlier than the rise post-CON exercise at 72 h, which also remained elevated above baseline 168 h post-CON exercise (P<0.001) (Figure 3.11 A). Only ECC exercise induced the phosphorylation of the transcription factor NFκβ $p65^{Ser536}$, a downstream effector of TNF-α, 72 h post-exercise (P<0.05) persisting with a trend at 168 h (P<0.08); no changes were observed between ECC vs. CON exercise (Figure 3.11 B). Histological muscle staining against $CD68^{+}$ (a pan macrophage marker) found the number of $CD68^{+}$ positive cells increased in the endomysial and perimysial space 72 h following ECC (P<0.0001) and CON exercise (P <0.01) but only persisted 168 h after ECC exercise (P<0.01) (Figure 3.11 C). No $CD68^{+}$ cells were observed within the muscle fibre (representative images in Figure 3.11 D-F). No difference in muscle $CD68^{+}$ expression existed between ECC vs. CON exercise.

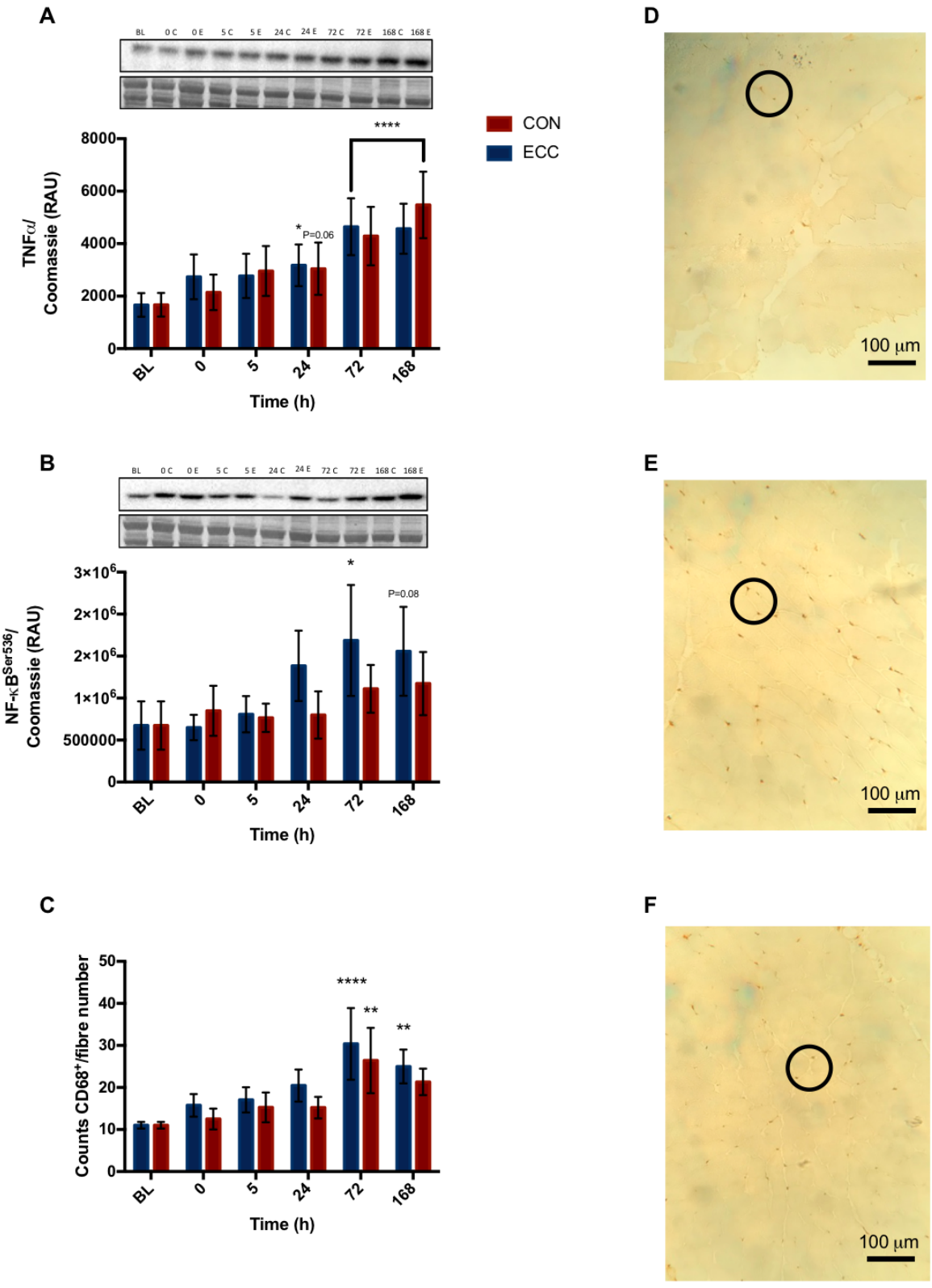


Figure 3.11. Temporal changes in local inflammatory markers; TNF-α protein abundance (A), phosphorylation of NFκβ p65^{Ser536} (B) and muscle macrophage

infiltration (C) in response to ECC versus CON exercise. Representative images of macrophage infiltration (black circles) at baseline (D), 72 h post-ECC (E) and 168 h post-ECC (F). *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. RAU relative arbitrary units. *indicates significant difference at that time point compared to baseline (P<0.05), ** (P<0.01) and **** (P<0.0001).*

3.4.5 Satellite cell response

No significant changes in mixed, type I or II specific $Pax7^+$ cells were observed at any post ECC or CON exercise time point (Figure 3.12). When these data were collapsed i.e. not taking into account the contraction mode, there was still no change in mixed or fibre type-specific $Pax7^+$ cells (data not shown). Thus, neither ECC or CON exercise induced SC activation up to seven days' post-exercise.

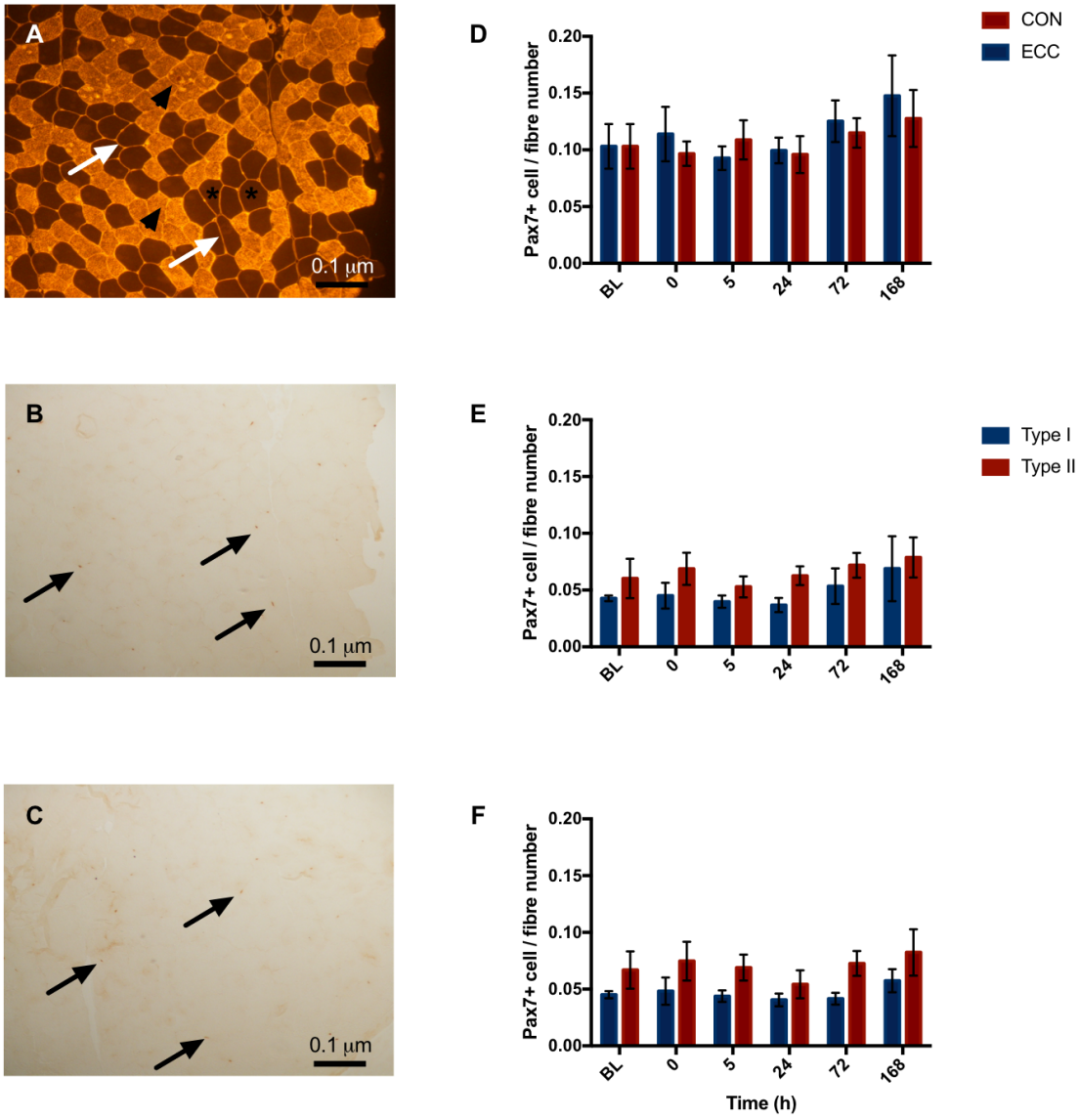
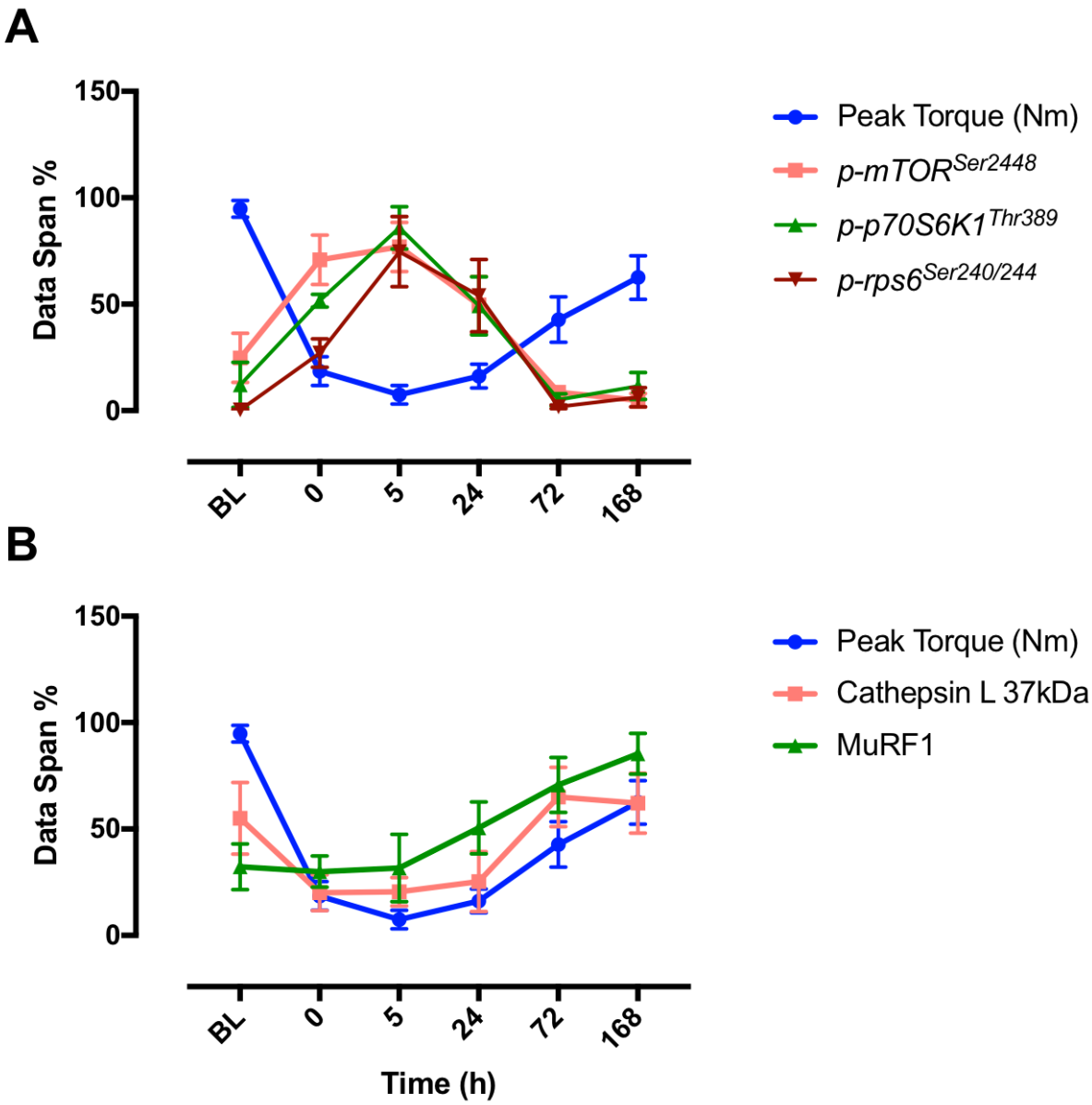


Figure 3.12. Lack of satellite cell response following an acute bout of ECC versus CON exercise. *(A) staining against type I fibres (black arrowheads) and laminin (white arrow) with unlabelled type II fibres (black asterisks), (B) baseline representative histochemical staining of SC's (Pax7$^+$ cells, arrows), (C) representative image of Pax7$^+$ cells 168 h post-CON exercise and (D) quantification of mixed muscle Pax7$^+$ cells pre-exercise and up to 168 h post-ECC vs. CON exercise (935±44 fibres analysed per time point), (E) quantification of type I and II specific Pax7$^+$ cells pre- and up to 168 h post-ECC exercise and (F) quantification of type I and II specific Pax7$^+$ cells pre and up to 168 h post CON exercise.*

3.4.6 Temporality of human muscle regeneration

Data spans were performed to investigate molecular events that precede the post-exercise functional recovery of muscle, as putative regulators of muscle regeneration. As anticipated, anabolic signalling presented as an early post-exercise response preceding functional recovery (Figures 3.13 A and C). Cathepsin L protease inactivation also occurred early post-exercise, whereas MuRF-1 was only activated when muscle function was near returning to basal values (Figures 3.13 B and D). Macrophage infiltration of the ECM and inflammatory signalling (TNF-α and the downstream activator NF-κBp65^{Ser536}) only increased once muscle function had past its nadir (Figures 3.14 A-D). Thus, concurrent activation of anabolic signalling and inactivation of lysosomal-mediated proteolytic signalling all present as early molecular responses preceding functional recovery to ECC exercise and, in general, CON exercise. The concomitant increase in the ubiquitin-proteasome system, macrophage infiltration and inflammatory signalling activation occurred once force had started to recover and thus may be required for longer-term repair/ augmentation of muscle ultrastructure and, ultimately, function.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p-rps6 Ser240/244
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Time (h)
Peak Torque (Nm)
Cathepsin L 37kDa
MuRF1

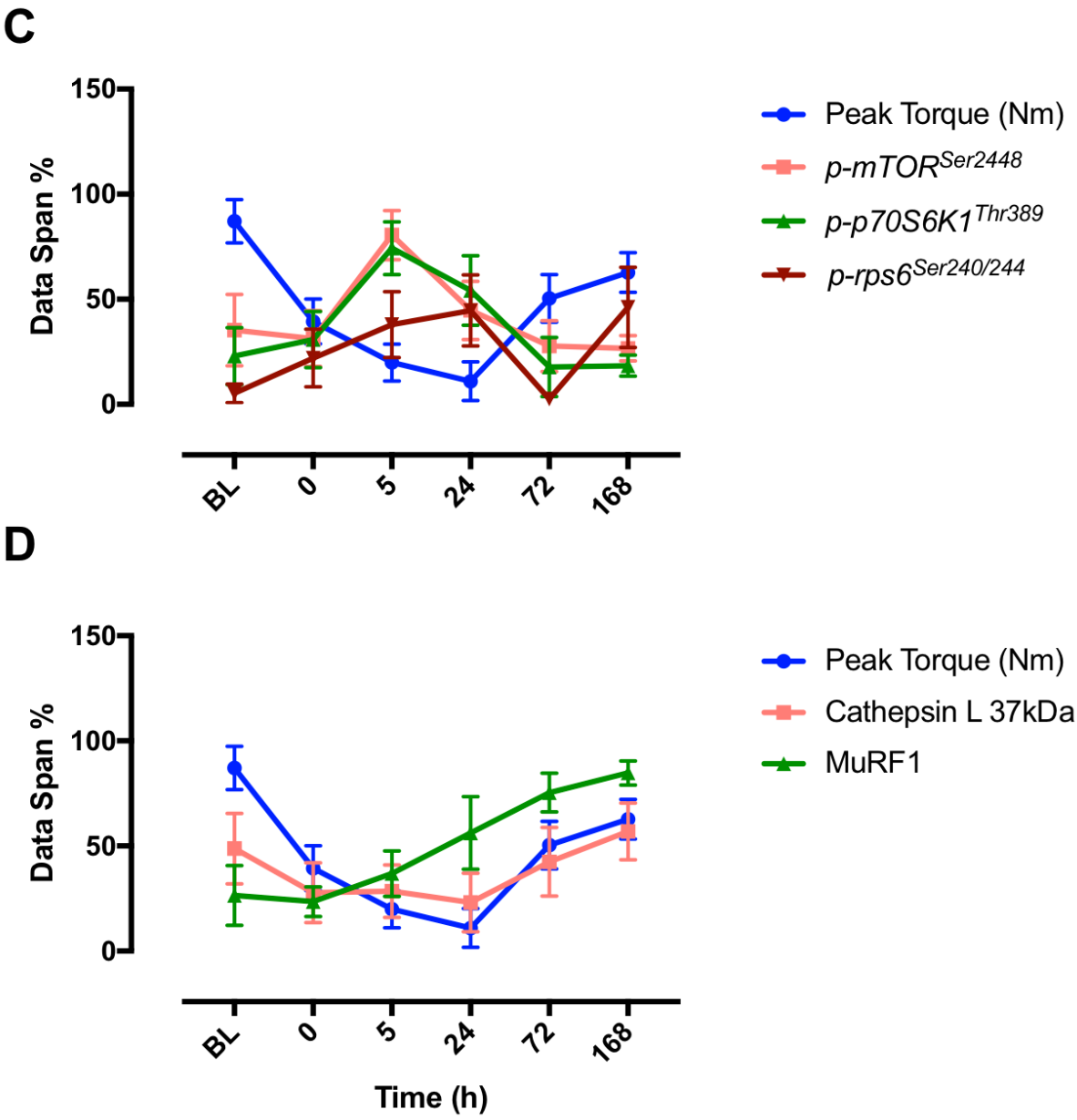
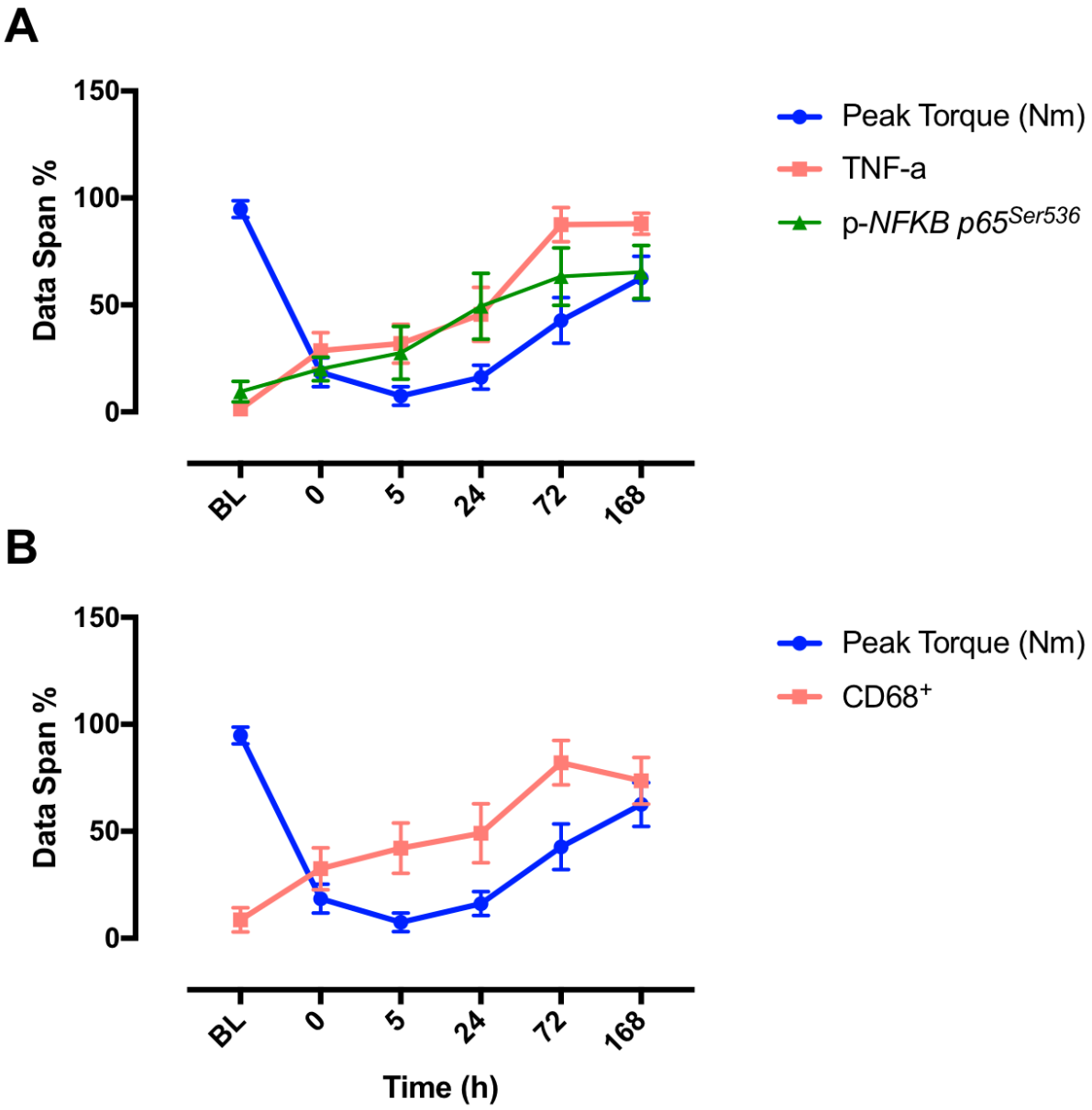


Figure 3.13. Data spans of anabolic signalling and functional recovery for ECC (A) and CON (C) exercise, and catabolic signalling and functional recovery for ECC (B) and CON (D) exercise. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively.* All significance values are presented in Figures 3.4, 3.9 and 3.10.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Time (h)
Peak Torque (Nm)
CD68+

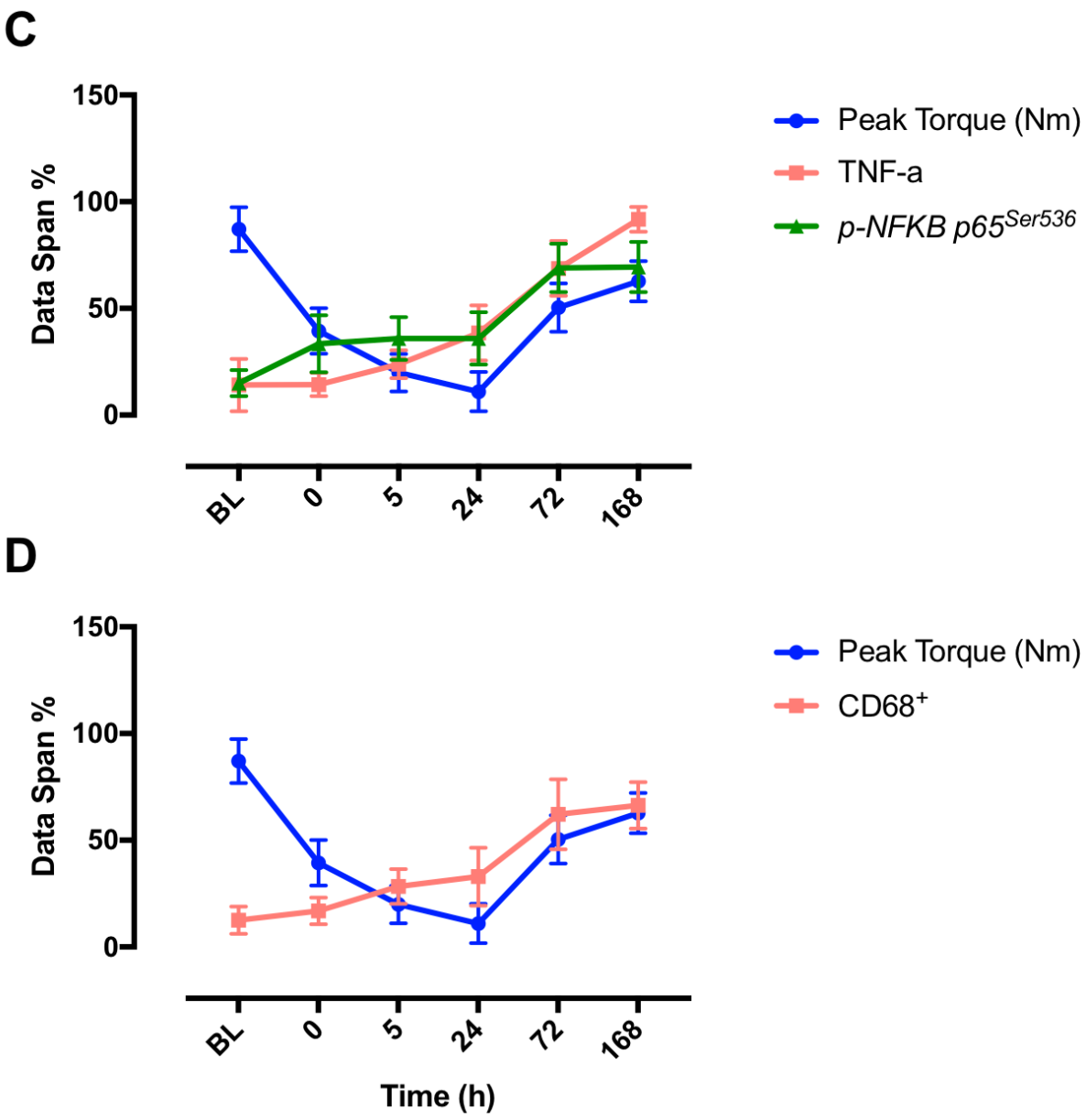

Figure 3.14. Data spans demonstrating the temporality of macrophages and functional recovery following ECC (A) and CON (C) exercise, and inflammatory signalling and functional recovery following ECC (B) and CON (D) exercise. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. All significance values are presented in Figure 3.4 and 3.11.*

3.5 Discussion

This study shows novel temporal data regarding the mechanistic underpinnings of muscle regeneration following ECC versus CON exercise in healthy young males. Both ECC and CON exercise resulted in reduced peak torque (which correlates to myofibrillar disruption (270)) indicating the presence of muscle damage, highlighting that both exercise modes were successful at initiating skeletal muscle regenerative processes. The key findings of this study demonstrate that i) increased anabolic signalling and repression of lysosomal proteolysis precedes functional recovery following both ECC and CON exercise and ii) subsequent inflammatory signalling, macrophage infiltration and up-regulation of the ubiquitin-proteasome system occur alongside functional recovery after both ECC and CON exercise. Additionally, compared to CON, ECC exercise is associated with: i) greater and prolonged declines in muscle function, ii) earlier onset and greater magnitude of anabolic signalling, iii) earlier onset and sustained increases in inflammatory markers and iv) an activated inflammatory transcriptional response.

Components of the anabolic mTORC1 signalling pathway were rapidly and transiently up-regulated during the early post-exercise phase following both ECC and CON exercise, preceding functional recovery. This is in agreement with an earlier study which found early and transient increases in mTORC1 signalling up to 5 h post ECC and up to 1 h post-CON exercise in young adults (272). Whilst the present study reports a return of anabolic signalling to baseline by 24 h post ECC and CON exercise, others have reported mTOR signalling persisted for up to 8 days following 300 ECC contractions (221). However, it is unclear whether the participants in that study were fed when they returned to the laboratory for post-

exercise measurements. Being in a fed state may confound the results since feeding alone can induce anabolic signalling and may induce a potentiation of the post-exercise anabolic effect (348). Early anabolic signalling contributes to increased MPS, which is critical for synthesising structural and contractile proteins that replace damaged and degraded proteins in response to exercise-induced damage, therefore remodelling/repairing muscle structure and function. This is somewhat substantiated in rodents, since functional recovery was prolonged when mTOR signalling was inhibited (23). Recent work in humans has shown the MPS response to a single bout of unaccustomed RE was higher than after an acute RE session performed after three and ten weeks of RET (153). When MPS was normalised to ultrastructure damage (i.e. Z-disk streaming), the relative increase in MPS was similar across the RET programme, further highlighting that increased anabolic events are directed towards the remodelling/repair of the damaged muscle protein (153).

Unexpectedly, no significant changes in myofibrillar MPS were shown following either ECC or CON exercise at any time-point investigated within this study. Previous work has shown increased MPS in response to a single bout of RE, ECC or CON exercise, a response which is well acknowledged (72, 172, 173, 224, 260). Herein, several instances were noted where the delta shift, representative of isotopic enrichment, was reduced at later time-points compared to earlier time-points. For example, in one young participant at 0 h post-ECC exercise mean delta was 260, which reduced to 203 5 h post-ECC exercise (all raw data can be viewed in Appendix 3.1). This drop in delta is unexpected since D_2O is cumulative, therefore physiologically the delta should increase even in the absence of an external stimulus

(i.e. exercise). Perhaps, sample preparation errors may have lead to erroneous data. Consequently, all of the MPS analysis will be re-run and analysed.

A novel finding of this study was the rapid repression (0 h) of the lysosomal protease Cathepsin L post-exercise, which occurred in tandem with increased mTOR and p70S6K1 signalling, preceding functional recovery. This repression was sustained for 24 h post-exercise, even once mTORC1 signalling had returned to baseline. Cathepsin L is implicated in the degradation of many myofibrillar proteins, including myosin heavy chain, alpha-actinin, actin, troponin T and troponin I (206). Suppression of Cathepsin L may indicate a reduced capacity for lysosomal-mediated dismantling of the contractile structure. Hypothetically, this suppression may reduce protein breakdown within the muscle, contributing to the accumulation of muscle proteins being synthesised. Few previous studies have investigated Cathepsin L regulation post-exercise. Feasson et al., (2002) reported no early change in muscle Cathepsin L enzyme activity, but did observe a late increase 14 days' post-exercise. This led the authors to conclude that Cathepsin L may contribute to post-exercise muscle remodelling as opposed to functional recovery. Interestingly, this early proteolytic response seems to be unique to the lysosmal breakdown pathway, since no early changes were observed in local Beclin-1, Calpain-1 or MuRF1. It is possible that additional markers of proteolysis, which have not been measured here, may regulate early muscle regenerative responses. For example, Calpain 3 is activated 24 h following ECC exercise (230), however Calpain 1 was chosen to be investigated herein since it is implicated in muscle protein degradation and subsequent shuttling to the UPS within physiological ranges of Ca^{2+}, but the role in human muscle regeneration is poorly defined (229). Thus, the early increase in anabolic signalling

and repressed lysosomal activity, which precede functional recovery, suggest that both processes may be involved in functional recovery.

Combining the data showing increased markers of anabolism and a repressed marker for protein degradation following exercise, suggests that there is a growth environment which facilitates muscle remodelling and possibly hypertrophy. Following ECC exercise, the onset of anabolic signalling was earlier than CON exercise, suggesting that this anabolic growth environment is greater following ECC exercise and thus may lead to greater muscle remodelling and may explain the greater hypertrophic adaptations reported (283). Since muscle is capable of adaptation after one bout of exercise in as short as seven days (i.e. the RBE) (55), it is likely that these processes are central for the rapid response of muscle to a single exercise-induced insult.

In tandem with the late induction of inflammatory signalling and macrophage infiltration, the E3 ubiquitin ligase MuRF1 increased 168 h following both ECC and CON exercise, at which point force had fully recovered. MuFR1 is implicated in the degradation of the contractile filaments and the structural protein titin via the UPS (121), and therefore may indicate the activation of UPS-mediated proteolysis of sarcomeric proteins during the later stages of muscle regeneration. This late proteolytic activation may degrade any exercise-induced damaged proteins, which may not have been dismantled and degraded during the earlier time-points since lysosomal degradation was repressed. Although, as previously mentioned it is possible that other proteolytic signals not measured herein (i.e. calpain 3) were

activated earlier and have been missed (230). This late activation of MuFR1 and potentially increased UPS activity 168 h post-exercise, once function had recovered, may thus be required for longer term remodelling. Similar reports of increased UPS activation during late stages of regeneration have been reported (98), others have reported no change in MuRF1 protein levels, although the time frame investigated was acute (< 48 h), and there for may have missed later increases (273, 311). Despite demonstrating an early suppression of the lysosomal marker Cathepsin L and the late increase in the ubiquiting ligase MuRF1, we did not measure MPB directly. There does exist a disassociation between the abundance of proteolytic protein markers and MPB (126), thus we cannot infer whether or not MPB changed throughout the duration of this study.

Based on the protein turnover related data (excluding MPS) presented herein, it may be suggested that where early anabolic processes are activated i.e. (0-5 h) the catabolic mechanisms are supressed, and once this anabolic environment has dissipated a catabolic environment is induced later (168 h) for the removal of damaged proteins. Furthermore, data from within this study suggests that the process of full muscle recovery/ regeneration at the molecular level takes longer than the typically investigated time-frame of 7 days' post-exercise (since functional recovery occurs within this time). Such markers which occur beyond 7 days (i.e. inflammation and UPS activation) may potentially be novel candidates that appear to specifically be associated with the delayed remodelling of muscle beyond acute functional recovery in response to a single bout of strenuous exercise.

Increased TNF-α becomes evident once functional recovery is initiated i.e. the nadir of force has passed, and persists once muscle function is fully regained following both contraction types. The source of the observed elevations in TNF-α is unknown (232), and since TNF-α activation preceded significant increases in macrophages, it is unlikely that this source of TNF-α was macrophage-derived. However, TNF-α can drive the activation of pro-inflammatory macrophages within skeletal muscle (323) and may explain the increased infiltration of macrophages observed 72-168 h post-exercise, in line with previous reports (247). Significant macrophage infiltration occurred after the most severe declines in muscle function, persisting once function had fully recovered. This observation may suggest a role for macrophages in promoting muscle remodeling for longer term muscle adaptation. In support of this notion, macrophage conditioned medium has been shown to increase muscle mass of regenerating muscle fibres (47), a consequence of increased SC proliferation (205), although this precise mechanisms remains to be shown in humans.

Interestingly, we report no significant changes in SC following ECC or CON exercise throughout the regenerative process (although numerical increases were present). The lack of SC activation post-exercise is in contrast to previous reported studies which show elevated SC activation as early as 24 h post-ECC exercise (49, 84). A number of reasons may explain the differential findings, primarily the total number of muscle fibres counted when enumerating SC content is important (197). This is because the SC content can vary across a single muscle cross-section with areas containing substantially more and substantially less SC, therefore it is recommended that as many fibre cross-sections as possible are counted (ideally the whole muscle cross section), with higher fibre numbers providing more reliable

results (197). Previous studies which have reported increased SC responses have used less than 400 fibres to analyse SC numbers (49, 63, 84). In the present study SC content was robustly determined at each time point by counting all of the SC on the muscle-cross section, averaging 935±44 fibres. It is also possible that SC activation may occur later than 168 h. In agreement with the current data, Farup et al (2014) report no changes in mixed or fibre type specific SC content 24, 48 or 168 h post-ECC exercise. Interestingly, the study by Farup et al (2014) study quantified SC content on >450 fibres at all time points. Thus, the present data herein suggest SC activation is not required for functional recovery or muscle remodelling following ECC or CON exercise in healthy young sedentary males. Although these data refute the established dogma that SC are a requirement for muscle regeneration, it may be explained by the fact that SC are not required for longer-term muscular adaptation i.e. muscle hypertrophy (210). As such, these data suggest that the muscle intrinsic molecular mechanisms (i.e. proteolytic suppression, increased anabolic and inflammatory signalling and inflammatory infiltration) that are exercise-responsive are the essential global (i.e. in response to both ECC and CON exercise, albeit to different extents) processes for muscular adaptation to exercise in young muscle. Furthermore, this lack of SC response may indicate the muscle possesses sufficient capacity within nuclear DNA to transcribe and translate the material required to repair and grow muscle, and/ or the efficiency by which chaperone-mediated repair mechanisms can cope with repairing exercise-induced damaged proteins. Thus the muscle stimulus herein may not have induced fibre necrosis to the extent that SC were needed to fuse and repair damaged fibres.

Despite the generally accepted notion that muscle inflammation occurs after unaccustomed exercise, there are very few investigations into the human responses of the inflammatory-related transcription factor NF-κB following exercise (151). Therefore investigation of the temporal response of NF-κB to ECC versus CON exercise are lacking. In the inactive state, NFκβ is sequestered in the cytoplasm bound to IκB inhibitor proteins (33). The presence of TNF-α (amongst other inflammatory stimuli) induces the phosphorylation and subsequent degradation of the NF-κB inhibitor, IκBα, through the UPS (228). This releases the p50-p65 NF-κB heterodimer for translocation to the nucleus where it binds to target genes to regulate gene expression (228, 244). When active, NF-κB promotes the expression of > 150 target genes, which relate to immunoreceptors, cytokines, cell adhesion molecules, cell surface receptors, apoptosis regulators, stress response and transcription factors (244). The present study is the first to show the activation of NF-κB 72 h post-exercise, which was unique to ECC exercise despite the up-regulation of TNF-α following both ECC and CON exercise. Furthermore, the activation of NF-κB was initiated after the nadir of force suggesting that NF-κB activation did not not contribute to functional recovery and instead may be involved in subsequent muscle remodelling. This regulation of remodelling is unknown, however in mice NF-κB binds to the MuRF1 promoter, increasing gene expression of the E3 ubiquiting ligase thus promoting proteolysis (46). Herein, MuRF1 increased 168 h post-exercise and may indicate increased proteolysis of exercise-induced 'damage' proteins, essential for longer-term muscle remodelling. Furthermore, following ECC-bias exercise in rodents, NF-κB has also been implicated in modulating gene expression of several nitric oxide synthase (NOS) genes which are implicated in blood flow regulation, which is believed to be essential to muscle regeneration (187), although this remains

to be shown in humans. One of the few studies in humans found NF-κB was activated and localised to pericytes 3 h post-ECC exercise, implicating NF-κB in vascular remodelling (151), although later time points were not investigated. These processes may regulate adaptation observed following chronic ECC exercise. Further work is needed to understand which genes are regulated by NF-κB during skeletal muscle regeneration in humans.

Herein both ECC and CON exercise elicited early anabolic signalling and early lysosomal inhibition which preceded functional recovery, followed by increased inflammatory signalling and macrophage infiltration once the recovery of force had been initiated. Despite general temporal similarities, contraction-specific differences were found. Compared to CON, ECC exercise resulted in the earlier onset of soreness and exacerbated and prolonged declines in peak torque indicating ECC exercise induced greater muscle disruption compared to CON exercise, which is a well acknowledged phenomenon (57). These data demonstrate the lengthening nature of ECC contractions leads to greater muscle damage. Interestingly, the nadir of force was 5 h post-ECC exercise, which to the authors knowledge has not been shown before in humans. One possible explanation may be fatigue, although typically this is subsided after 2 h (265). The high levels of tension placed on the fibres due to the lengthening nature of ECC contractions most likely explains the decline in force at 5 h (6). In addition, the mechanical lengthening of myofilaments likely explains the greater Z-line streaming observed following ECC versus CON exercise (224), although this was not investigated in this study.

In addition to greater muscle dysfunction, ECC exercise induced an earlier onset and greater magnitude of mTOR signalling proteins compared to CON exercise suggesting ECC exercise induces a greater net anabolic environment. This may be due to the greater and more prolonged declines in function, thus requiring a greater anabolic signalling response in order to restore the greater deficits in muscle function. Similar to the current study, a recent report found the phosphorylation of $mTOR^{Ser2448}$, $p70S6K1^{Thr389}$ and $rps6^{Ser235/236}$ to increase following both ECC and CON exercise in humans, which was more persistent following ECC (up to 24 h post) compared to CON exercise (1 h post) (273). This finding provides further evidence of a greater anabolic signalling environment post-ECC exercise. This greater anabolic environment may culminate over successive exercise bouts of ECC exercise and translate into and explain the greater gains in muscle mass and strength observed following chronic ECC compared to CON training (283). Interestingly, ECC exercise lead to the unique up-regulation of NF-κB, despite similar levels of TNF-α. Perhaps other mechanisms not measured herein were responsible for the activation of NF-κB, such as Calpain 3 (228), although this needs to be investigated further. The fact that NF-κB was only activate post-ECC exercise may unique transcriptional responses which may mediate contraction-specific adaptation although this remains poorly defined.

Collectively, these data suggest that anabolic, catabolic and inflammatory molecular responses are critical in orchestrating human skeletal muscle regeneration following exercise. However, the extent of anabolic and inflammatory responses was greater following ECC exercise which might elicit greater muscular adaptation.

This study is not without limitations. The absence of measuring MPB precludes our understanding into how protein catabolism is regulated throughout an extended post-exercise regenerative period in relation to force and other regenerative mechanisms. The dynamic measurement of MPB is difficult and invasive in nature and thus static surrogates of the proteolytic pathways were investigated as an alternative. Furthermore, the lack of investigative time points beyond 168 h precludes our understanding of MPB, inflammatory and SC regulation during the remodelling phase. Future work should address a more comprehensive time course between 0-5 and 24-168 h, where we observe anabolic and inflammatory environments, respectively, in order to further characterise the temporality of the mechanisms regulating regeneration. Additionally, future investigations should characterise the inflammatory response beyond 168 h.

3.6 Conclusion

Herein, we provide one of the most comprehensive studies to date investigating the temporality of established and novel regulators of muscle regeneration simultaneously following ECC vs. CON exercise in young exercise naïve males. This study shows anabolic signalling and lysosomal repression precede functional recovery and therefore regulate rapid adaptation, whilst inflammatory signalling, macrophage infiltration and UPS activation occur after functional recovery is initiated, likely mediating longer term/ chronic muscle adaptation. Despite similar temporality between contraction types, ECC exercise was associated with magnified muscle soreness, dysfunction, anabolic signalling and inflammation, augmenting the net anabolic environment which may explain the greater strength and mass

adaptations previously observed following chronic ECC training compared to CON training (283).

4 Exploring the Mechanisms Underpinning Age-Related Human Skeletal Muscle Regeneration Following Acute Eccentric versus Concentric Exercise

4.1 Abstract

Background: Ageing is associated with reduced skeletal muscle mass and function, which may be a result of impairments in post-exercise skeletal muscle regenerative processes. However, the age-related differences in exercise-induced skeletal muscle regeneration remain poorly defined. The aim of this study was to investigate multiple metabolic and molecular mechanisms implicated in human skeletal muscle regeneration over a comprehensive time-course following 'damaging' eccentric (ECC) versus 'non-damaging' concentric (CON) exercise in young and older participants. **Methods:** Eight young (22±1 y) and eight older (70±1 y) healthy exercise naïve participants performed a single bout of unilateral ECC exercise (7×10 repetitions at 80% of ECC one-repetition maximum) and unilateral CON exercise (7×10 repetitions at 80% of CON one-repetition maximum). Functional (muscle soreness, sensitivity to pain, peak torque, power and lower body function), biochemical (plasma creatine kinase) and molecular responses (mTORC1 signalling, proteolytic activation, inflammatory signalling, macrophage infiltration, satellite cell proliferation) were measured at baseline (BL), immediately (0), 5, 24, 72 and 168 h after ECC and CON exercise. **Results:** Both exercise modalities resulted in reduced peak torque, which onset earlier (0 & 5 h, $P<0.05$), was greater (0 & 5 h, $P<0.05$) and persisted for longer (72 h, $P<0.01$) following ECC exercise. p70S6K1 and rps6 (5 h, $P<0.05$) increased following ECC exercise preceding functional recovery. Satellite cell proliferation increased 24 h post- ECC exercise ($P<0.05$), when functional restoration was underway. As expected, basal ECC and CON torque was higher in younger versus older participants ($P<0.05$). Older participants displayed blunted anabolic (mTOR and p70S6K1) and catabolic (cathepsin L (37 kDa)) signalling following exercise. Interestingly, older participants displayed higher basal

levels of muscle TNF-α compared to the young (P<0.05). TNF-α did not change at any post-ECC or CON time-point in the older participants, but was increased 24-168 h after ECC and 72-168 h after CON exercise in the young (compared to BL, P<0.05). Similarly, basal macrophage infiltration was higher in the older versus younger participants (P<0.05), and only increased in the young post-ECC (72 and 168 h, P<0.01) and CON (P<0.01) exercise. SC content was greater in older versus younger participants 24 h post-ECC exercise (P<0.01). **Conclusion:** In older adults, ECC exercise stimulated SC activation and induced greater anabolic signalling compared to CON exercise and is thus potentially a more potent exercise stimulus of muscle growth in ageing muscle. Blunted anabolic and catabolic signalling and higher basal inflammation in older adults may underlie the blunted adaptations to RET programmes and age-related loss of skeletal muscle mass and strength in older adults. However, these perturbations in regenerative mechanisms did not impede functional recovery.

Specific acknowledgements

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4.2 Introduction

The plasticity of skeletal muscle is diminished with advancing age, demonstrated by blunted anabolic responses to acute exercise (112) and attenuated absolute increases in muscle growth and strength in response to chronic RET (128). Although, it should be noted that some studies have shown equal growth and strength adaptations between young and older adults (136, 208). Over time, blunted anabolic responses accumulate presenting as age-related losses in muscle mass (i.e. sarcopenia) and strength (i.e. dynapenia) (223, 286). Sarcopenia is associated with functional impairments (154), falls, physical disability, morbidity (24), reduced quality of life (281) and premature mortality (175). Therefore, identifying regulators of sarcopenia is critical to developing effective countermeasures.

Currently, the most effective intervention for counteracting age-related losses in skeletal muscle mass and strength is RET (36, 100). Conventional RE can be divided into two phases: ECC phase where the muscle contracts whilst lengthening, and the CON phase where the muscle contracts while shortening (106). Work-matched (e.g., 80% 1-RM) ECC exercise produces greater forces than CON exercise, translating into superior gains in muscle mass and strength (283) at a reduced metabolic cost (i.e. reduced oxygen consumption) over the course of a training period (1). As such, ECC exercise interventions may be more efficacious than CON exercise for promoting muscle growth, and therefore, may harness potential as an intervention in populations characterised by low muscle mass, such as the elderly.

However, the safety of ECC exercise in older adults has been questioned since ECC contractions induce muscle damage (i.e. morphological disruption and the loss of force) to a greater extent than CON contractions (118). This damage necessitates a regenerative response in order to restore muscle homeostasis and promote muscular adaptation. Based on evidence in rodent models (41, 97, 271, 359), it has been hypothesised that the intrinsic muscle regenerative capacity in ageing is diminished. It is suggested that functional and structural deficits are not repaired effectively, which over subsequent bouts of exercise culminates losses of muscle mass and strength (97), further contributing to sarcopenia. However, the combination of mechanisms regulating human exercise-induced muscle damage and regenerative responses, especially in older adults; remain poorly defined.

Some human research has shown greater muscle damage denoted by Z-disk streaming (201) and slower functional recovery (73) in older compared to younger adults. Conversely, others have shown similar damage and repair responses between the ages (56); thus, the age-related damage-regeneration response is humans remains unclear. Conceptually, age-related perturbations in human muscle regenerative capacity are plausible since ageing *per se* has been shown to be associated with attenuations in the basal population of SC (160, 328) and heightened basal muscle inflammation (219). Furthermore, in response to acute RE anabolic signalling, MPS (112, 173), inflammation (137) and the rise in SC activation (84) responses are blunted; although, contradictory evidence does exist (44, 287). Typically, previous studies have been limited to 24-48 h follow up periods measuring limited regenerative mechanisms, precluding a comprehensive understanding of the no-doubt complex regenerative process in ageing.

The most comprehensive human investigation to date explored the regenerative process up to 7 days' post exercise, although only 2 and 7 days' post-exercise were assessed. This work found no age-related heightened damage susceptibility following ECC exercise denoted by similar changes in peak torque, muscle soreness and plasma CK (a marker of muscle membrane damage) in young and older adults (44). Furthermore, serum inflammatory markers, myogenic markers and angiogenic regenerative responses were similar between the ages, thus suggesting that ageing *per se* does not impair muscle regeneration (44). These results go against the generally accept dogma in rodents, which suggests that there are age-related attenuations in muscle regenerative mechanism. Consequently, further research into age-related human regenerative mechanisms is required. In addition, the central role of age-related protein turnover responses in degrading exercise-induced 'damaged' proteins and depositing new functional proteins has been overlooked since no studies have measured MPS and/ or MPB >48 h post-exercise (112, 172, 173).

With these identified knowledge gaps, further research which investigates key exercise-induced regenerative mechanisms simultaneously over the time-course of functional decline and subsequent recovery in older versus younger adults is required. Such investigation may highlight age-related alterations in muscle regenerative capacity which may contribute to the progression of sarcopenia. Furthermore, this knowledge may aid the development of interventions aimed at recovering age-associated losses in regenerative capacity.

Therefore, the first aim of this study was to investigate multiple metabolic and molecular mechanisms implicated in human muscle regeneration over a comprehensive time-course following ECC 'damaging' exercise versus CON 'non-damaging' exercise in older adults. A second aim of this study was to compare the regenerative responses between young and older adults. Combined, such data will enhance current understanding of muscle regeneration during ageing, and will highlight whether there are any differences in the regenerative responses between the ages which may go some way in explaining age-related muscle loss.

4.3 Methods

4.3.1 Study Design

Eight older healthy exercise naïve male participants were recruited for the study (70±1y, 26±1kg·m-^{2} body mass index, 190±13kg ECC 1-RM, 95±6kg CON 1-RM), with all recruitment methods fully detailed in Chapter 3 (3.3.2). In brief, participants attended a health screening session to ensure suitability for the study and baseline data were collected (SPPBT, peak torque and power). Upon study inclusion, participants were required to visit the laboratory on five separate occasions over twelve days. On the first visit (baseline), participants arrived fasted at 9 am and muscle soreness (VAS) and PPT were measured. Participants provided a baseline saliva sample followed by a baseline muscle biopsy and a venous blood sample. Afterwards, participants consumed 3ml/kg of D_2O (70% APE) in order to measure MPS, and provided daily saliva samples at mid-day everyday (collected at least 30 minutes after eating or drink) throughout the duration of the study to determine body water enrichment.

Ninety-six hours later, participants arrived at ~8.30 am for visit 2 (0 h), having consumed a 250 ml liquid high energy nutritionally complete drink (Fortisip, Nutricia, Netherlands) at 07:00 am but remained fasted thereafter until visit 2 was complete, at ~5.30 pm. Participants acted as their own internal controls by performing ECC exercise on one leg and CON exercise on the contralateral leg using the MLP. Participants underwent a familiarisation, warm up and ECC/CON 1-RM testing prior to performing unilateral ECC/CON exercise on a MLP (7 sets of 10 repetitions at 80% of ECC/CON 1-RM). Immediately following cessation of the exercise participants reported their soreness (VAS) and PPT, followed by a blood sample and muscle biopsy. Thereafter, peak torque, power and SPPBT were performed. After the initial exercise bout, participants followed the same regime on the contralateral leg doing the opposing randomised exercise.

Five hours following the cessation of the exercise protocol (visit 2, 5 h) participants reported muscle soreness (VAS), PPT was measured, a blood sample taken and a muscle biopsy was taken from each leg. Immediately following the biopsy, peak torque, power and the SPPBT were performed. Participants arrived to the laboratory overnight fasted 24 h post-exercise for visit 3, 72 h post-exercise for visit 4 and 168 h post-exercise for visit 5. During these visits, participants reported muscle soreness (VAS), PPT was measured, a single blood sample was obtained, a muscle biopsy was taken from each leg and the same functional exercise tests were performed.

4.3.2 Sample analysis

All sample analysis methods are detailed fully in Chapter 3. In brief, saliva samples were used to determine body water enrichment (TC/EA- IRMS). Blood samples were used to determine plasma CK concentration (clinical chemistry analysis). Muscle biopsy samples were used to measure FSR (GC-pyrolysis-IRMS) anabolic, catabolic and inflammatory signalling (immunoblotting), inflammatory infiltration and SC activation (histology and immunohistochemistry).

4.3.3 Statistical Analysis

The distribution of all data was analysed for normality using the Kolmogorov-Smirnov test (accepted if $P > 0.05$). Data are presented as mean ± S.E.M. Paired t-tests were performed to determine differences between ECC vs. CON 1-RM. Ordinal data (visual analogue scale) and data which failed normality testing (plasma CK) were analysed using the non-parametric Friedman one-way ANOVA. In the event of significance being detected, Dunn's multiple comparisons test was used to determine the point of significance. The Dunns test was chosen as it is the appropriate multiple comparisons test for data that is not normally distributed. Repeated-measures (time) two-way (exercise type x time) ANOVA with a Bonferroni post-hoc analysis was used to compare the effect of contraction type (GraphPad Prism 6, La Jolla, CA, USA). A log transformation was performed for the phosphorylation of $mTOR^{Ser2448}$ to achieve normal distribution, followed by a RM two-way ANOVA. To draw temporal comparisons between functional, metabolic and molecular outputs, data sets were normalised over a range of 0-100% according to the data span (i.e. for each set of data, 0% represented the lowest whilst 100% represented the highest value). The number of $Pax7^{+}$ (SC) was more than 2 standard deviations away from the mean

for one older adult and was therefore excluded as an outlier. Three sections across two participants for $Pax7^+$ (SC) staining must be repeated and thus were excluded, therefore an n = 5 was used herein for $Pax7^+$ (SC) analysis. Mixed model ANOVA (within: time, between: age) with Bonferroni post-hoc analysis was used to determine the age-related differences at baseline and in response to contraction type, using young participants from Chapter 2 as the young comparison (SPSS 23, Illinois Chicago, USA). The α-level of significance was set at $P < 0.05$.

4.4 Results

4.4.1 Muscle function, soreness and plasma CK responses to ECC versus CON exercise in older participants

No effects of time or contraction type were observed for the SPPBT (data not shown). Compared to baseline, peak torque significantly declined immediately post-ECC exercise (0 h) (P<0.0005) and remained significantly decreased at 72 h (P<0.01) post-ECC exercise (Figure 4.1 A). Post-CON exercise, peak torque declined at 24 h post-exercise only (P<0.01); thus ECC exercise resulted in a more rapid onset of functional decline which persisted for longer when compared to CON exercise. When comparing the declines in torque between contraction modes, the reduction in peak torque was significantly greater at 0 (P<0.05) and 5 h (P<0.01) post-ECC compared to CON exercise. Therefore, unaccustomed ECC exercise caused greater declines in peak torque versus CON exercise. Peak power was only impaired 5 h post-ECC exercise (P<0.01) with a significantly greater reduction compared to CON exercise at the same time (P<0.05) (Figure 4.1 B). Surprisingly, peak power significantly increased 168 h post-CON exercise (P<0.005).

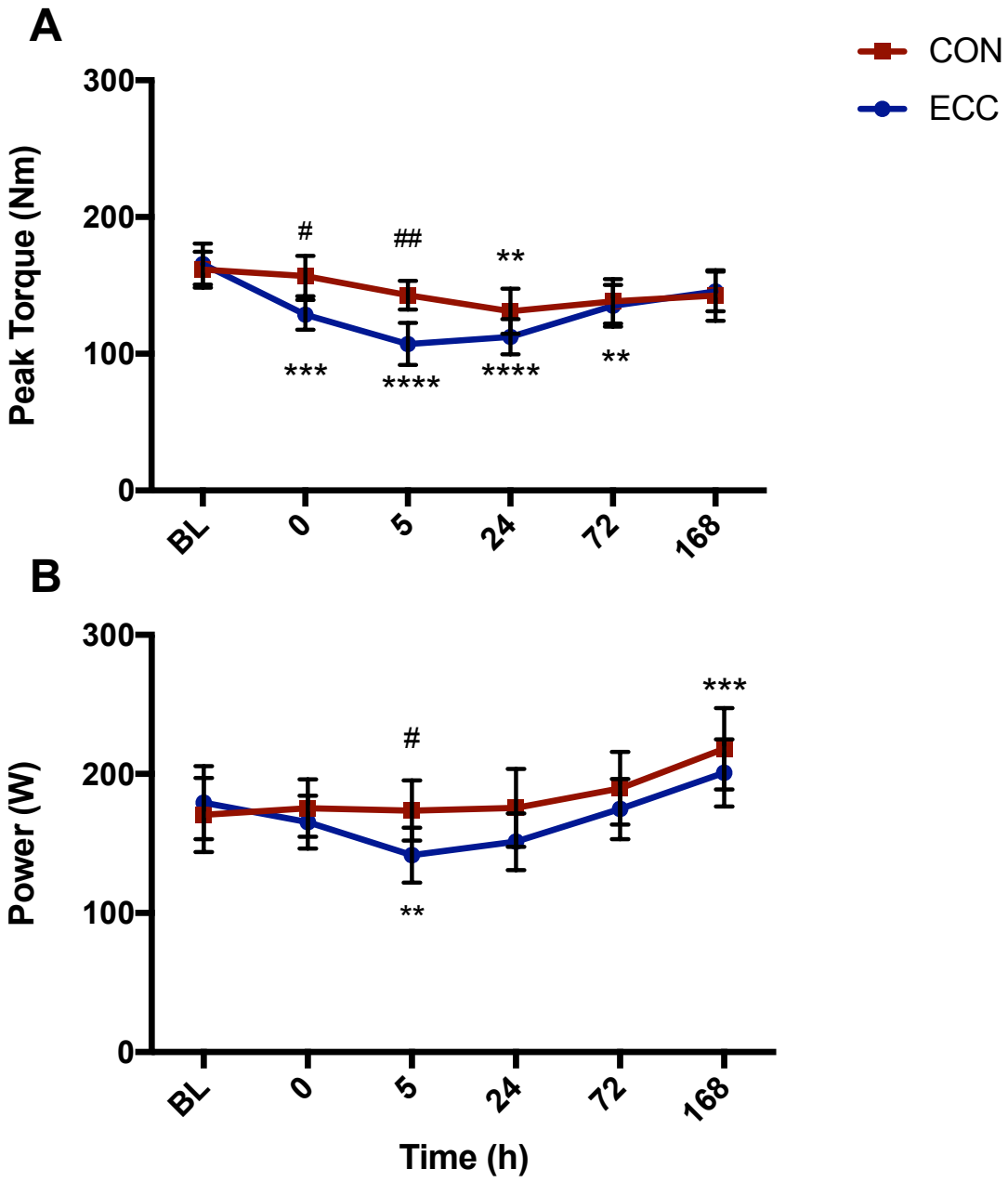


Figure 4.1. Declines in peak torque (A) and power (B) following ECC vs. CON exercise in older participants. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. * indicates significant difference at that time point compared to baseline (P<0.05), ** (P<0.01), *** (P<0.0005), **** (P<0.0001), # indicates significant difference between contraction types at that time point (P<0.05) and ## (P<0.01).*

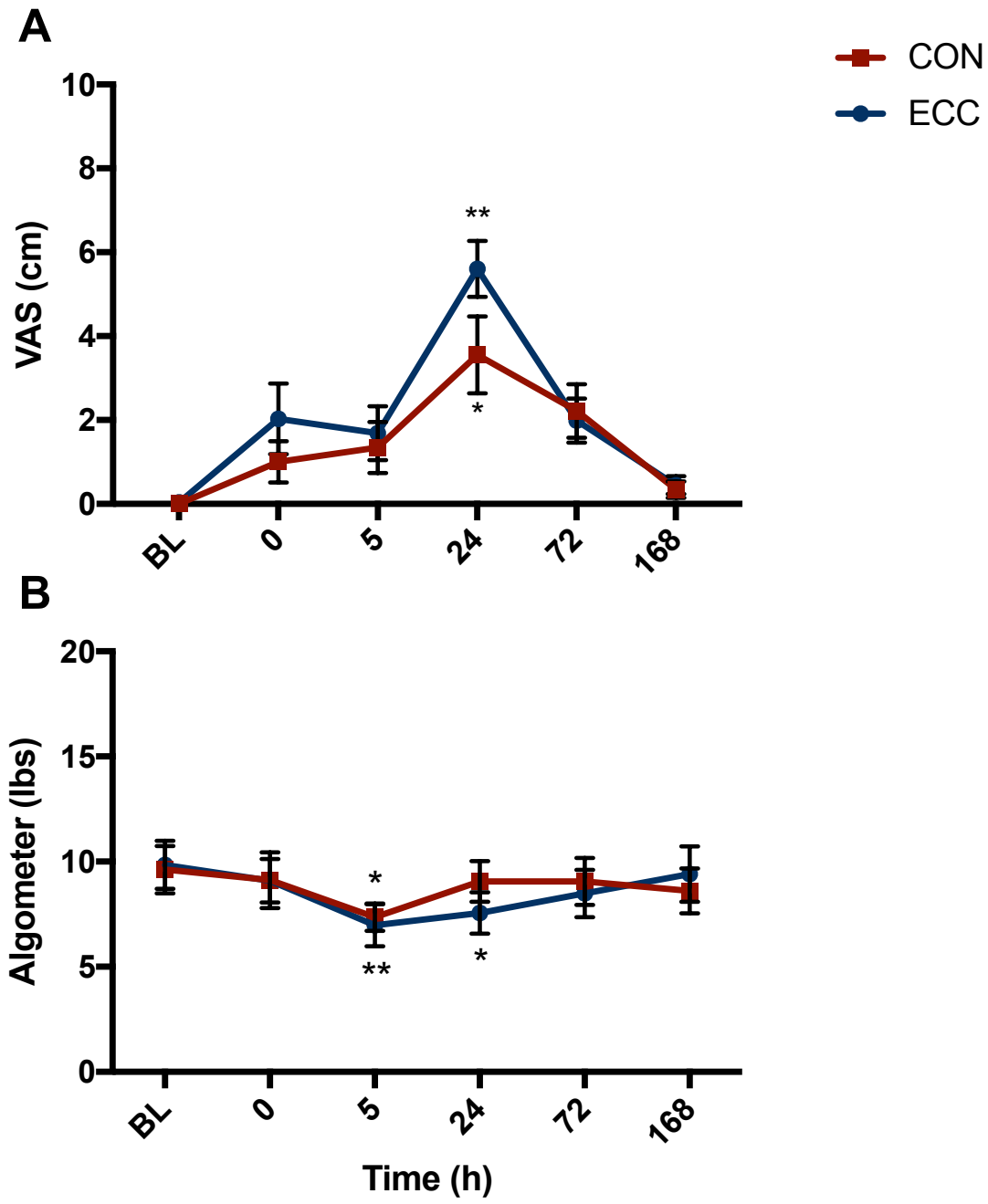


Figure 4.2. Perceived muscle soreness measured using the VAS (A) and changes in the PPT (B) pre and post ECC vs. CON exercise in older participants. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. * indicates significant difference from baseline (P<0.05) and ** (P<0.005).*

Increased sensations of muscle soreness were evident at 24 h post both CON (P<0.05) and ECC exercise (P<0.0005) (Figure 4.2 A). Reduced tolerance to pressure was present at 5 h post-CON (P<0.05) and ECC exercise (P<0.005), remaining reduced at 24 h post-ECC exercise only (P<0.05) (Figure 4.2 B). Despite similar reporting of muscle soreness in both exercise contraction types, ECC exercise resulted in sustained impairments of pressure tolerance versus CON exercise. Overall, no significant differences between the two contraction types were found.

Consistent with reports in the literature (56), plasma CK concentration increased 72 h following exercise (P<0.05) (Figure 4.3), indicative of muscle membrane disruption. However, contraction-specific effects could not be delineated as CK content was measured in the plasma and not in the skeletal muscles.

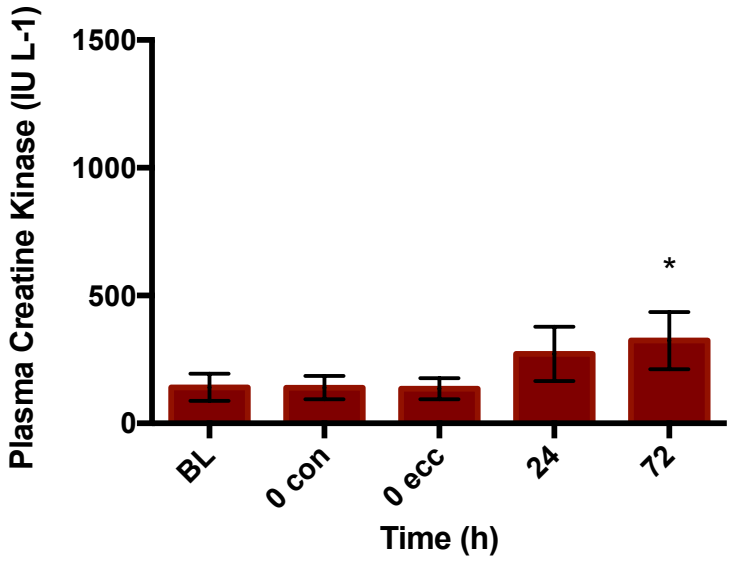


Figure 4.3. Plasma creatine kinase levels prior to and up to 72 h following exercise in older participants. *BL, 0 con, 0 ecc, 24 and 72 refer to baseline, 0 h post-CON exercise, 0 h post-ECC exercise, 24 and 72 h post-exercise, respectively. * indicates significant difference at that time point compared to baseline (P<0.005).*

Taken together, these data demonstrate increases in soreness and CK, coupled with declines in pressure tolerance and muscle function, suggestive that both ECC and CON exercise are capable of inducing muscle disruption in older adults; although ECC exercise elicits an earlier onset, exacerbated and prolonged disruptive response versus CON exercise.

4.4.2 Muscle protein synthetic response to ECC versus CON exercise

A single D_2O bolus (70 atom percent) of 3ml/kg led to a peak in body water enrichment of 0.338 ± 0.006% 3 h post consumption and an enrichment of 0.147 ± 0.008% on the final day (Figure 4.4 A). Body water enrichment followed an

exponential decay pattern throughout the trial at ~0.017% per day (Figure 4.4 B). Myofibrillar MPS did not significantly change in response to ECC or CON exercise throughout the time course of the study, although the data was highly variable between 0-5 h (Figure 4.5 A). When the data was collapsed independent of contraction type i.e. n = 16 for each time-point, there were still no exercise-induced increases in MPS (Figure 4.5 B).

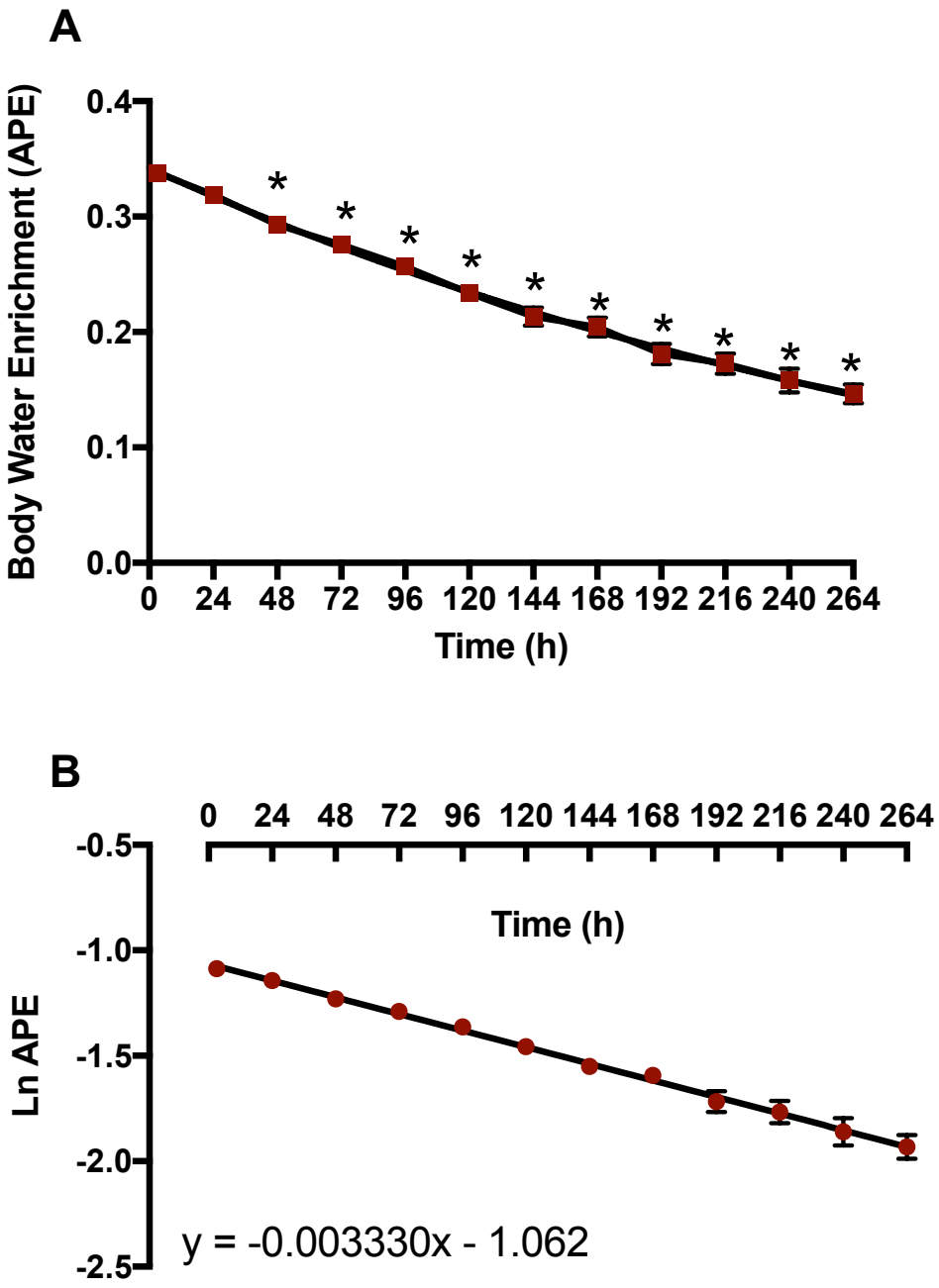


Figure 4.4. Deuterium body water enrichment and muscle protein incorporation A) time course of body water enrichment over 11 days following the oral consumption of D_2O and B) natural logarithm transformed body water enrichment to determine decay constant and half life. *0, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 refer to 0, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 h post-ingestion of D_2O, respectively. * indicates significant difference from initial day 0 body water enrichment ($P<0.005$).*

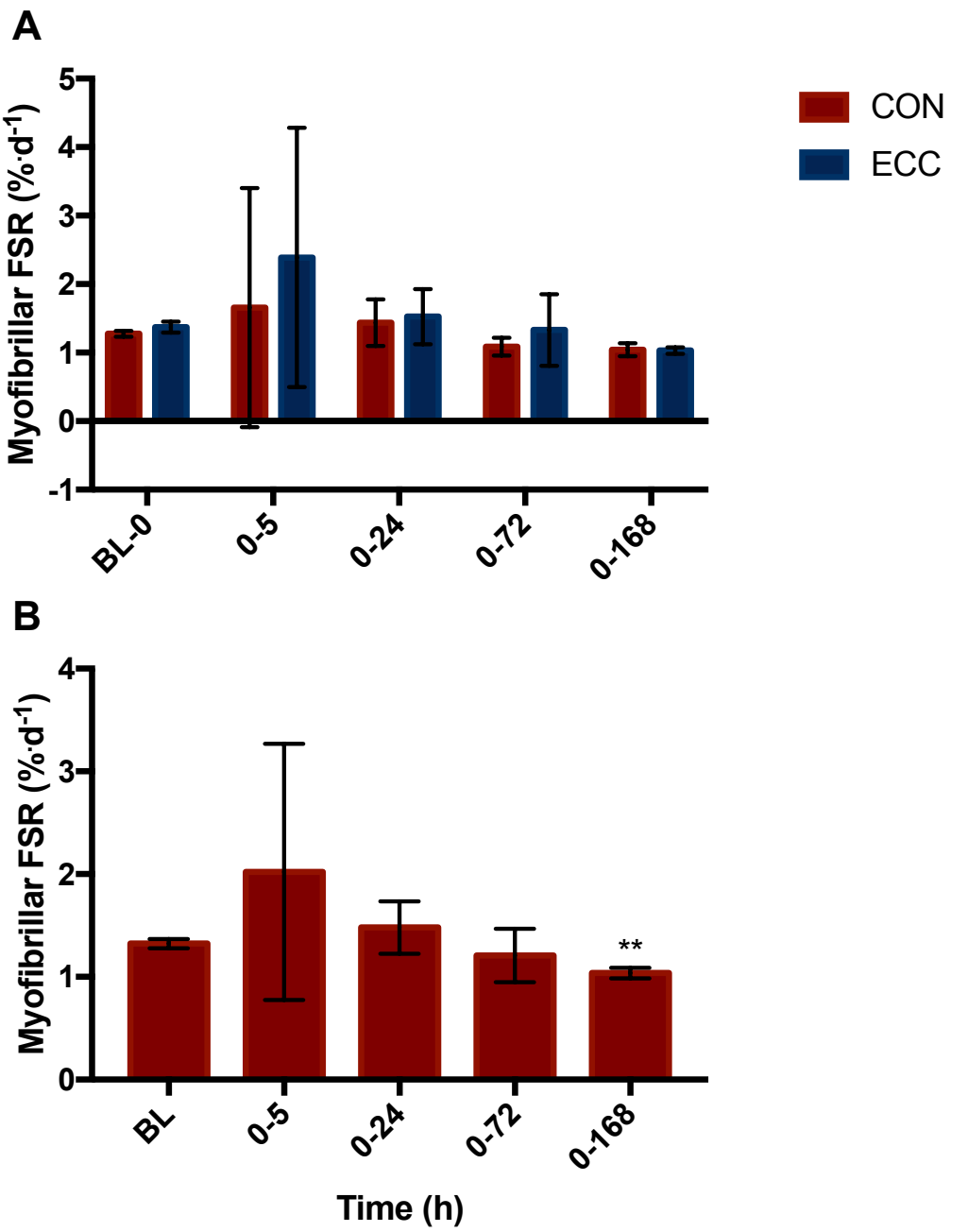


Figure 4.5. Baseline and temporal response of myofibrillar FSR in older participants up to 168 h post-exercise A) following ECC versus CON exercise and B) when these data were collapsed i.e. independent of contraction-type. *BL-0, 0-5, 0-24, 0-72, 0-168 refers to BL-0, 0-5, 0-24, 0-72, 0-168 post-exercise, respectively. ** indicates significant difference from BL (P<0.01).*

4.4.3 Anabolic and catabolic signalling responses to ECC versus CON exercise

Only ECC stimulated mTORC1 pathway signalling, which demonstrated a significant increase in *p70S6K1*^{Thr389} (P=0.050) (Figure 4.6 A) and rps6$^{Ser240/244}$ 5 h post-exercise (P<0.05) (Figure 4.6 B). No significant changes in the phosphorylation of anabolic signalling targets; mTOR^{Ser2448}, 4EBP1$^{Thr37/46}$ and eEF2^{Thr56} were observed, nor were there any changes in catabolic targets; pro Cathepsin L (42 kDa), Cathepsin L (25 kDa), Beclin 1 (autophagy) or Calpain 1 (calcium-dependent cysteine protease) at any time point following either ECC or CON exercise (data not shown).

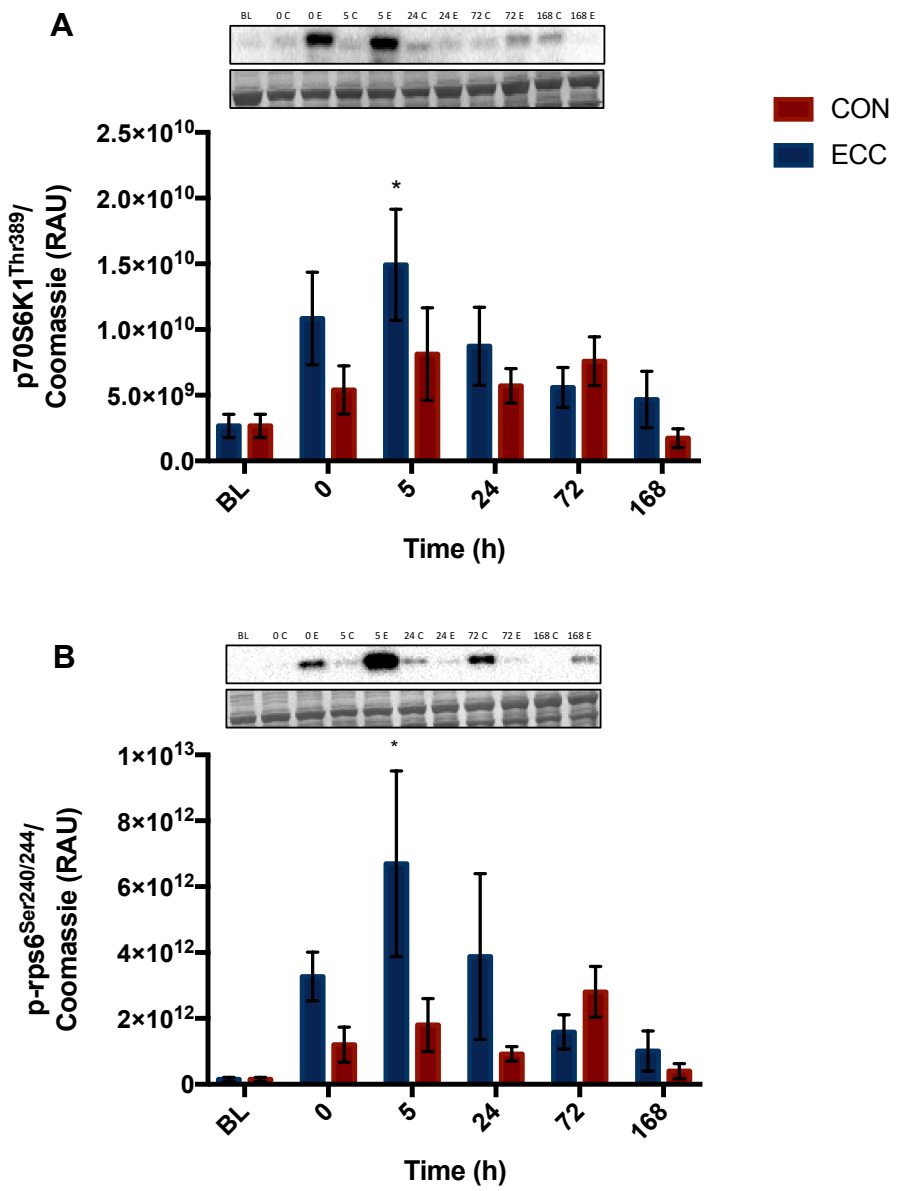


Figure 4.6. Temporal response of anabolic mTOR pathway signalling markers; p70S6K1^{Thr389} (A) and rps6$^{Ser240/244}$ (B) in response to ECC vs. CON exercise in older participants. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. RAU, relative arbitrary units. * indicates significant difference at that time point compared to baseline (P≤0.05).*

Therefore, ECC exercise is capable of inducing a greater anabolic signalling response in ageing muscle compared to CON exercise.

4.4.4 Inflammatory responses

Immunoblotting against the inflammatory protein TNF-α and the phosphorylation of NFκβ $p65^{Ser536}$ revealed no changes following either ECC or CON exercise (data not shown). Muscle macrophage infiltration of the endomysial and perimysial space did not significantly increase, although there was a trend for increased infiltration 24 h post-ECC exercise (P=0.07) (Figure 4.7). Thus, there was no significant inflammatory response following exercise.

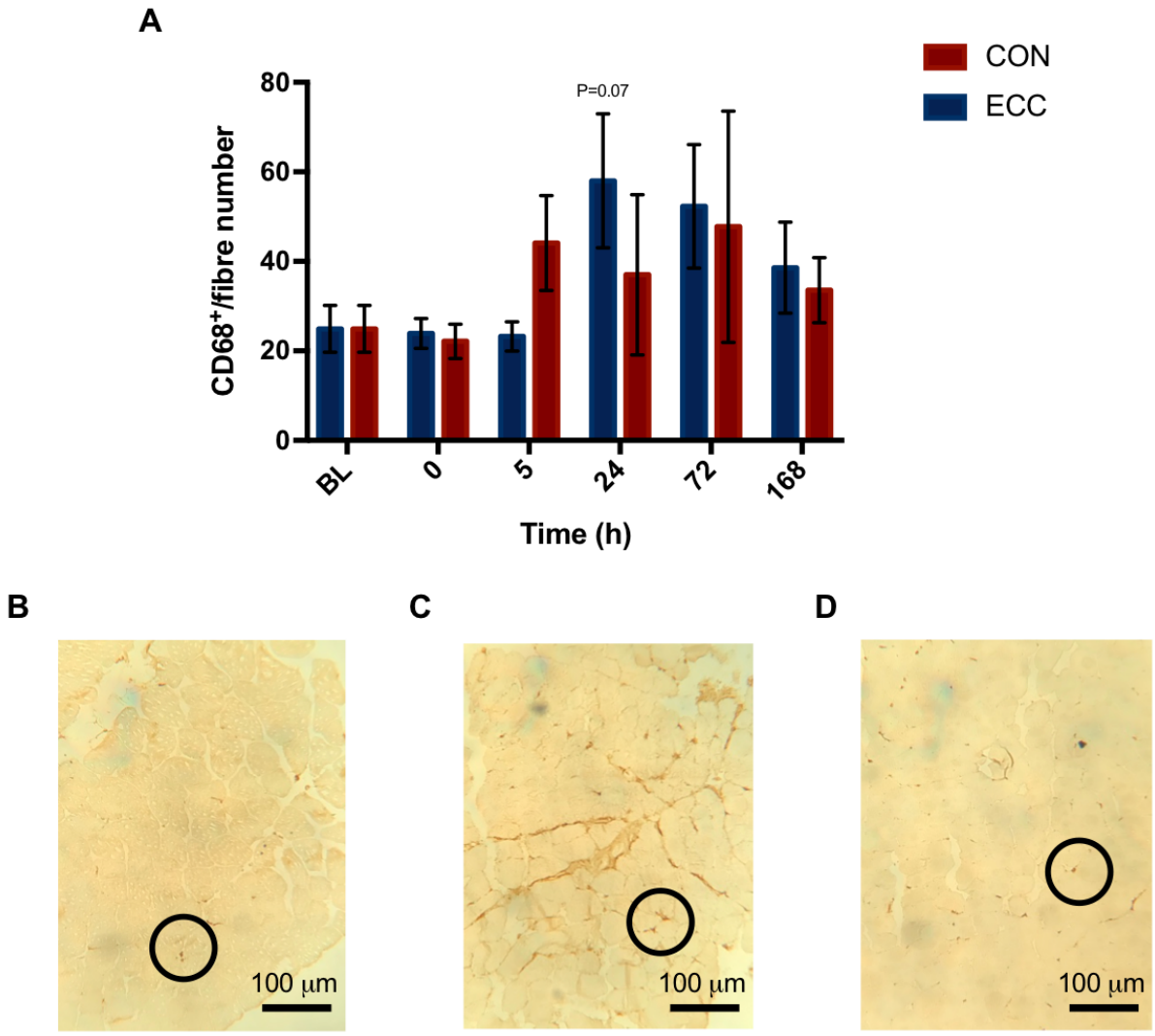


Figure 4.7. Temporal response of muscle macrophage infiltration in response to ECC versus CON exercise in older participants (A). Representative images of macrophage infiltration (black circles) at baseline (B), 72 h post-ECC (C) and 168 h post-ECC (D). *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively.*

4.4.5 Satellite cell response

ECC exercise induced SC activation 24 h into the regenerative period (Figure 4.8 C, D), determined by increased $Pax7^{+}$ cells. As a result of assessing temporal

relationships between different response parameters, it was observed that SC activation occurred once functional decline had past its nadir and recovery had started.

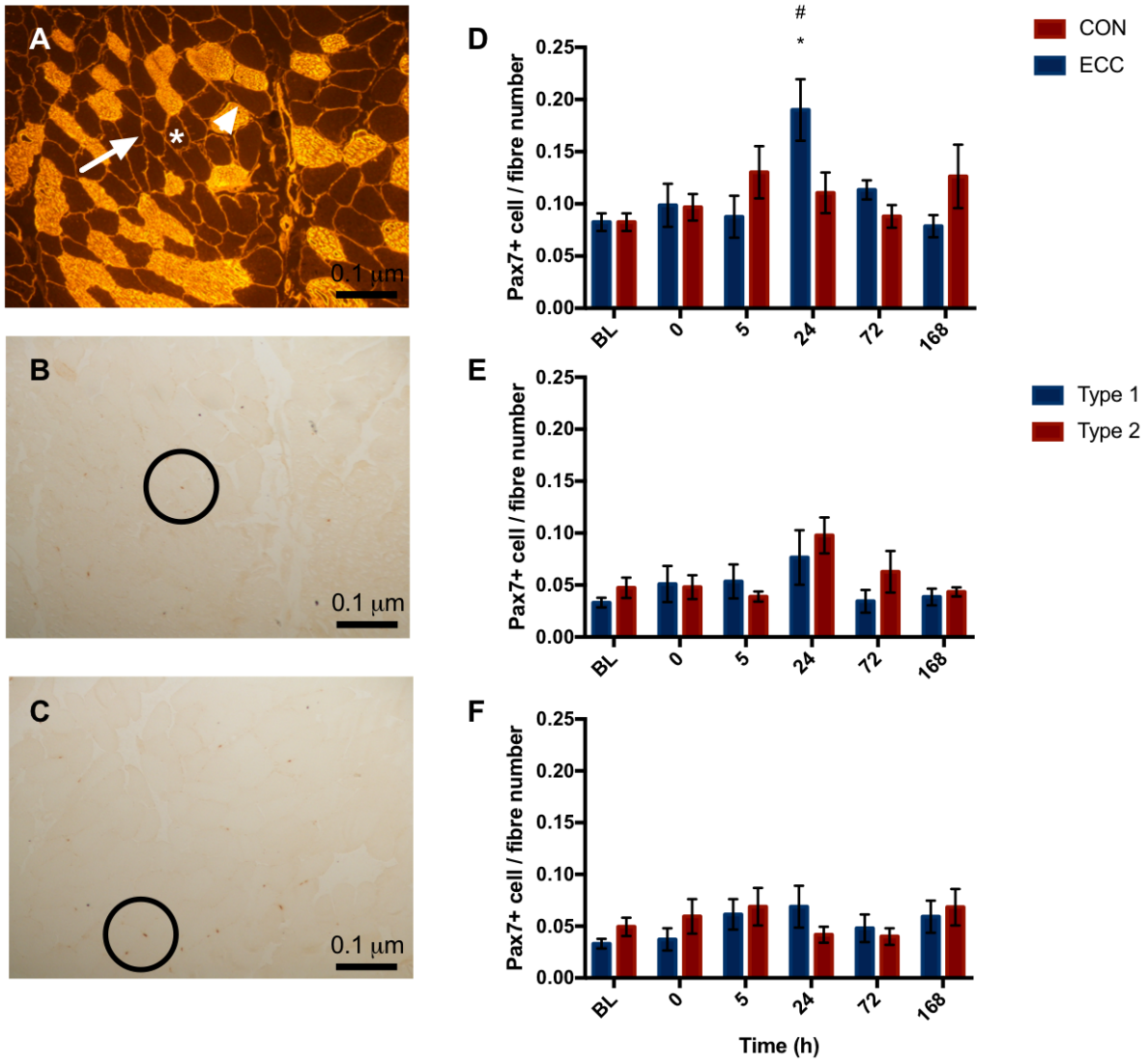
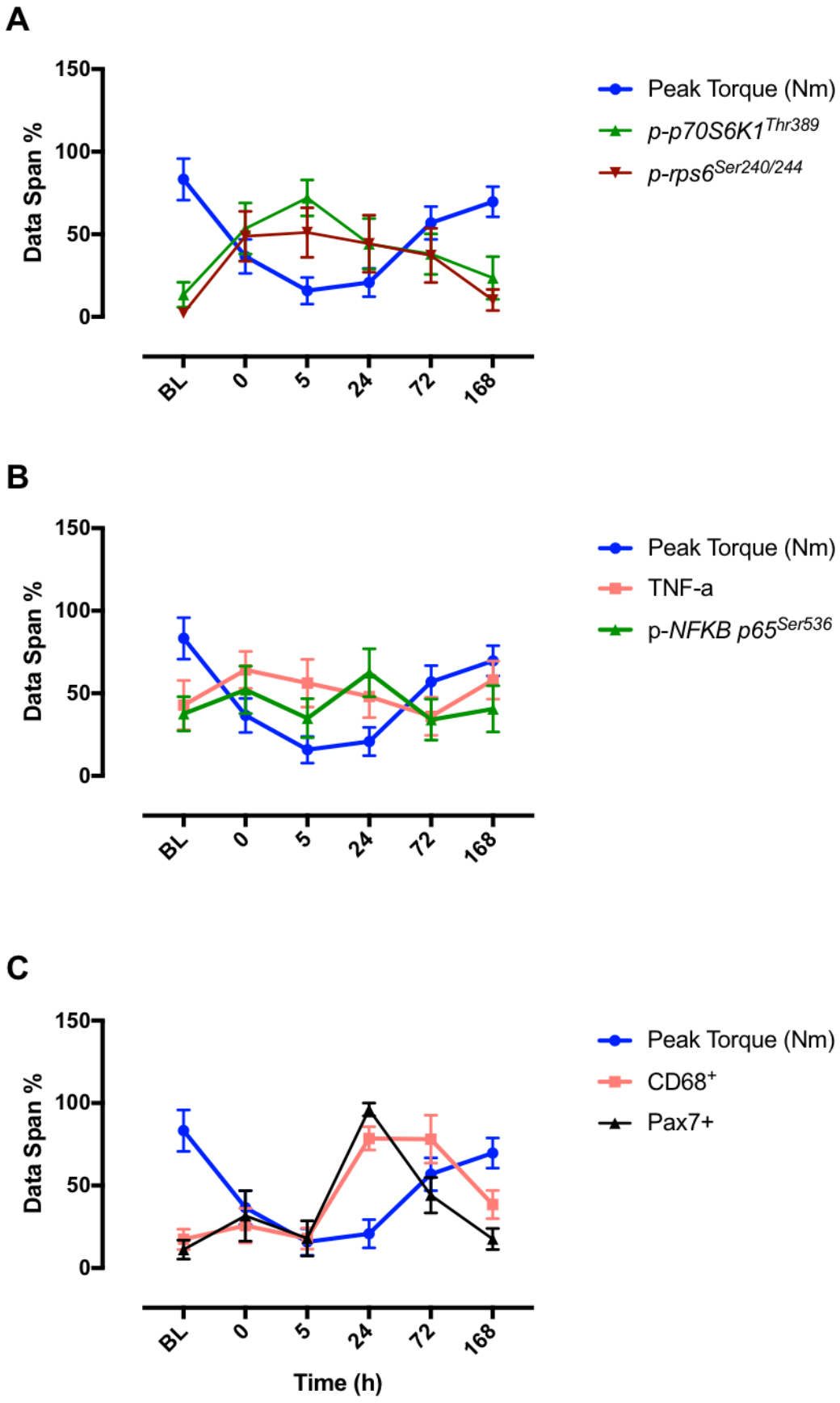


Figure 4.8. Temporal profile of the satellite cell response following an acute bout of ECC versus CON exercise in older participants. *(A) representative image of staining against type I fibres (white arrowheads) and laminin (white arrow) with unlabelled type II fibres (white asterisks), (B) multiplex staining of the baseline section with histochemical staining of SC's (Pax7^{+} cells, black circle), (C)*

representative image of Pax7$^+$ cells 24 h post-ECC exercise*, (D) quantification of mixed muscle Pax7$^+$ cells pre-exercise and up to 168 h post-ECC versus CON exercise (1001±46 fibres analysed per time point), (E) quantification of type I and II specific Pax7$^+$ cells pre- and up to 168 h post-ECC* exercise *and (F) quantification of type I and II specific Pax7$^+$ cells pre and up to 168 h post CON exercise. N=5. BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. *indicates significant difference at that time point compared to baseline (P<0.05).*

4.4.6 Temporality of muscle regeneration

Data spans were performed to demonstrate the temporality of molecular events preceding post-exercise muscle functional recovery during the regenerative period, as putative regulators of muscle recovery in ageing muscle. Anabolic signalling responses precede post-ECC exercise functional recovery (Figure 4.9 A). However, post-CON exercise non-significant peaks in anabolic signalling display a rightward-shift after the nadir in function (Figure 4.9 D). Similarly, the unexpected lack of inflammatory response to both ECC and CON exercise fails to provide insight into the temporal relationship between inflammatory molecular events in ageing muscle and functional recovery (Figure 4.9 B and E). Consequently, the mechanisms regulating post-exercise functional recovery in ageing muscle remain obscure.


A
150
100
50
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72
168
Peak Torque (Nm)
p-p70S6K1Thr389
p-rps6Ser240/244
B
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Data Span %
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Peak Torque (Nm)
TNF-a
p-NFKB p65Ser536
C
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Pax7+

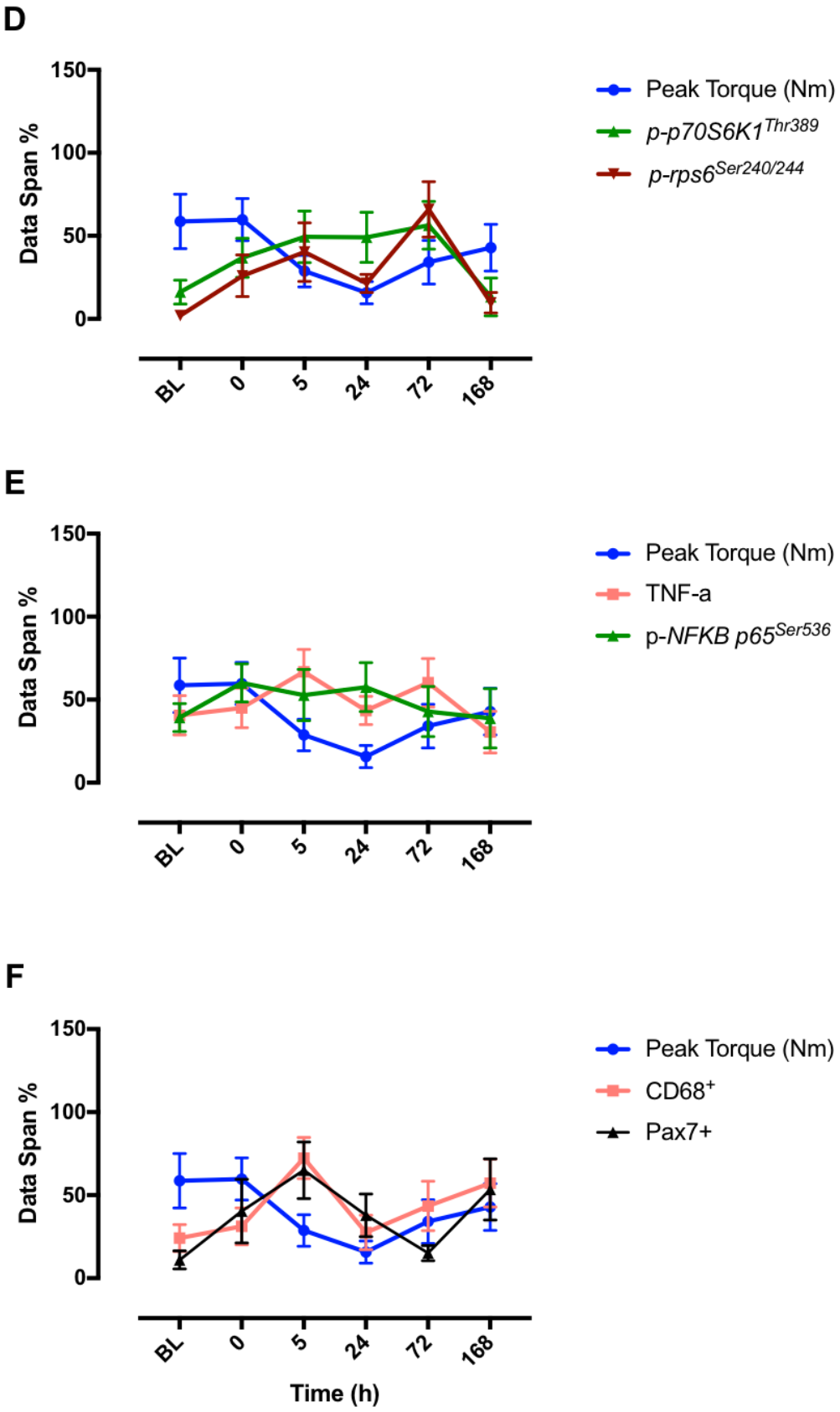

Figure 4.9. Comparisons between normalised (data span=100%) peak torque with markers of anabolic signalling (A, D), inflammatory signalling (B, E) and

macrophage and SC infiltration (C, F) following ECC (A-C) and CON (D-F) exercise in older participants. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. ** denotes significant difference at that time point from baseline for peak torque (P<0.01), *** (P<0.005), **** (P<0.0001). All significance values are presented in Figure 4.1, 4.6 and 4.8.*

4.4.7 Regenerative responses in younger versus older participants

4.4.7.1 Muscle function, soreness and plasma CK responses in young versus older participants

Despite age-related differences in baseline ECC peak torque production (P<0.005), older participants showed a similar temporal peak force decline to the young, both of which had recovered to baseline by 168 h. Peak torque following ECC exercise was significantly higher in the young at all time points except 5 h (P<0.05), where a trend for this difference was apparent (P=0.06) (Figure 4.10 A). As with ECC, age-related differences were apparent for baseline CON peak torque production (P<0.05). Older participants displayed a delayed decline in peak torque post-CON exercise versus the young group (Figure 4.11 A), which recovered to baseline by 72 h in both young and older groups. In addition to baseline, age-related differences in peak torque were observed at 168 h only (P<0.05) (Figure 4.11 A).

The temporal response of reductions in peak power production following ECC exercise in the older group displayed a delayed onset and earlier recovery to peak power versus the young (Figure 4.10 B), with peak power returning to within baseline by 24 h in the older and 72 h in the younger participants. No peak power decline was observed in either age-group, and no age-related difference were

observed following CON exercise (Figure 4.11 B). Thus, there is no evidence of impaired functional recovery in older participants after a single bout of either ECC or CON exercise.

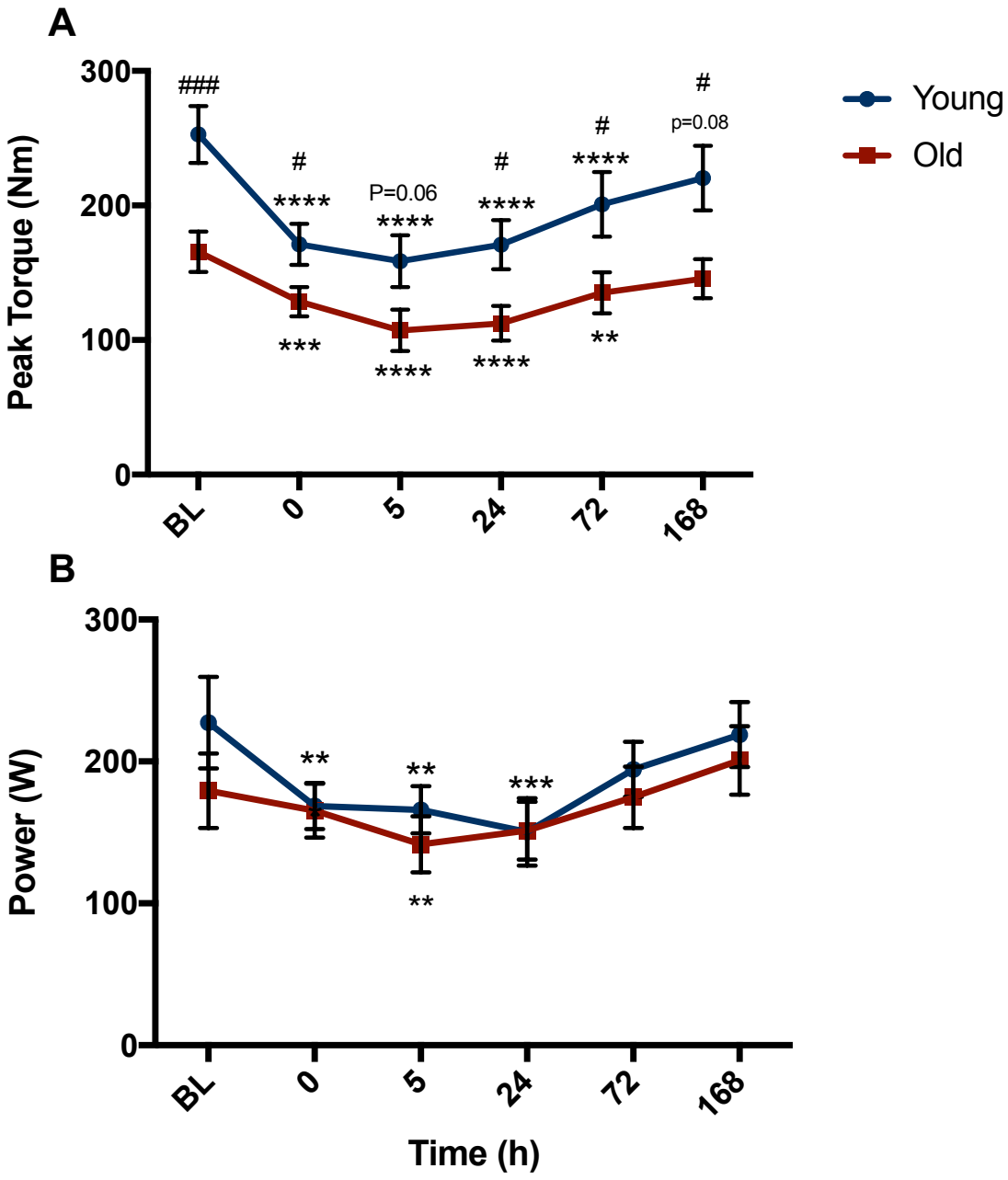

Figure 4.10 Temporal recovery of peak muscle torque (A) and power (B) after ECC exercise in young and older participants*. BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. ** indicates significant difference versus baseline (P<0.01) *** (P<0.001) **** (P<0.0001). # indicates significant difference between young and older age groups at that time point (P<0.05), ### (P<0.005).*

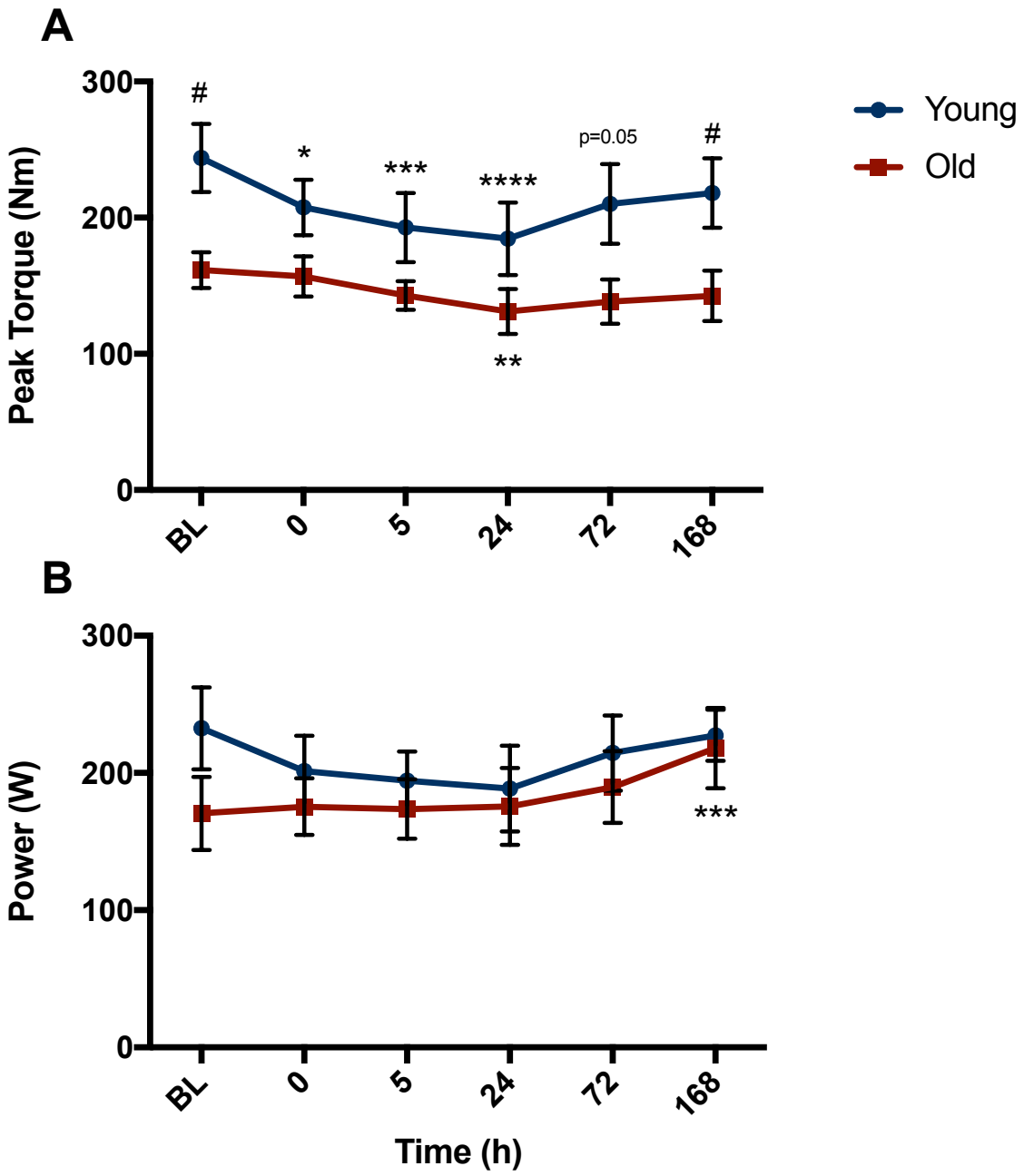

Figure 4.11 Temporal recovery of peak muscle torque (A) and power (B) after CON exercise in young and older participants. *BL, 0, 5, 24, 72, 168 refer to baseline, 0, 5, 24, 72, 168 h post-exercise, respectively. * indicates significant difference versus baseline (P<0.05), ** (P<0.01) *** (P<0.001) **** (P<0.0001).* [#] *indicates significant difference between young and older age groups at that time point (P<0.05).*

Post-ECC exercise perceptions of muscle soreness displayed earlier onset and sustained increases in the young versus older participants, whilst post-CON exercise soreness increased at 24 h only in both age groups (Appendix 4.1). Reduced tolerance to pressure-induced discomfort was also prolonged in young versus older participants post-ECC exercise. Post-CON exercise tolerance to pressure-induced discomfort occurred earlier in the older versus younger participants (Appendix 4.2). PPT was significant different between young and older participants only at 5 h post-CON exercise (P<0.05). The increase in plasma CK was delayed in older adults, increasing 72 h post-exercise versus the increase at 24 h post-exercise observed in younger participants (Figure 4.12).

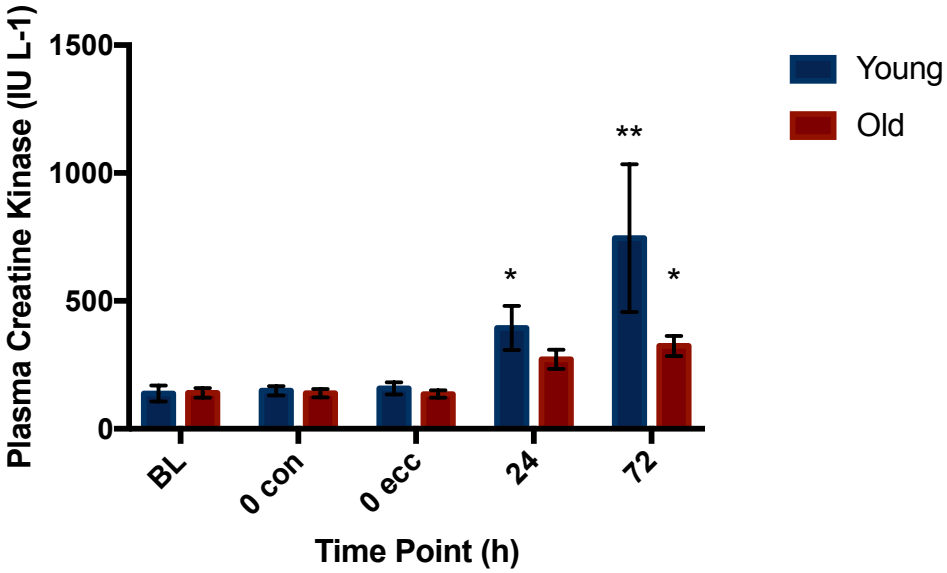

Figure 4.12. Plasma creatine kinase content post-ECC/CON exercise in young and older participants. *BL, 0 con, 0 ecc, 24 and 72 refer to baseline, BL, 0 h post-CON exercise, 0 h post-ECC exercise, 24 and 72 h post-exercise,*

*respectively. * indicates significant difference versus baseline (P<0.05), ** (P<0.005).*

The combination of these age comparisons suggest that older participants were not more susceptible to exercise-induced muscle 'damage' and that the functional post-exercise muscle remodelling response to both ECC and CON exercise was not impaired in older healthy participants.

4.4.7.2 Muscle protein synthetic response to ECC versus CON exercise in younger versus older participants

Myofibrillar MPS was not significantly different between young versus older participants at any time-point post-ECC (Figure 4.13 A) or post-CON (Figure 4.13 B). When these data were collapsed i.e. independent of age and contraction type (*n*=31), no significant changes were observed in myofibrillar MPS post-exercise (Figure 4.13 C).

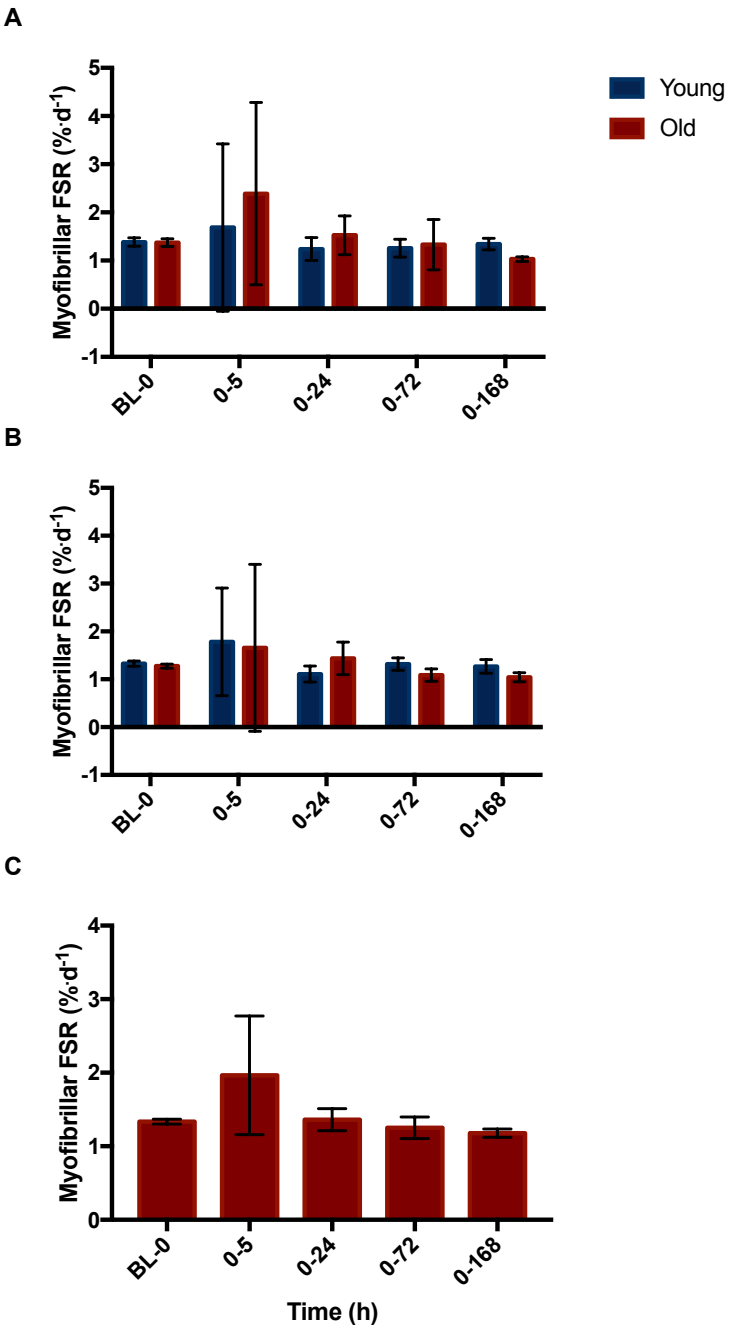

Figure 4.13. Baseline and temporal response of myofibrillar FSR in young versus older participants up to 168 h post-ECC (A), post-CON exercise (B), and when the data was collapsed i.e. independent of age and contraction-type (C). *BL-0, 0-5, 0-24, 0-72, 0-168 refers to BL-0, 0-5, 0-24, 0-72, 0-168 post-exercise, respectively.*

4.4.7.3 Anabolic and catabolic signalling responses in young versus older participants

Post-ECC exercise, anabolic signalling (mTOR^{Ser2448}, *p70S6K1*^{Thr38} and rps6$^{Ser240/244}$) activation status was not increased in older participants, with the exception of *p70S6K1*^{Thr38} and rps6$^{Ser240/244}$ at 5 h post (Figure 4.14 A-C). Post-CON exercise, no activation of anabolic signals was observed in the older group, although rps6$^{Ser240/244}$ phosphorylation was higher in older versus young participants at 72 h following CON exercise (P<0.01) (Figure 4.14 F). No age-related differences were observed at any time point for eEF2^{Thr56} or 4EBP1$^{Thr37/46}$. Therefore, ECC exercise may rejuvenate some of the exercise-induced anabolic signals in older participants, although the extent and duration is blunted compared to younger counterparts.

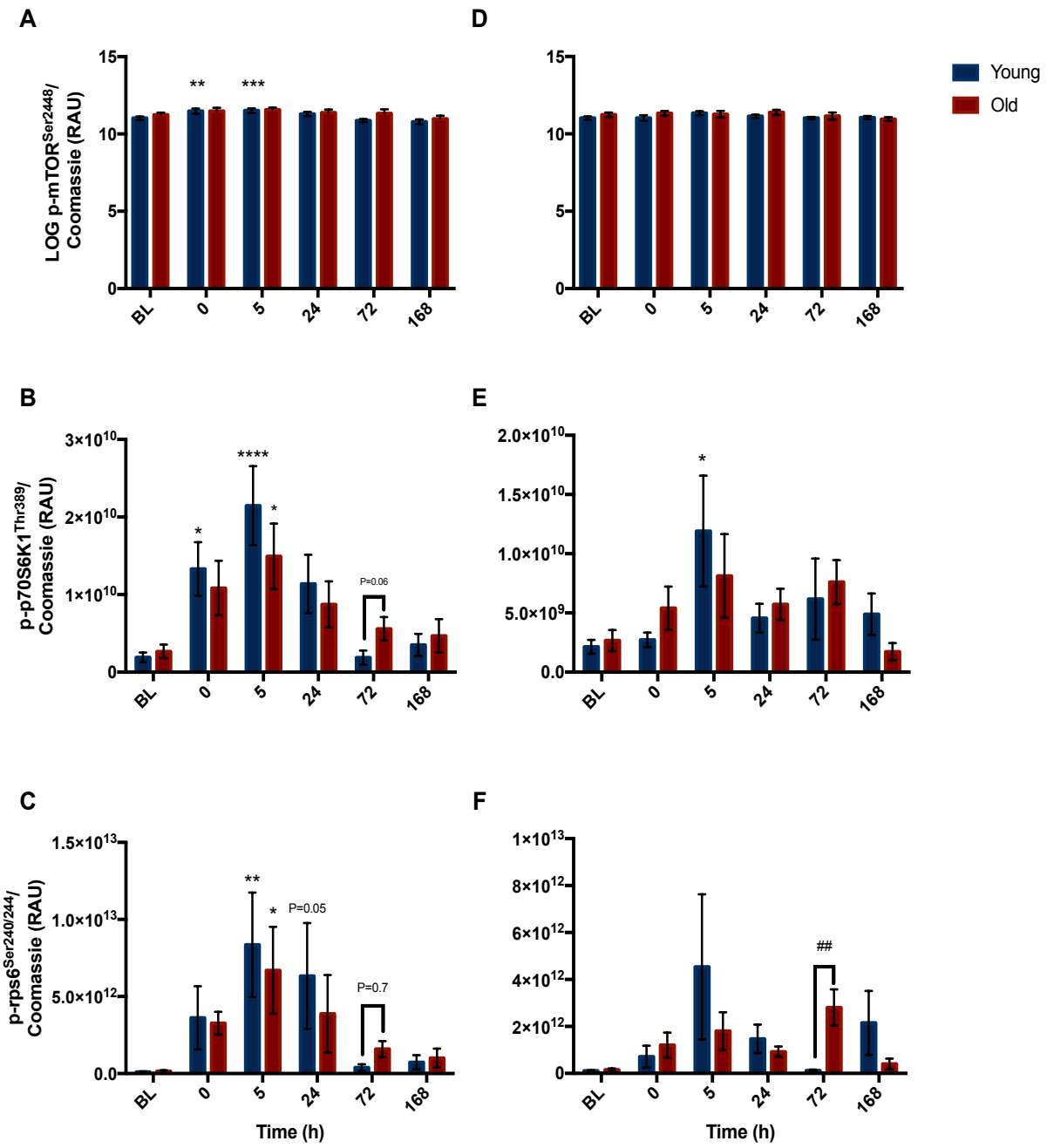

Figure 4.14. Temporal response of molecular anabolic signalling markers post-ECC (A-C) and –CON (D-F) exercise in young and older participants; mTOR^{Ser2448} (A, C) p70S6K1^{Thr389}(B, E) and rps6$^{Ser240/244}$ (C, F). *BL, 0, 5, 24, 72 and 168 refer to baseline, 0, 5, 24, 72 and 168 h post-exercise, respectively. RAU, relative arbitrary units. * indicates significant difference versus baseline (P<0.05),*

*** (P<0.01), *** (P<0.001) **** (P<0.0001),* ## *indicates significant differences between groups at that time point.*

Of the four proteolytic systems examined (25, 37 and 42 kDa Cathepsins, MuRF1, Beclin 1 and Calpain 1), young demonstrated suppressed lysosomal activity (0-24 h post-ECC/CON exercise) and increased UPS activity (168 h post-ECC/CON exercise). In contrast, compared to baseline, older participants displayed no expression changes in any proteolytic markers post-ECC or post-CON exercise at any time point. Despite differences in the regulation of breakdown pathways, the only age-related difference identified was Calpain 1 content, which was higher 5 h post-ECC exercise in the older versus younger group (P<0.05) (Figure 4.15). Since the activity of proteolytic markers was regulated in the young but not the old, it might be that older participants display facets of impaired muscle remodelling/ protein turnover, though this cannot be confirmed and does not appear to translate to an acute functional deficit.

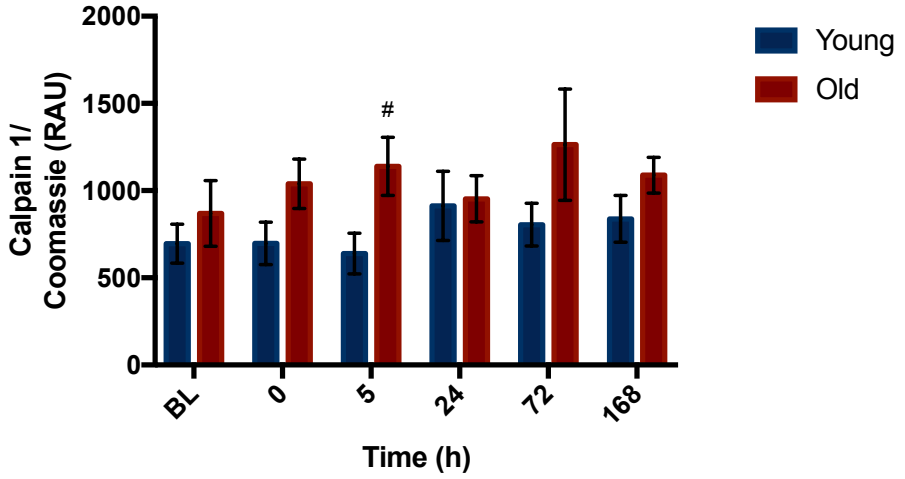

Figure 4.15. Temporal response of calpain 1 post-ECC exercise in young and older participants. *BL, 0, 5, 24, 72 and 168 refer to baseline, 0, 5, 24, 72 and 168 h post-exercise, respectively. RAU, relative arbitrary units.* #indicates significant difference between groups at that time point (*P<0.05)*.

4.4.7.4 Inflammatory responses

Consistent with the literature, older participants demonstrated elevated levels of TNF-α at baseline, which remained significantly higher than the young immediately and 5 h post-ECC (Figure 4.16 A) and CON exercise (Figure 4.17 A). Whilst the young group increased TNF-α signalling post-ECC (24, 72 and 168 h) and post-CON exercise (72 and 168 h), levels of inflammatory markers in older muscle remained unchanged after either contraction mode, resulting in levels that were not significantly different between the age-groups between 24-168 h post-exercise (Figure 4.16 A and Figure 4.17 A). The inflammatory transcriptional response was unique to young post-ECC only (Figure 4.16 B and Figure 4.17 B), although greater amounts of phosphorylated NFκβ p65^{Ser536} were found in older participants 5 h post-CON exercise (P<0.05) (Figure 14.7 B).

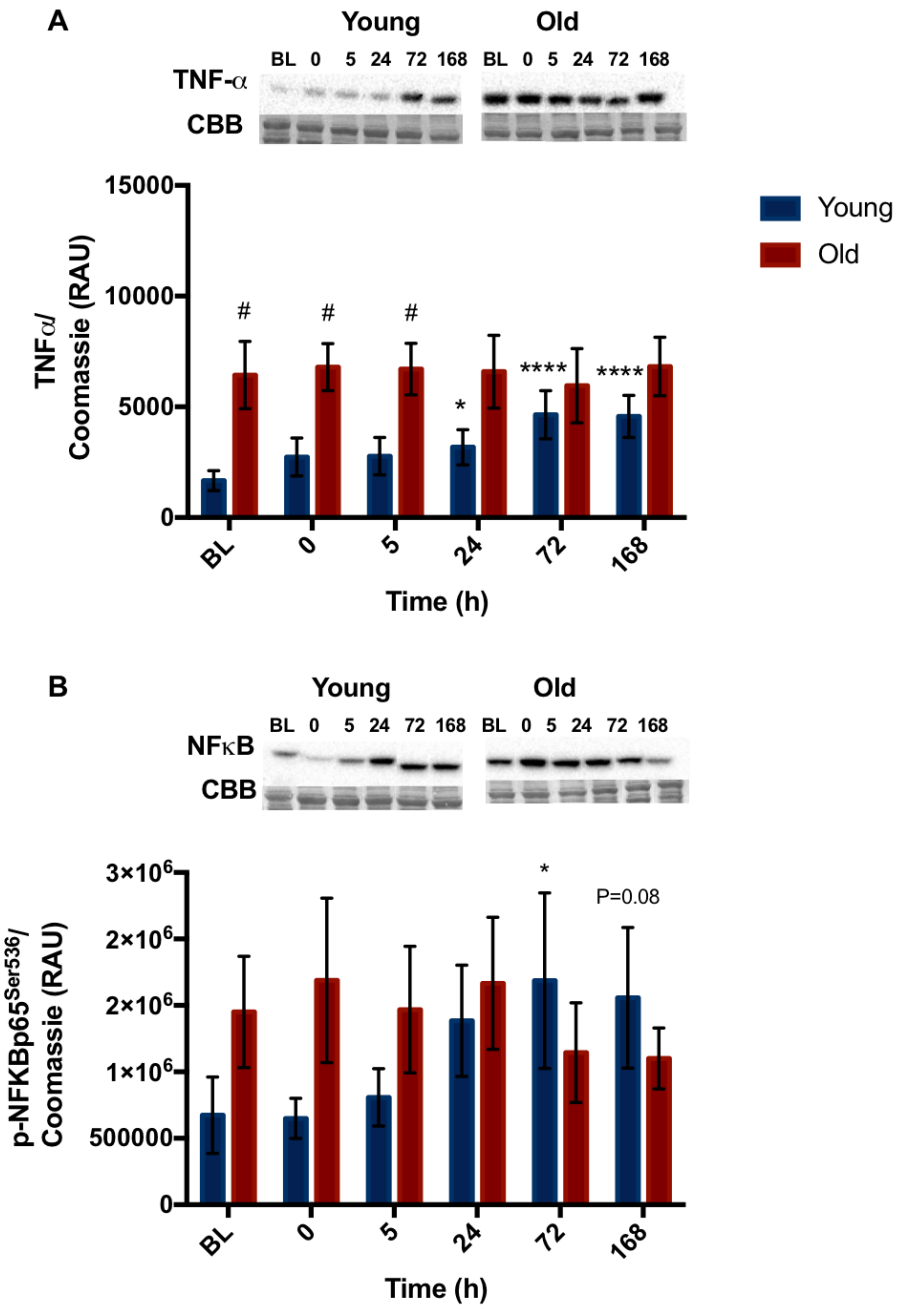

Figure 4.16. Temporal response of muscle inflammatory mediators post-ECC exercise in young and older participants; **TNF-α (A) and NFκβ p65^{Ser536}(B).** *BL, 0, 5, 24, 72 and 168 refer to baseline, 0, 5, 24, 72 and 168 h post-exercise, respectively. RAU, relative arbitrary units. * indicates significant difference versus baseline (P<0.05) **** (P<0.0001). # indicates significant difference between young and older groups at that time point (P<0.05).*

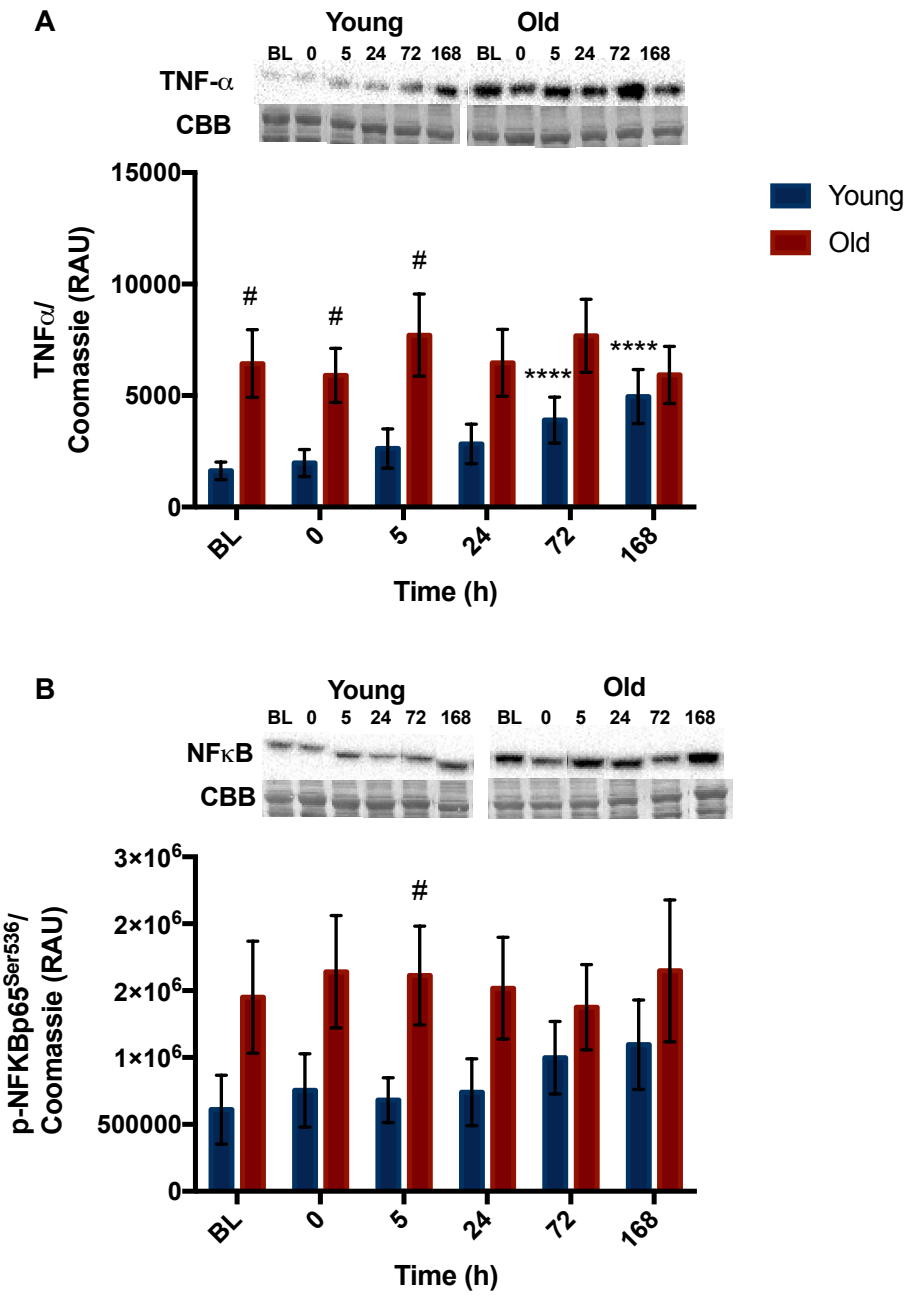

Figure 4.17. Temporal response of muscle inflammatory mediators post-CON exercise in young and older participants; **TNF-α (A) and NFκβ p65^{Ser536}(B).** *BL, 0, 5, 24, 72 and 168 refer to baseline, 0, 5, 24, 72 and 168 h post-exercise, respectively. RAU, relative arbitrary units. **** indicates significant difference versus baseline (P<0.0001). # indicates significant difference between young and older groups at that time point (P<0.05).*

Similarly, older participants had elevated basal levels of macrophage infiltration compared to younger participants (P<0.05) (Figure 4.18 A, B), which did not increase after ECC or CON exercise, unlike the young who demonstrated increased infiltration at 72 h post both exercise types (ECC P<0.001, CON P<0.01) and 168 h post-ECC (P<0.01). Macrophage infiltration was higher in older versus younger participants at 5 h post-CON and 24 h post-ECC exercise (P<0.05). Thus, age-related increases in basal inflammation does not increase susceptibility to exercise-induced inflammation, and inflammatory status does not impair the functional remodelling of ageing muscle to acute ECC and CON exercise.

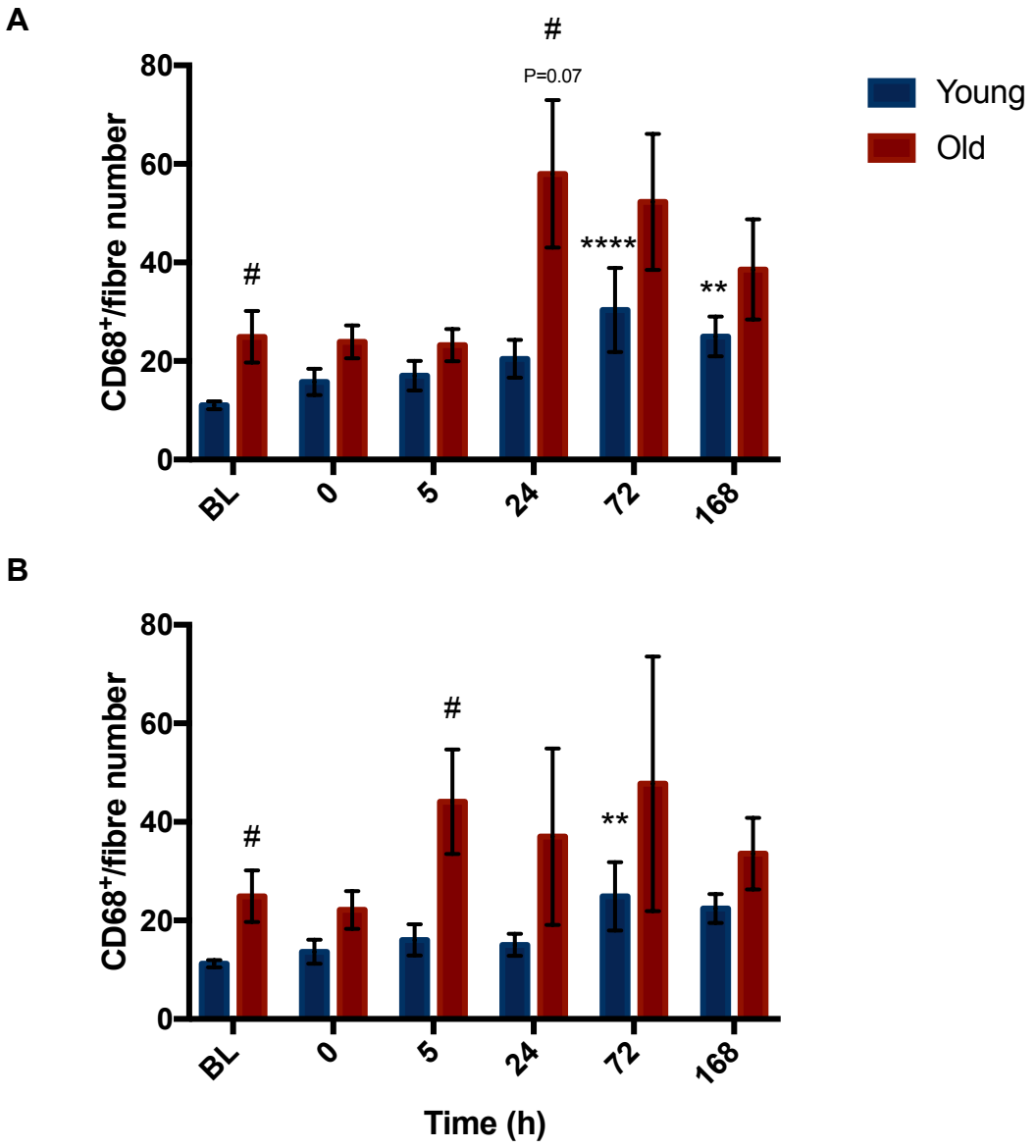

Figure 4.18. Temporal response of muscle macrophage infiltration post-ECC (A) and –CON (B) exercise in young and older participants. *BL, baseline; 0, 5, 24, 72, 168 refer to 0, 5, 24, 72 and 168 h post-exercise. * indicates significant difference versus baseline (P<0.05), ** (P<0.01), **** (P<0.001), # indicates significant between groups at that time-point (P<0.05).*

4.4.7.5 Satellite cell response

Baseline SC content was not different between the age-groups. SC content was greater in older versus younger participants at 24 h post-ECC exercise ($P<0.01$) (Figure 4.19 A). This observation is likely due to significant SC activation at this time-point in the older participants. SC content was assessed in type 1 and type 2 fibres and no post-exercise fibre-type specific SC changes were observed in either age-group (data not shown).

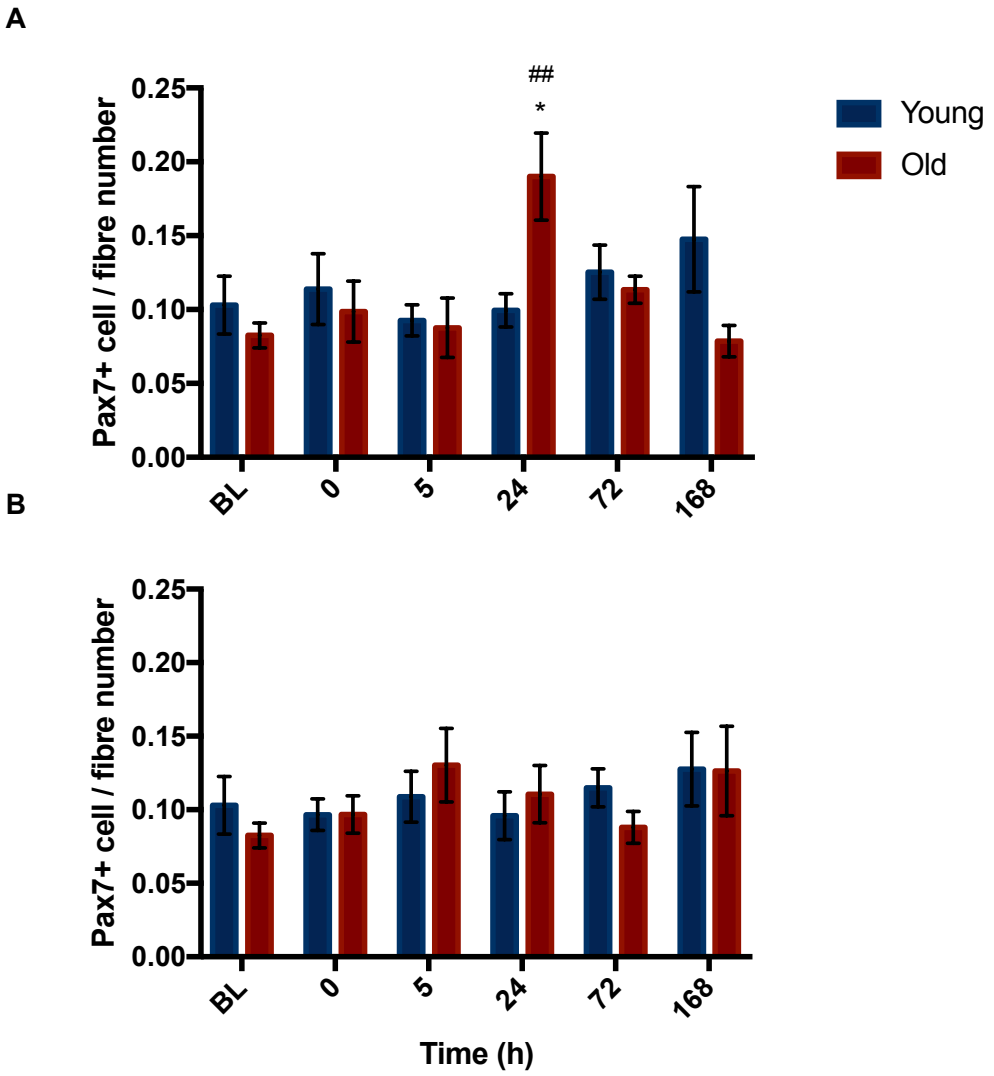

Figure 4.19. Temporal response of muscle satellite cell activation post-ECC (A) and –CON (B) exercise in young and older participants. *BL, 0, 5, 24, 72 and 168 refer to baseline, 0, 5, 24, 72 and 168 h post-exercise, respectively. * indicates significant difference versus baseline (P<0.05), ## indicates significant difference between groups at that time point (P<0.01).*

4.5 Discussion

This study shows novel data regarding the age-related mechanisms regulating skeletal muscle regeneration in older adults following ECC versus CON exercise. Both ECC and CON exercise resulted in reduced peak torque indicating the presence of muscle damage, highlighting that both exercise modes were successful at initiating regenerative processes in older adults. The key findings of this study are that i) preceding functional recovery ECC exercise induces anabolic signalling, which is absent following CON exercise, ii) despite no inflammatory or proteolytic responses, muscle function was restored and iii) SC activation occurred once functional repair was initiated post-ECC exercise only. Additionally, compared to younger adults, older adults displayed i) blunted anabolic (mTOR and p70S6K1) and catabolic (cathepsin L (37 kDa)) signalling following exercise, ii) higher basal levels of inflammatory markers and iii) increased SC activation following ECC exercise. These age-related perturbations in metabolic processes (anabolic, catabolic and inflammatory) did not hamper functional recovery.

In older adults following acute RE, mTOR signalling has been shown to increase for up to 24 h (208) or remain unchanged (112, 173). Herein, we show no change in phosphorylation status of mTOR pathway constituents up to 168 h after CON exercise. Since contraction-induced increases in MPS are dependent upon mTOR signalling (85), this may indicate that CON exercise did not increase MPS due to the absence of increases in anabolic signals, although discordance between anabolic signalling and MPS has been reported (126). This lack of change in the phosphorylation of mTOR pathway constituents and potential blunting of MPS may also suggest that CON exercise-induced damaged proteins are not effectively

replaced since it is the process of MPS that is responsible for synthesizing *de novo* proteins that replace degraded proteins (301). Conversely, ECC exercise was able to induce some level of anabolic signalling in older adults, therefore potentially creating a more anabolic environment during recovery compared to CON exercise. This difference between contraction modes may be related to the greater mechanical stress associated with ECC contractions further stimulating mTOR signalling, and therefore, may explain the enhanced gains in muscle mass observed following ECC versus CON exercise training (283). As further support for this notion, the extent of phosphorylation of mTOR signalling pathway constituents in the acute post-exercise period correlates with gains in muscle mass over time (316). Interestingly, muscle function recovered following both ECC and CON exercise despite disparate anabolic signalling, suggesting that anabolic signalling is not necessarily critical for functional recovery in older adults; a premise in contrast with previous rodent data (23). Furthermore, functional recovery following both exercise types highlights the safety of ECC and CON exercise in older adults. However, since anabolic signalling is absent following CON but somewhat restored following ECC exercise, ECC exercise may offer a more effective exercise intervention for promoting muscle growth in older adults.

Previous studies have shown blunted mTOR signalling in older adults from 1 hour after RE up to 24 h compared to younger counterparts (112, 173). To our knowledge, this is the first study to investigate age-related differences in mTOR signalling more than 24 h post-ECC versus CON exercise. Herein, the exercise-induced increase in anabolic signalling was blunted in older adults following CON exercise, and was shorter lived following ECC exercise in the older versus younger adults. Thus, a

perturbed anabolic environment is present following both ECC and CON exercise in older adults, which may go some way to explain the attenuated increases in muscle mass and strength in response to RET when compared to younger adults (37). To fully elucidate the role of this blunted anabolic signalling in adaptive responses the chronic effect of ECC versus CON training in younger versus older adults needs to be tested.

No significant changes in myofibrillar MPS were shown following either ECC or CON exercise at any time-point throughout the study. These data were unexpected since the acute stimulation of MPS in response to a single bout of RE, ECC or CON exercise has been well established and demonstrated in numerous previous studies in both young and older adults (72, 172, 173, 224, 260). Several instances were noted where the delta shift, representative of isotopic enrichment, was smaller at later time-points compared to earlier time-points. For example, in one older adult at 0 h post-CON exercise mean delta was 247 and reduced to 228 5 h post-CON (all raw data has been shown in Appendix 4.3). Since D_2O is cumulative, this drop in delta is not physiologically possible, therefore sample preparation errors may have led to inaccurate data; all of the MPS analysis will be re-run and analysed.

No changes in proteolytic markers were observed in older adults up to 168 h following either ECC or CON exercise. In contrast, previous research has shown similar changes in breakdown markers between young and older adults up to 24 h following RE (113). However, this finding was following conventional RE and was assessed as levels of mRNA (as opposed to protein), which may explain the

discrepant findings. The lack of proteolytic changes appears to be an unexpected age-related phenomenon, since suppression of lysosomal markers and increased UPS markers were observed in the young. However, defects in breakdown systems i.e. reduced removal of damaged organelles and proteins, are deleterious to muscle homeostasis and can lead to myofibre degeneration, muscle weakness and loss of muscle mass (166, 204). As such, perturbed proteolytic responses in older adults may suggest impaired muscle protein turnover and could impair muscle adaptation. Ultimately, in this acute study, the lack of proteolytic systems did not appear to translate to impairments in functional restoration.

Interestingly, no local inflammatory response was evident up to 168 h following either ECC or CON exercise in older adults, despite heightened basal inflammatory marker levels. When compared to younger muscle, levels of inflammation (TNF-α and macrophages) became similar between the age-groups as inflammation increased in response to exercise in the young. It is generally accepted that systemic inflammation is heightened in ageing and the wealth of research in this area has lead to the term "inflammaging" (105). However, only a few studies have confirmed increased levels of muscle inflammatory markers with ageing *per se* (129, 219), where others have shown no basal age-related differences (80, 137). In response to exercise, various studies have shown blunted or no detectable inflammatory responses in ageing muscle (80, 137). Thus, these novel data show that local skeletal muscle inflammation is up-regulated by ageing *per se* and is subsequently dysregulated following exercise in older adults. However, despite this, muscle function was restored by 168 h post-exercise. This is suggestive that inflammation is not necessary for functional repair, but instead mediates adaptive processes such as

ECM remodelling; a system in which maladaptation may accumulate over time to present as age-related losses in muscle mass. This premise is supported by data in young adults which shows that inflammation occurs once peak torque has past the nadir and persists once function has fully recovered.

Herein, increased SC activation was unique to ECC exercise and occurred once functional decline had past the nadir. Thus, SC activation in older adults is most likely not necessary for functional recovery. This notion is consistent with findings by Dreyer et al., (2006) who found an increased number of SC per muscle fibre area 24 h post-ECC exercise in older adults, although no measures of functional repair were taken. Increases in SC following a single exercise bout may, over the course of an exercise training programme, culminate in the greater numbers of SC observed in older adults in response to RET (290). Since SC activity increased following ECC exercise only, this may contribute to the greater gains in mass observed following ECC versus CON training (283). No basal differences were observed in the mixed or fibre type specific SC pool in young compared to older adults, and this is in line with previous reports (84, 287). Interestingly, SC activation was only increased in the older adults post-ECC exercise, a divergent finding compared to previous work where increases have been observed in both young and older participants, or there had been a blunted response in the older adults (84, 216). As alluded to previously (Chapter 3), SC content varies across a single muscle cross-section, so the most reliable way of detecting SC changes is to count as many fibres as possible (ideally the whole muscle cross section) (197). As such, divergent finding may be explained by the total fibre count. Herein, each whole cross-section was counted (1001±46 fibres), compared to previous reports which used <150 muscle fibres (84), and

another study which reported ≥125 fibres (216), although the maximum number of total fibres was undisclosed (216). Nonetheless, data generated in the present study is suggestive that SC activation is required for muscle regeneration and adaptation following ECC exercise in older but not younger muscle, and may imply that that the intrinsic regenerative capacity in older adults is not sufficient compared to younger adults. Therefore, an additional mechanism i.e. SC activation, is required in order to facilitate muscle regeneration via the satellite cell nuclei incorporating into the fibre and thus increasing the capacity for mRNA synthesis required to regenerate the fibre (161).

4.6 Conclusion

Both ECC and CON exercise in older adults induced muscle dysfunction which was restored within the 168 h time-frame investigated. Compared to CON, ECC exercise induced a greater anabolic environment with regard to anabolic signalling proteins and stimulated SC activation. Therefore, ECC exercise in older adults is safe, tolerable and potentially a more potent stimulator of muscle growth in ageing muscle. In comparison to younger adults, older adults demonstrate higher basal inflammation and blunted anabolic, catabolic and inflammatory responses to exercise which do not affect functional recovery. The activation of SC is unique to older adults. Therefore, the regenerative mechanisms following acute exercise differ with advancing age. These age-related perturbations in key regenerative processes may underlie the blunted adaptations to RET programmes often seen in older muscle and ultimately may contribute to losses and/ or impaired maintenance of skeletal muscle mass and strength in older adults.

5 Age-related Skeletal Muscle Transcriptomic Response to Acute Eccentric and Concentric Contractions

5.1 Abstract

Background: The molecular mechanisms mediating the divergent functional adaptations to eccentric (ECC) versus concentric (CON) exercise may initiate within hours' post-exercise, but are poorly understood. Age-related abnormalities in post-exercise molecular responses may contribute to attenuated muscle adaptation in response to chronic ECC and CON exercise training. **Methods:** Eight young (22±1 y) and eight older (70±1 y) healthy exercise naïve participants performed a single bout of unilateral ECC exercise (7×10 repetitions at 80% of ECC one-repetition maximum) and unilateral CON exercise (7×10 repetitions at 80% of CON one-repetition maximum). Muscle biopsies were collected at baseline and 5 h post-ECC and post-CON exercise. Total RNA was extracted and subjected to next generation sequencing and differentially expressed genes tested for pathway enrichment using Gene Ontology (GO). **Results:** Older participants displayed 952 differentially expressed genes enriched for blood vessel development, plasma membrane and cell-cell junction GO terms which were down-regulated at baseline. Independently of age, ECC and CON exercise elicited a similar post-exercise transcriptional response. Age-dependent post-ECC transcriptional profiles resulted in no GO term enrichment in young (81 up-regulated, 23 down-regulated) or older (151 up-regulated, 19 down-regulated) participants. Post-CON exercise, no clear pattern of GO term enrichment was present in young (13 up-regulated, 2 down-regulated). Whereas post-CON exercise in the older participants displayed 147 uniquely up-regulated genes enriched for cell adhesion, extracellular organisation and blood vessel development pathways, and 28 uniquely down-regulated genes relating to the metabolism and/or catabolism of amino acids, lipids, carboxylic acid and DNA. **Conclusions:** Ageing is associated with distinct transcriptomic profiles at baseline and following ECC and CON

exercise. Such differences may contribute to the known impaired chronic adaptation/ remodelling that occurs in response to exercise training in ageing adults.

Specific acknowledgements

I would like to acknowledge each clinician that performed muscle biopsies for this study: Miss Catherine Boereboom, Dr. Haitham Abdulla and Dr. Syed S I Bukhari. I would like to acknowledge Beijing Genomics Institute for extracting the RNA from the muscle samples and for performing RNA sequencing. Finally, I would like to acknowledge Dr. Ryan Ames who taught me how to analyse data produced from RNA sequencing and for playing a major role in the data analysis.

5.2 Introduction

Associated with ageing is the progressive loss of muscle mass and function (sarcopenia and dynapenia, respectively) (223, 286), which compromises locomotory capabilities (25), metabolic health (306, 307) and ultimately increases the risk of premature mortality (175). RET is currently the most safe and effective countermeasure to enhance muscle mass and function in older adults (36, 100). Previous work and data within this thesis has shown that by segmenting conventional RE into ECC and CON contractions, the functional, metabolic and molecular responses are divergent, which might explain differences observed in chronic adaptations (283). For example, ECC exercise is associated with more muscle damage (117), pro-longed acute anabolic signalling (Chapter 3,4), greater muscle protein accretion (224), pro-longed inflammation (Chapter 3) and greater SC activation (Chapter 4). Furthermore, divergent responses to contraction-type have been noted between the ages. For example, anabolic signalling is blunted in older versus younger adults in response to both ECC and CON exercise (Chapter 4). Therefore, research is warranted which aims to elucidate the molecular regulators of divergent responses to different contraction-types in young and older adults. Such research may highlight opportunities for therapeutic intervention aimed at ameliorating muscle loss with ageing.

Targeted molecular analysis such as real-time reverse transcriptase polymerase chain reaction (RT-qPCR), western blotting and/ or immunohistochemical/fluorescent staining are limited to the investigation of just one or a few target mRNA's or proteins, and thus provide valuable insight but only into a limited number of putative molecular regenerative mechanisms. However, the true extent of molecular

mechanisms in the skeletal muscle regenerative processes are likely far more extensive and complex than currently understood. As such, more innovative analytic approaches are necessary in order to identify the complexity of such processes.

The development of 'OMIC' analysis, of particular interest transcriptomics, permits the detection of hundreds, tens of thousands or even all mRNA (depending on the method used) at once within a biological sample. As such, utilising transcriptomic analysis facilitates the identification of more global molecular networks involved in regulating muscle regeneration. Still today the most popular transcriptomic method for investigating gene expression is using microarrays (362). This is due to the combination of affordability and the capability to detect tens of thousands of transcripts simultaneously (362). However, there are limitations associated with microarrays, most notably the detection of only genes that transcripts have been designed for (362). Recently, there has been an increase in the number of studies implementing RNA sequencing which allows the discovery, profiling and quantification of all RNA transcripts in a biological sample (329). Although currently more expensive compared to microarrays, RNA sequencing allows the unbiased detection of novel transcripts including splice variants (i.e. no need for transcript specific probes). The enhanced specificity and sensitivity of RNA sequencing increases the detection of differential expression and low-abundance transcripts can be detected. The benefits of RNA sequencing over microarray suggest there is the potential that RNA sequencing will soon become the chosen method of choice to perform transcriptomics profiling (362).

In an attempt to understand the contraction-specific molecular mechanisms regulating muscle regeneration, global transcriptomic profiling has been implemented in both rodent (19, 51) and human studies (50, 169, 199). Genes related to ECM, cytoskeletal, hypertrophy, angiogenesis, signal transduction and stress response processes have been shown to be up-regulated, whereas genes related to gene transcription and translation, protein metabolism, mitochondrial structure and oxidative phosphorylation activity are down-regulated four hours after traditional RE in males (190). As such, the authors suggested that the transcriptional data might imply inhibited mitochondrial activity and increased protein accretion following acute RE (190). In order to define ECC-induced transcription responses, Chen and colleagues (2003) compared ECC-CON versus CON exercise responses 4-8 h post-exercise. They found genes uniquely up-related following ECC-CON (and thus unique to ECC contractions) were related to cell growth, stress response, DNA damage, inflammation, ECM remodelling and signalling, whereas no genes were consistently down regulated (50). This may be related to the 'damaging' nature of ECC exercise, which is known to induce inflammation (Chapter 3) and ECM remodelling (149) in young healthy muscle. However, the lack of specific biopsy time point (i.e. biopsies were taken between 4-8 h) precludes understanding into the precise temporal responses, since remodelling responses can be rapid and might be different at 4 h versus 8 h post-exercise. Others have identified individual up and down-regulated differentially expressed genes 3 and 48 h after isolated ECC exercise, which were characterised into several biological categories; apoptosis, growth, proteolysis, metabolism, stress management and transcription (i.e. up and down-regulated genes in all categories) (199). These data highlight that many cellular processes are regulated during muscle regeneration. However, the small

subject population of only four males in this study likely prevented the detection of many gene changes that occurred (199). Only a handful of studies have directly compared the contraction-specific transcriptomic response in humans. Kostas and colleagues (2006) reported divergent transcriptional responses as early as 3 h following ECC versus CON exercise in healthy young males. For example, FBXO32 (also known as MAFbx), which targets proteins for degradation, was down-regulated 6 h post-ECC in relation to CON exercise and certain heat shock proteins, which are involved in rescuing proteins from misfolding, were up-regulated following ECC exercise (169). These regulatory responses might be due to the greater muscle damage associated with ECC contractions, and may underlie divergent chronic adaptations.

Utilising OMIC approaches, several studies have highlighted genes which might mediate the ageing of skeletal muscle (86, 120, 258, 305, 313, 339). For example, ageing is associated with reduced expression of genes related to mitochondrial function (313). This might contribute to age-related reductions in mitochondrial protein synthesis and function (284). When compared to younger adults, older adults displayed down-regulated differentially expressed genes encoding energy metabolism (i.e. mitochondrial protein synthesis, tricarboxylic acid cycle activity), whereas differentially expressed genes encoding proteasome components were up-regulated in older versus younger adults (339). This might suggest that ageing *per se* is associated with impaired metabolic processes, that contribute to muscle dysregulation during ageing. Drummond and colleagues (2011) identified small non-coding microRNA (miRNA) Let-7 family members (Let-7b and Let-7e), which are involved in reducing cellular replication and were expressed at higher levels in older

versus younger adults. Further, increased Let-7 was associated with down-regulated cell cycle regulators and Pax7 (satellite cell) mRNA expression (86), that might contribute to reduced regenerative potential in ageing muscle. As such, there seems to be an age-related transcriptional regulation during ageing which differs from the young.

Currently, only a handful of studies have utilised microarray techniques to investigate the age-related transcriptomic response to acute exercise. Twenty-four hours following acute RE 318 genes were differentially expressed in the old compared to only 87 in the young, although plasma creatine kinase was similar between the ages denoting similar levels of exercise-induced muscle 'damage' (317). In the older adults, up-regulated transcripts related to stress and cellular compromise, inflammation and immune responses, necrosis, and protein degradation (317). These data suggest older muscle induce a greater transcriptional response to exercise despite similar levels of 'damage' compared to the young, and thus may induce an altered regenerative response which is suboptimal to that of the young. However, this was following conventional RE, and so the age-related contraction-specific transcriptome response is unknown. Furthermore, this study investigated the 24 h post-exercise time-point, which misses the nadir of muscle function (post-ECC exercise), peaks in anabolic signalling and repressed proteolytic signalling, as shown in Chapter 3. As such, this study may have missed important transcriptional responses that are rapidly up-regulated as part of the rapid regeneration process.

Therefore, the aim of this study was to utilise RNA sequencing to identify age-related transcriptional differences at baseline, and changes following acute ECC versus CON exercise in young versus older adults. Specifically, differential gene expression 5 h post ECC and CON was investigated since the nadir of force was at 5 h post-ECC and repressed catabolic signalling and peak anabolic signalling occur 5 h post-ECC and CON (Chapter 3), indicative of transcriptional activity at this time.

5.3 Methods

5.3.1 Study Design

Participants for this analysis were the same eight young (21±1 y, body mass index 23±2 kg/m^2; 80% ECC 1-RM 211±14 kg and 80% CON 1-RM 122±11 kg) and eight older (70±1y, body mass index 26±1 kg/m^2; 80% ECC 1-RM 155±9 kg and 80% CON 1-RM 79±6 kg) healthy exercise naïve males described in Chapter 3 and Chapter 4, respectively. The experimental protocol is fully described in Chapter 3, with analysis for this chapter based around the protocol from baseline until 5 h post exercise (visit 2), excluding the 0 h time-point (Figure 5.1). In brief, participants arrived for their first (baseline) visit fasted (for 11 h) at ~9 am for a baseline (*m. vastus lateralis)* muscle biopsy taken under local anesthesia. Ninety-six hours later, participants arrived at ~8.30 am for visit 2 having consumed a 250 ml of a liquid high energy nutritionally complete drink (Fortisip, Nutricia, Netherlands) at 07:00 am. Participants then remained fasted until visit 2 was complete at approximately 5.30 pm. Participants performed ECC exercise on one leg and CON exercise on the contralateral leg using a MLP. Participants underwent a familiarisation, warm up and ECC/CON 1-RM testing prior to performing unilateral ECC/CON exercise (7 sets of

10 repetitions at 80% of ECC/CON 1-RM). Five hours after the cessation of the exercise, participants had an additional muscle biopsy taken from each leg.

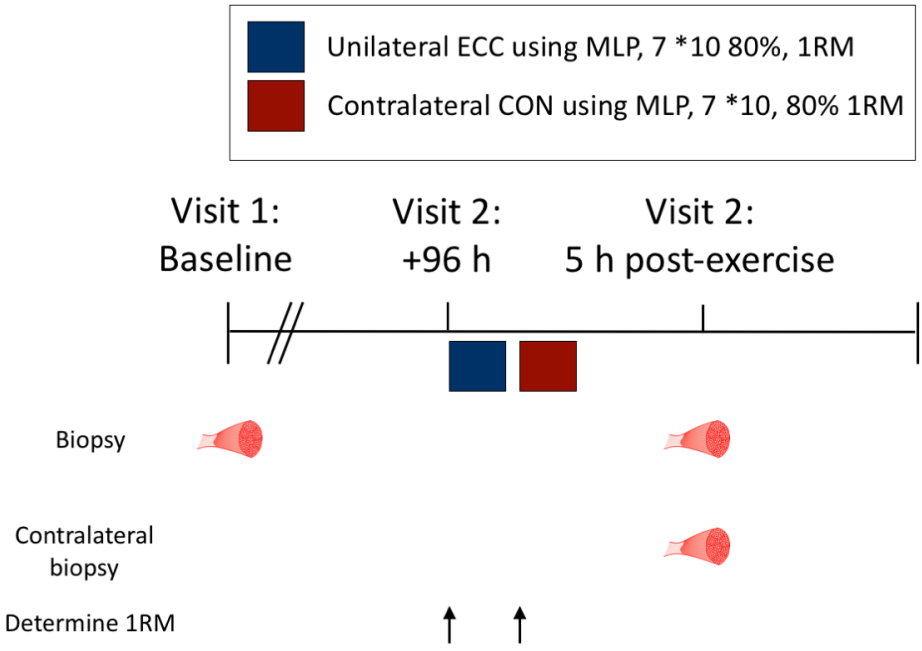

Figure 5.1. Schematic of the study protocol for transcriptomic analysis

5.3.2 RNA extraction and concentration

Muscle biopsy tissue taken at baseline and 5 h post-ECC and CON exercise was shipped to the Beijing Genomics Institute (BGI) for RNA isolation. According to the institute's instructions, muscle was homogenized in 1.5 ml of TRIzol (Invitrogen) for 2 minutes using a TissueLyser II (Qiagen), and then left to rest for 5 minutes to allow the breakdown of cells and cellular components whilst maintaining the integrity of RNA. Afterwards, samples were centrifuged at 12,000 g for 5 minutes at 4ºC. The RNA-containing supernatant was transferred into a new Eppendorf and 300 µl of Chloroform/ isoamyl alcohol (24:1) was added and the tubes were vigorously

shaken. Samples were centrifuged at 12,000 g for 10 minutes at 4ºC after which the sample separated into 3 phases, the lower phenol-chloroform phase containing cell debris, an interphase containing DNA and protein and the upper aqueous phase containing RNA. The aqueous phase was decanted into a new Eppendorf and an equal volume (matching that of the supernatant) of isoamyl alcohol was added, mixed and left at -20 ºC for 2 hours for precipitation. Samples were centrifuged at 13,600 rpm for 20 minutes at 4ºC, and the supernatant was discarded. The remaining RNA containing pellet was washed by adding 1 ml 75% ethanol and centrifuged at 13,600 rpm for 3 minutes at 4ºC, this step was repeated. All ethanol was removed from the Eppendorf without disturbing the pellet, any excess ethanol was left to air dry off the RNA pellet. Between 20-30 µl of diethylpyrocarbonate-treated (i.e. RNase-free) water was added to dissolve the RNA pellet.

To determine the quality of the RNA, samples were run on an Agilent 2100 bioanalyser (Agilent Technologies). Only samples with an RNA integrity number (RIN) of ≥ 5.7 and RNA mass of >200 ng were accepted for RNA sequencing.

5.3.3 RNA sequencing

All processes involved for performing next generation RNA sequencing were performed by BGI. Library preparations were performed using TruSeq RNA library preparation kits. The first step in the library preparation work flow was to purify the poly-A containing mRNA, and thus most mature mRNA, from 0.1-1 µg total mRNA using Poly-T oligo attached magnetic beads. This removes ribosomal rRNA which accounts for >90% of total RNA (358), which would thus drown out the presence of

RNA of interest. A second round of purification fragments RNA to minimise secondary structure formation and reduce end biases (358). RNA fragments were reverse transcribed using random primers into the first cDNA strand, then the RNA template is removed and replaced by a second cDNA strand, this reverse transcription is necessary since sequencing technologies require DNA libraries (358). cDNA fragments then go through an end repair process where overhangs (i.e. unpaired nucleotides) were converted into blunt ends, and a single 'A' nucleotide is added to the 3' end of the blunt fragments to prevent ligation with one another and there is the ligation of the adapters. Products were then purified and enriched with PCR to compile the cDNA library.

Next generation sequencing was performed using the HiSeq 3000/HiSeq 4000 Illumina sequencing by synthesis chemistry systems, using advanced patterned flow cell technology for maximised level of sample throughput.

5.3.4 Sequence quality and the alignment of sequencing data

The quality of raw sequencing reads was evaluated using FastQC (Babraham Bioinformatics), a quality control tool for high throughput sequencing data. In particular, the per base sequence quality, which shows the range of quality values across all bases at each point was only accepted if the median of the base was ≥ 20. Additionally, sequence duplication levels, which creates a plot showing the relative number of sequences with different degrees of duplication, was only accepted if non-unique sequences make up less than 50% of the total. As the quality of reads was judged to be high no additional filtering or trimming to get rid of low quality reads

or sequences containing adaptor sequences was necessary. Reads were aligned to the human reference genome (hg38 NCBI - iGenomes) using Bowtie2 (176). A 'local' alignment was performed on single end reads with the 'very-sensitive' pre-set parameters. Further processing of the alignment files was performed using samtools (182).

5.3.5 Identifying differential expression

Reads mapping to known exons were counted using (184) in an unstranded fashion and using the human genome annotation as a reference (hg38 NCBI – iGenomes). To characterise the variation between sequencing samples, edgeR (282) was used to produce a multi-dimensional scaling (MDS) plot. The MDS plot identified the presence of two outliers in the samples (Appendix 5.1 A). The samples, a young baseline sample and an older 5 h post-ECC exercise sample, were removed from all subsequent analysis. The MDS plot of the remaining data showed a more clustered set of samples with no obvious outliers (Appendix 5.1 B). Differential expression was inferred between groups (i.e. young baseline vs young CON exercise) using edgeR. In the edgeR analysis genes were only included in the analysis if the counts per million (CPM) for that gene was greater than 2 in more than 10 samples. Counts were normalised by library size and biological and technical variation (dispersion) was estimated. Significance of differential expression was estimated using the Fisher's exact test and P-values were corrected for a false discovery rate using the method of Benjamini and Hochberg (28) with a significance cutoff of $P\text{-}corr < 0.05$.

To identify changes in expression between the age groups, first differentially expressed genes in the specific contraction groups (ECC and CON) were compared to baseline for each age group and compared the baseline of each age group. Then these differentially expressed genes were compared across the age groups in order to identify genes that are differentially expressed in both age groups or are unique to either the young or older groups.

5.3.6 Pathway analysis

Significantly differentially expressed genes were tested for pathway enrichment using Gene Ontology (GO) (10) and BINGO (198). This allowed the identification and visualisation of common functions, pathways and/or processes of differentially expressed genes. In this analysis the sample was the set of differentially expressed genes and the background was all genes that passed the filtering criteria in the edgeR analysis (as outlined above). To test for enrichment the hypergeometric test with correction for a false discovery rate was used, using the method of Benjamini and Hochberg (28) and a significance cutoff of $P\text{-}corr < 0.05$. The hypergeometric test was used in order to reduce the number of false positives identified. For this analysis the 'Biological Process' section of the GO was primarily focused on and utilised all annotations for human genes and GO terms. Significantly enriched terms were visualised as a network using BINGO and Cytoscape (297) a rank-rank hypergeometric overlay (RRHO) was performed prior to applying a *P*-value cut-off ($P<0.05$).

5.4 Results

5.4.1 Age-related regulation of the baseline transcriptomic profile

Initially, differences in baseline transcriptomic expression between age groups were compared. A total of 952 genes displayed significant differential expression in older versus younger participants; of these 328 were up-regulated and 624 were down-regulated. Full lists of the differentially expressed genes can be found in Supplementary File 1. Using the Gene Ontology (GO) to identify the functions of differentially expressed genes, up-regulated genes clustered to several high-level and diverse GO terms. However, GO terms enriched for genes down-regulated in older muscle identified a pattern of reduced blood vessel development (using 'Biological Process' ontology, Figure 5.2 A), and of decreased plasma membrane, and cell-cell junction expression (using 'Cellular Component' ontology, Figure 5.2 B). Complete lists of enriched GO terms for all differentially expressed genes between older and younger individuals at baseline can be found in Supplementary File 2.

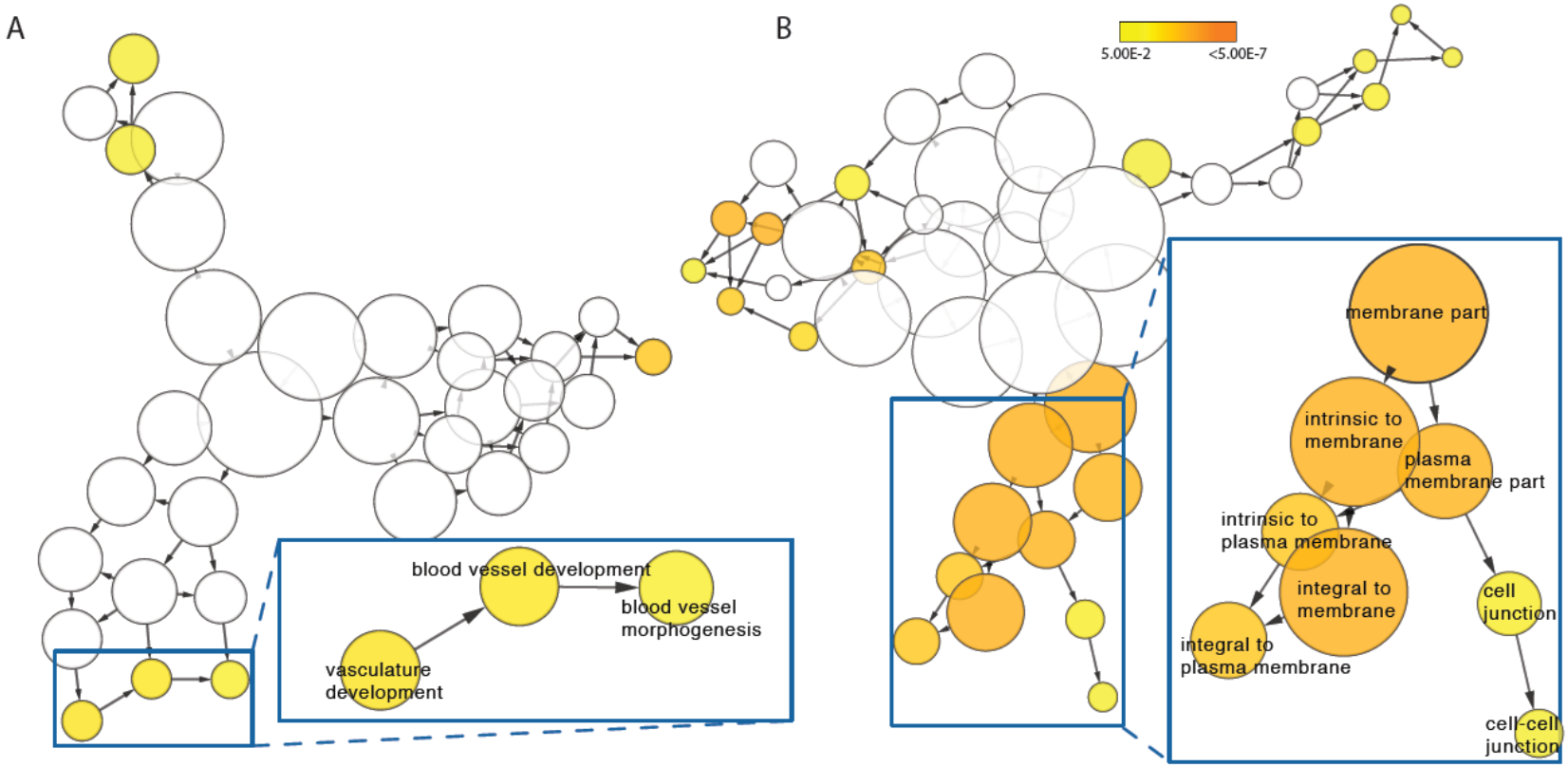

Figure 5.2. Reduced differential expression of genes related to blood vessel development and cell membrane gene expression in older versus younger participants at baseline. *(A) Reduced expression of terms associated with blood vessel development, using the 'Biological Processes' ontology. (B) Reduced expression of terms relating to the cell membrane and cell-cell junctions, using the 'Cellular Component' ontology. Each node in the network represents a GO term with the size of each node corresponding to the number of genes associated with that term. Significantly enriched terms are coloured yellow with more significant terms a deeper shade.*

5.4.2 Common transcriptomic responses characterise CON and ECC exercise, independent of age

Since catabolic repression and peaks in anabolic signalling occurred after 5 h after both CON and ECC exercise, this relevant time point (i.e. 5 h post-exercise) was chosen to compare the transcriptional response to different contraction modes. Comparing baseline to 5 h post-CON and -ECC exercise, the muscle transcriptome displayed a pattern of significant differential gene expression in both age groups (Table 5.1).

Table 5.1. Total numbers of significantly differentially expressed genes in skeletal muscle of young and older participants 5 hours following ECC and CON exercise.

	Concentric		**Eccentric**	
	Up-regulated	**Down-regulated**	**Up-regulated**	**Down-regulated**
Young	227	63	590	204
Old	665	328	724	198

Transcriptomic profiles between post-CON and post-ECC exercise differentially expressed genes were directly compared, within age groups (Figure 5.3). In young participants the up- and down-regulated transcriptional profiles of CON and ECC exercise are virtually identical. Similarly, in older participants there is large overlap between CON and ECC differentially expressed genes. Despite the higher number of contraction-unique up-regulated genes in older participants, these gene sets show no enrichment for GO terms suggesting that the genes do not coherently represent any specific functions or that these sets comprise poorly

annotated genes. Full lists of differentially expressed genes from baseline to post-exercise, and for the overlap between the contraction types can be found in Supplementary File 1. Complete lists of enriched GO terms for all contraction mode-associated differentially expressed genes can be found in Supplementary File 3.

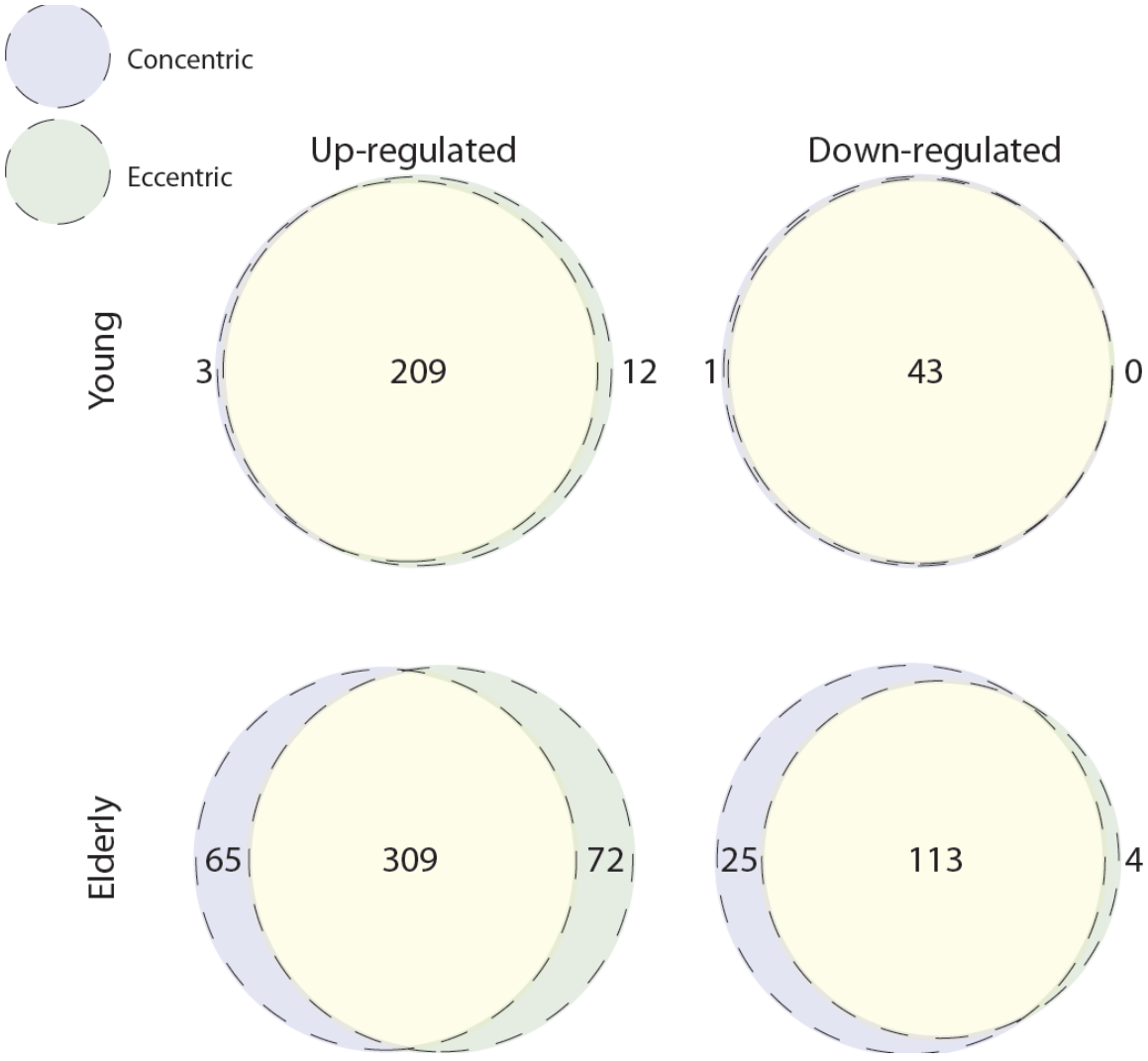

Figure 5.3. CON and ECC exercise induce a common transcriptomic response in young and older participants. *Overlap of contraction mode-associated differentially expressed genes within age groups. Near complete overlap exists between up-regulated (top left Venn) and down-regulated (top right Venn) genes, 5 h after CON and ECC exercise in young*

participants. Up-regulated (bottom left Venn) and down-regulated (bottom right Venn) genes in older participants after CON and ECC exercise also display predominant overlap.

5.4.3 The effect of ageing on the muscle transcriptomic response to CON and ECC exercise:

To determine whether older participants display a disparate post-exercise transcriptional profile compared to the young, GO term enrichment was first assessed for differentially expressed genes between baseline and post-CON exercise and baseline and post-ECC exercise within age groups. In the young, the 'Biological Processes' ontology identified between 6-115 high-level terms as up- or down-regulated after CON and ECC exercise, thus no consistent functional pattern emerges for the post-exercise transcriptional response in young participants. The response in older participants to ECC exercise similarly identified numerous high-level GO terms for up-regulated (138) and down-regulated (39) terms. Full lists of these GO terms can be viewed in Supplementary File 3. However, a post-CON exercise specific pattern of GO term enrichment arose in older participants: terms associated with blood vessel development and cell adhesion were up-regulated (Figure 5.4 A), and several terms relating to mitochondrial respiration were down-regulated (Figure 5.4 B).

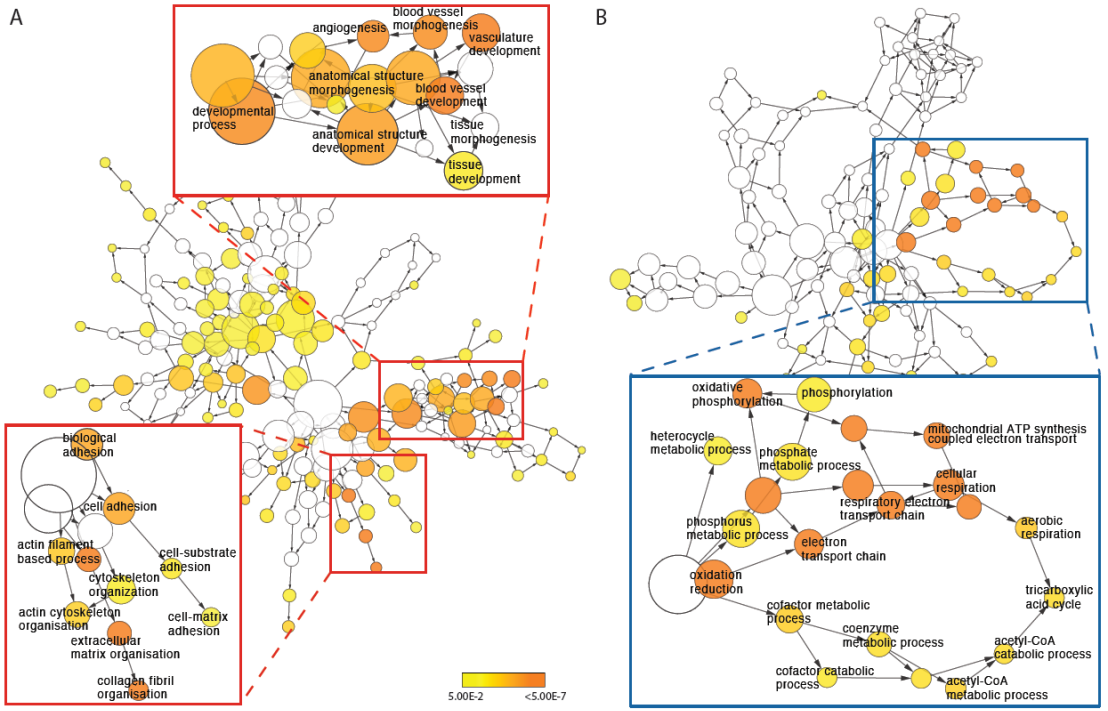

Figure 5.4. Transcriptomic profile of older participants 5 h post-CON exercise. *(A) increased expression of GO term networks associated with cytoskeletal, cell adhesion and extra-cellular matrix (bottom left red insert) and blood vessel development (top right) (B) reduced expression of GO term network associated with mitochondrial metabolism (blue insert). Each node in the network represents a GO term with the size of each node corresponding to the number of genes associated with that term. Significantly enriched terms are coloured yellow with more significant terms a deeper shade.*

Post-CON and post–ECC exercise differentially expressed genes between young and older participants were overlaid to directly compare age-specific transcriptomic responses (Figure 5.5). After CON exercise, few genes were uniquely up-regulated (13 genes) and down-regulated (2 genes) in young participants, with no clear pattern of GO term enrichment. In contrast (and in line with older baseline to 5 h post-CON expression changes), post-CON older participants displayed: i) unique up-regulation of 147 genes enriched for terms associated with cell adhesion, extracellular organisation and blood vessel development and, ii) unique down-regulation of 28 genes enriched for terms relating to the metabolism and/or catabolism of amino acids, lipids, carboxylic acid and DNA (Table 5.2). Despite comparably high numbers of uniquely up-regulated and down-regulated genes after ECC exercise in both young and older groups, there was no GO term enrichment for any age-dependent ECC gene sets. Complete lists of enriched GO terms for post-exercise differentially expressed genes between age groups can be viewed in Supplementary File 3.

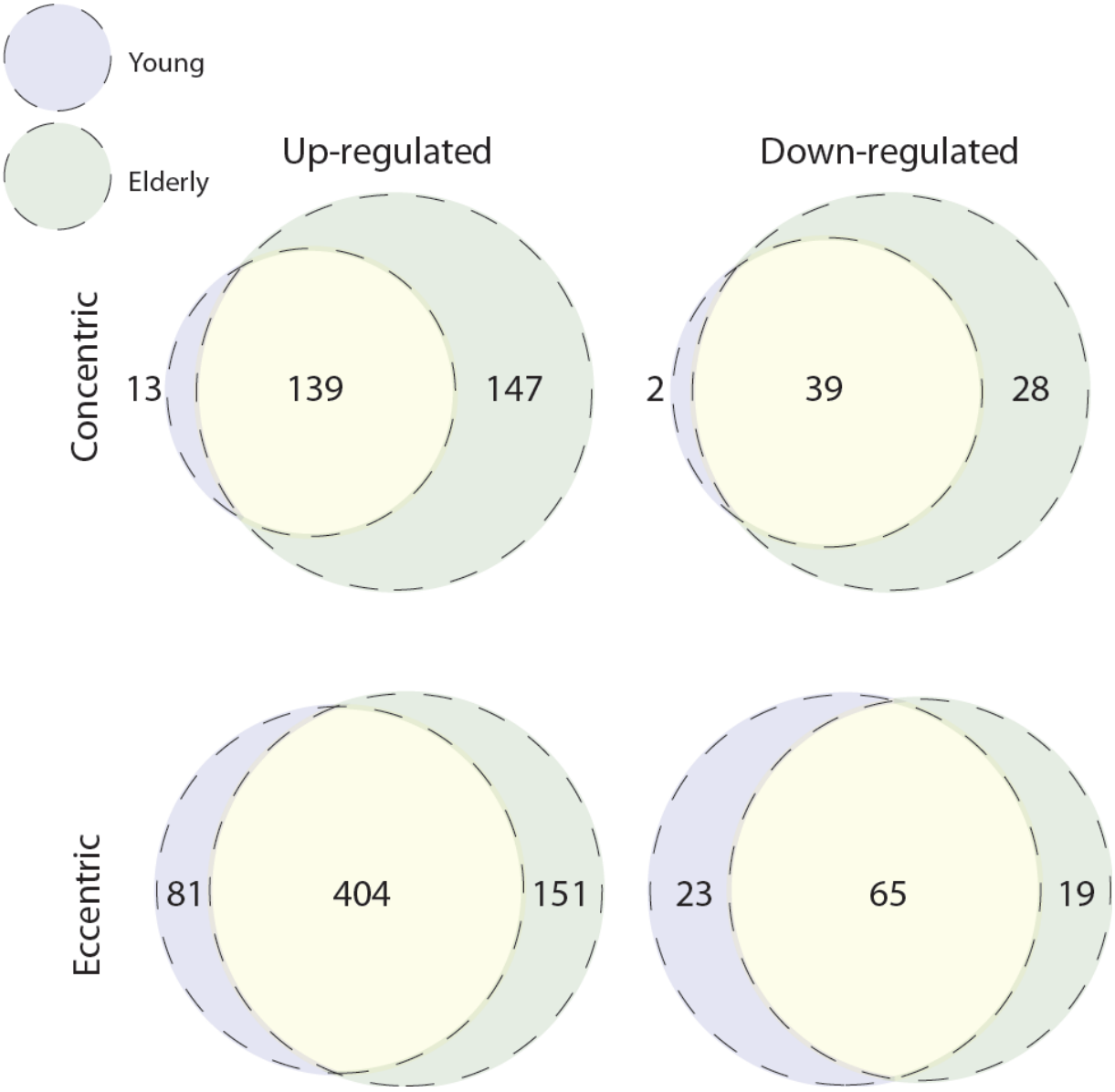

Figure 5.5. Unique age-related transcriptomic response to CON and ECC exercise. *Overlap of age-associated differentially expressed genes within contraction modes. Both young and older participants display unique signatures of differential gene expression 5 h post-CON (up-regulated, top left Venn; down-regulated, top right Venn) and post-ECC (up-regulated, bottom left Venn; down-regulated, bottom right Venn) exercise.*

Table 5.2. Gene Ontology (GO) terms enriched for significantly differentially expressed genes in older participants, 5 h post-CON exercise. *Dashed lines separate broad functional GO term classes.*

GO-ID	GO description	Observed DE genes	Total genes in GO-ID	*P*-raw	*P*-corrected
Up-regulated post-concentric exercise in elderly muscle only:					
7155	cell adhesion	14	355	2.12E-04	3.24E-02
22610	biological adhesion	14	355	2.12E-04	3.24E-02
43062	extracellular structure organization	9	96	4.18E-06	1.71E-03
30198	extracellular matrix organization	8	72	4.08E-06	1.71E-03
30199	collagen fibril organization	6	22	2.81E-07	3.44E-04
1568	blood vessel development	12	210	1.83E-05	4.47E-03
1944	vasculature development	12	215	2.31E-05	4.71E-03
43588	skin development	5	21	6.14E-06	1.88E-03
Down-regulated post-concentric exercise in elderly muscle only:					
9081	branched chain family amino acid metabolic process	2	16	5.24E-04	3.10E-02
9082	branched chain family amino acid biosynthetic process	1	2	4.33E-03	4.60E-02
9098	leucine biosynthetic process	1	2	4.33E-03	4.60E-02
19482	beta-alanine metabolic process	1	1	2.17E-03	4.60E-02
9063	cellular amino acid catabolic process	2	42	3.64E-03	4.60E-02
9083	branched chain family amino acid catabolic process	2	12	2.90E-04	2.86E-02
6550	isoleucine catabolic process	1	2	4.33E-03	4.60E-02
19484	beta-alanine catabolic process	1	1	2.17E-03	4.60E-02
6574	valine catabolic process	1	1	2.17E-03	4.60E-02
34440	lipid oxidation	2	34	2.39E-03	4.60E-02
19395	fatty acid oxidation	2	34	2.39E-03	4.60E-02
6635	fatty acid beta-oxidation	2	29	1.74E-03	4.60E-02
9062	fatty acid catabolic process	2	37	2.83E-03	4.60E-02
46395	carboxylic acid catabolic process	4	80	2.21E-05	3.27E-03
42732	D-xylose metabolic process	1	1	2.17E-03	4.60E-02
5997	xylulose metabolic process	1	1	2.17E-03	4.60E-02
45072	regulation of interferon-gamma biosynthetic process	1	2	4.33E-03	4.60E-02
45078	positive regulation of interferon-gamma biosynthetic process	1	2	4.33E-03	4.60E-02
46113	nucleobase catabolic process	1	2	4.33E-03	4.60E-02
6208	pyrimidine base catabolic process	1	2	4.33E-03	4.60E-02
19859	thymine metabolic process	1	2	4.33E-03	4.60E-02
6210	thymine catabolic process	1	2	4.33E-03	4.60E-02
9439	cyanate metabolic process	1	2	4.33E-03	4.60E-02
9440	cyanate catabolic process	1	2	4.33E-03	4.60E-02
6082	organic acid metabolic process	5	371	9.19E-04	4.53E-02
16054	organic acid catabolic process	4	80	2.21E-05	3.27E-03
44282	small molecule catabolic process	4	179	5.06E-04	3.10E-02
44270	cellular nitrogen compound catabolic process	2	46	4.35E-03	4.60E-02

5.5 Discussion

This study shows novel data regarding the age-related acute transcriptomic response to a single bout of ECC and CON exercise. The key findings of the study demonstrate: i) ageing *per se* is associated with a down-regulation of genes involved in blood vessel development, plasma membrane and cell-cell junction expression, ii) post-CON and post-ECC exercise

transcriptional profiles were virtually identical in both young and older adults, iii) differentially expressed genes between baseline and post-CON and baseline and post-ECC within age groups revealed post-CON in older muscle up-regulated genes related to blood vessel development and cell adhesion and down-regulated mitochondrial respiration related genes and iv) when post-CON and post-ECC differentially expressed genes between young and older age groups were overlaid, post-CON exercise in older adults displayed the unique up-regulation of genes enriched for terms related to cell adhesion, extracellular organisation and blood vessel development and unique down-regulation genes enriched for terms relating to the metabolism and/or catabolism of amino acids, lipids, carboxylic acid and DNA.

5.5.1 Age-related basal muscle transcriptome

Herein, ageing *per se* affected the muscle transcriptome since genes related to blood vessel development, plasma membrane and cell-cell junction were down regulated. Sufficient delivery of growth factors and nutrients to the muscle is purported to be necessary for the maintenance of muscle mass (157). A growing body of evidence suggests that ageing is associated with reduced capillarisation (59, 67), reduced angiogenic factors (i.e. vascular endothelial growth factor (VEGF)) and fewer capillaries per type II fibre's (67), although attenuated capiliarisation in older adults is not always found (115, 335). In turn, this might limit the perfusion of skeletal muscle thereby restricting the delivery of essential nutrients, culminating in perturbed anabolic responses (157). Herein, age-related reductions in transcripts related to blood vessel development were reported which might underlie the purported age-related changes in angiogenesis. Such age-related alterations in angiogenesis have been hypothesised to contribute to sarcopenia (5). However, recent work has shown that restoring vascular responses in ageing was not associated with improved MPS responses (257). The maintenance of the muscle vasculature is also essential for muscle regeneration

(174) since increased VEGF, an important regulator of blood vessel formation, was associated with improved angiogenesis, regeneration and muscle strength combined with reduced fibrosis following injury, albeit in rodents (242). Therefore, it could be hypothesised that lower basal blood vessel development transcripts with advanced age may reduce the ability of muscle to regenerate from exercise.

Other down-regulated genes were related to plasma membrane and cell-cell junction terms, including variants of integrin genes. Integrin's form parts of attachment complexes, which are are protein-dense structures that physically tether the ECM to the contractile muscle proteins. Other proteins within these complexes are signalling molecules, receptors and structural proteins (68). Such complexes are required for the maintenance of muscle structure and function in worms (94), force transfer in rodents (274) and regulate exercise-induced muscle growth (104) and disuse atrophy (32) in humans. Indeed, mutations in attachment complex-related genes leads to muscular dystrophy (207), which is typically characterised by the loss of muscle mass and increased muscular weakness (253). Thus the maintenance of such complexes is essential for structural and metabolic integrity of muscle. Basal down-regulation of these genes (as observed herein) may compromise structural integrity and metabolic homeostasis via reduced force transfer and metabolism, which chronically, may explain compromised skeletal muscle mass and function observed in ageing. Indeed, old rodents with disrupted dystrophin-associated glycoprotein complex display impaired force transmission (274). Such comprehensive work remains to be done in humans.

A down-regulation in plasma membrane transcripts (including collagen transcripts) has been reported previously (246), and although this may perhaps suggest reduced EMC in ageing,

ageing is associated with increased collagen/ECM (274). This has been suggested to be the result of impaired degradation due to increased cross-linking of collagens, as opposed to increased gene expression and thus synthesis, has been suggested to the driver behind age-related accumulation of ECM (122). This ECM dysregulation may underlie the progression of sarcopenia, since older adults display attenuated basal levels of matrix metalloproteinase-2 (MMP-2) (80), which is involved in ECM breakdown, and basal MMP-2 gene expression correlates with muscle mass and strength gains following RET in older adults (81). Thus, chronic lower level expression could culminate and contribute to the loss of muscle mass and strength seen in ageing.

5.5.2 Similar transcriptional responses following CON and ECC exercise

The transcriptional responses 5 h post-CON and ECC exercise were similar (independent of age), which is in contrast to previous work reported in recreationally active males (169). This finding was somewhat unexpected since the extent of acute post-translational events (Chapter 3 and 4) and chronic adaptations (gains in muscle mass and function) (283) to ECC exercise are generally more pronounced than following CON exercise. It is speculated herein that the post-exercise transcriptomic profiles are similar because participants were unaccustomed to exercise and therefore elicited a similar early phase non-specific post-exercise transcriptional response (213). This transcriptomic response is expected to become more refined overtime (i.e. in response to RET), coding for proteins that are specific to the resultant phenotypic adaptation (213). To support this idea, it was shown that in response to RE in untrained young healthy males, the protein synthetic response was non-specific increasing both myofibrillar and mitochondrial MPS, demonstrating the idea of a non-specific response during the early phases of RE during which generic proteins required for structural and metabolic adaptations are increased. Within the same study, after 10 weeks of RET this MPS

response was refined, such that only myofibrillar MPS increased, which contributes to the gains in muscle mass seen with RET (349). This highlights the refinement of the response over time, which may occur at the gene level.

Furthermore, since transcriptional regulation is rapid, demonstrated by the varying number of differentially regulated transcripts over a small number of hours (169), it is plausible the presence of contraction-specific regulation of the transcriptome was missed due to only one post-exercise time-point being investigated. Herein, the 5 h post-exercise time-point was chosen since post-ECC exercise, the nadir of force was at 5 h and repressed catabolic signalling and peak anabolic signalling occurred 5 h post-ECC and CON exercise, indicative of transcriptional activity at this time. However, it is postulated that at times where other molecular mechanisms are disparate between the contraction types, for example the unique phosphorylation of NFκβ^{Ser536} at 72 h following ECC exercise in young participants (Chapter 3), transcriptional regulation between contraction types may differ, and should be investigated. Moreover, because ECC versus CON exercise training do elicit different chronic adaptations in muscle mass and function (283), it is expected that transcriptional responses are divergent at some point, which should be investigated further.

5.5.3 Age-related differences in the transcriptomic response to CON and ECC exercise

Differentially expressed genes between baseline and post-CON and baseline and post-ECC within age groups revealed post-CON in older adults up-regulated genes related to blood vessel development and cell adhesion and down-regulated genes related to mitochondrial respiration. The increase in blood vessel development related genes might be a compensatory response in order to restore vascular homeostasis, since blood vessel development related

genes were down-regulated at baseline in older adults. Additionally, the increased blood vessel development-related genes may go some way in explaining the enhanced limb blood flow observed in older adults in response to RET (256). Furthermore, since the transcriptional response was investigated after an acute bout of exercise in adults unaccustomed to exercise, perhaps the increase in blood vessel development is necessary to remodel exercise-induced damage to the vascular system, since sites of injury typically need re-vascularised (157). However, if this was the case it would be expected that blood vessel development transcripts would increase after both ECC and CON exercise in both age groups, which was not observed. It is possible that temporal changes in blood vessel development related genes are regulated differently in ageing and in response to different contraction modes, and thus may have been missed since only one post-exercise time point was measured herein.

The observed increases in cell adhesion transcripts may counteract age-related decreases in cell adhesion proteins (144, 274, 278) or on the other hand may contribute to the pathophysiological accumulation of adhesion proteins. An accumulation of such proteins has been reported in ageing as a compensatory mechanism to support the maintenance of muscle structural integrity since the contractile network undergoes re-organisation during ageing (i.e. different muscle protein isoforms) (18, 119).

The down-regulation of mitochondrial respiration related genes in response to CON exercise in older adults may contribute to the age-related perturbations in mitochondrial metabolism. During ageing, mitochondrial protein synthesis and mitochondrial enzymes are decreased (284). These blunted responses likely contribute to age-related decline in mitochondrial function (284), which is hypothesised to contribute to sarcopenia (191). Additionally, the

down-regulated transcriptional response may translate into blunted exercise-induced adaptations. For example, following exercise (albeit endurance exercise), age-related perturbations were observed in mixed muscle protein synthesis which may have been driven through declines in mitochondrial protein synthesis (39, 90, 299), although this remains to be confirmed. It is therefore hypothesised that the down-regulation of genes related to mitochondrial metabolism following CON exercise contribute to the impaired remodelling/ chronic adaptation to exercise training in older adults via impairments in mitochondrial protein synthesis. Ultimately this contributes to mitochondrial dysfunction-induced declines in muscle function and may underlie sarcopenia (300, 339).

When post-CON and post-ECC exercise differentially expressed genes between young and older age groups were overlaid, post-CON exercise in older adults displayed the unique up-regulation of genes enriched for terms related to cell adhesion, extracellular organisation and blood vessel development and unique down-regulation genes enriched for terms relating to the metabolism and/or catabolism of amino acids, lipids, carboxylic acid and DNA. Collectively these data suggest that post-CON exercise in older adults there is a general up-regulation of structural transcripts whilst there is the general down-regulation of genes related to metabolism. Although speculative, this is suggestive of impaired metabolism post-CON exercise may contribute to the often observed age-related blunting of muscle mass adaptations in response to RET (37). Furthermore, the up-regulated structural genes may result in a compensatory increase in adhesion proteins to maintain structural integrity during ageing (18, 119).

Additionally, in previous chapters (Chapter 3 and 4), it was noted that the transcriptionally induced response (NF-κβ) was unique to ECC exercise in the young and markers of inflammation were basally higher in the older adults. Therefore, the individual gene data was examined to see if any of the NF-κβ targets were regulated. Interestingly, differentially expression of the Myc (c-Myc) gene was up-regulated following ECC exercise in young and ECC and CON exercise in older adults (Supplementary File 3). NF-κβ mediates the transcription of c-Myc, which subsequently activates genes promoting muscle hypertrophy (344) and also enhances ribosomal biogenesis and protein synthesis (280). More recently, RE increased c-Myc gene expression, which strongly correlated to protein synthesis (342). Since c-Myc was up-regulated 5 h post-exercise, this may be one of the many genes implicated in regulating the anabolic responses observed during skeletal muscle regeneration.

5.6 Conclusions

Herein, we provide novel data regarding the age-related transcriptomic response to acute ECC and CON exercise. This study shows that ageing *per se* is associated with a basal repression of genes related to blood vessel development, plasma membrane and cell-cell junctions, which might be considered regulators of the ageing phenotype. Interestingly, the muscle transcriptome responds similarly to CON and ECC contractions, possible reflecting untrained adults. Post-CON exercise in ageing muscle there was a unique differential gene expression profile which was enriched for the up-regulation of blood vessel development and cell adhesion and down-regulation of mitochondrial respiration. Post-CON exercise compared to the young, older muscle displayed the unique up-regulation of genes enriched for terms associated with cell adhesion, extracellular organisation and blood vessel development and unique down-regulation genes enriched for terms relating to the metabolism and/or catabolism of amino acids, lipids, carboxylic acid and DNA. The down-regulation of

metabolism genes might contribute to the known impaired chronic adaptation/ remodelling that occurs in response to exercise training in ageing adults. Furthermore, up-regulated structural genes may be a compensatory mechanisms required to maintain the integrity of muscle structure during ageing.

6 General Discussion

Skeletal muscle is critical for the production of force, permitting the performance of necessary tasks for daily living such as rising from a chair and the more complex activities such as RE. In addition to necessary functional outputs, skeletal muscle contributes to the regulation of whole body metabolism. Indeed, skeletal muscle is the principle store of AA (111, 354), provides the largest site for glucose uptake (74, 318) and is a key store for triglycerides (162). Therefore, the maintenance of skeletal muscle mass is critical for the preservation of contractile function, metabolic health and ultimately survival. The loss of skeletal muscle mass and function with age (i.e. sarcopenia and dynapenia, respectively) is associated with poor physical function (330), falls (355) and higher all-cause mortality (315). This places a large economic burden on society due to increased hospitalisation and healthcare expenditure (156). As the ageing population continues to rise, major financial challenges will continue to be placed upon the economy (53). As such, delineating the underlying mechanisms and finding effective interventions to offset the progression of sarcopenia and dynapenia are key research priorities.

Skeletal muscle is a highly plastic tissue capable of structural (i.e. mass) and functional (i.e. force) remodelling in response to RET (37). Unaccustomed exercise can induce muscle dysfunction (i.e. reduced force producing capacity) and structural 'damage' (i.e. Z-disk streaming), which is exacerbated following ECC (lengthening) versus CON (shortening) exercise (118). Functional deficits are restored within ~7 days owing to the intrinsic muscle regenerative capacity (270). This regenerative response underpins the adaptation of muscle in response to RE, whereby progressive accumulation of repeated regenerative responses to individual exercise bouts ultimately translates into muscle mass and functional gains. It has been hypothesised that impairments within the post-exercise regenerative process might contribute to the loss of muscle mass and function that is associated with ageing (97). Despite

the importance of muscle regeneration following unaccustomed exercise, the precise temporality of key regenerative mechanisms remains to be fully elucidated in human skeletal muscle, particularly in response to ECC versus CON exercise. Furthermore, whether there are age-related changes in the muscle regenerative processes remain poorly defined.

Previous studies have shown protein turnover (260), anabolic signalling (91, 272), catabolic signalling (311), inflammation/ inflammatory signalling (221), and the activation of satellite cells (49, 84, 150) are all metabolic or molecular processes implicated in the post-exercise regenerative response. However, knowledge regarding the temporality and interplay of these mechanisms is limited due to the investigation of only one or a few mechanisms simultaneously and limited sampling times. In addressing these limitations, work within this thesis found increased anabolic signalling and repressed lysosomal signalling in the early period following both ECC and CON exercise and prior to the nadir of muscle function in young adults. The onset of anabolic signalling was earlier and the magnitude was greater following ECC exercise, potentially inducing a greater net anabolic environment than CON exercise. Once muscle function was past the nadir and repair had been initiated, inflammation increased following both exercise types. Although, post-ECC exercise the inflammatory response occurred earlier, remained active for longer, and led to an inflammatory transcriptional response (NF-κβ), which did not occur after CON exercise. Once function was fully recovered, UPS activity increased regardless of contraction-type. Thus molecular regenerative/adaptive processes continue despite the full recovery of muscle function. How long such molecular regenerative processes (i.e. inflammation and UPS-related activity) persist for after the full recovery of muscle function in young adults is unknown, and should be investigated in order to broaden our understanding of the processes regulating full muscle regeneration. Collectively, these data suggest that anabolic signalling and lysosomal

repression might regulate rapid adaptation, whilst inflammatory processes and UPS activation likely mediate longer term muscle adaptation in young healthy muscle following acute unaccustomed exercise. The greater anabolic and inflammatory responses following ECC may possibly translate into greater muscle adaptation and may go some way in explaining the greater gains in muscle mass and function observed following ECC versus CON exercise training (283), however this remains to be investigated. Furthermore, mechanisms regulating the unique activation of NF-κβ following acute-ECC exercise and the subsequent effects on target gene's are unknown and should be investigated further.

An important application of these data are the contribution towards optimising muscle hypertrophy. To demonstrate, it is shown that the protein turnover related responses i.e. anabolic and catabolic signalling, which contribute to changes in muscle mass, are increased and repressed early after exercise, respectively. Therefore, this presents a key time-frame in which nutritional interventions such as protein or other anabolic food stuffs known to stimulate muscle protein synthesis (12, 348) could be ingested in order to heighten the anabolic response and thus promote net protein balance. A greater net protein balance may have important implications on functional recovery (93) and longer term adaptation (333). Finally, these results provide a benchmark of healthy young human muscle regenerative responses to acute ECC 'damaging' and CON 'non-damaging' exercise, which has important implications for detecting impaired regenerative responses in clinical populations.

Using this benchmark of healthy muscle regeneration, data within this thesis found an age-related increase in local muscle inflammation at baseline, in line with previous reports (129, 219), and blunted anabolic, catabolic and inflammatory responses to exercise in older healthy

adults. The blunted molecular responses might underlie the previously reported impaired acute muscle protein turnover (173) and chronic muscle mass and functional adaptation in ageing (37). As such, it might be accumulations of these impaired responses to individual exercise bouts which explain age-related losses in mass and function. Despite age-related impairments in the molecular regenerative processes measured, functional recovery was not impaired. This implies that other regenerative processes must regulate functional recovery, which may not have been measured herein and should be investigated further. Additionally, it would be interesting to investigate the impact of anti-inflammatory substances/nutraceuticals on the ageing regenerative response since ageing *per se* was associated with chronic local inflammation.

SC activation was unique to older adults, a result which is in contrast to Dreyer et al., (2006) who found SC increase in both young and older adults but the response was blunted in the old versus the young adults (84). However, more contractions performed at a greater intensity were employed (84) compared to the protocol used in the present study, and thus may underlie the different responses. The precise reasons why SC were uniquely activated in the older adults cannot be deciphered from this study, although is it hypothesised that inadequate intrinsic molecular mechanisms specific to the older adults i.e. blunted anabolic, catabolic and inflammatory responses, necessitated additional molecular mechanisms i.e. SC, in order for muscle regeneration and adaptation following ECC exercise. This data has important implications for the development of countermeasures (i.e. nutritional, exercise or therapeutic) against cases of poor muscle regenerative capacity. To demonstrate, anabolic nutritional interventions (i.e. protein ingestion) could be implemented within a time-frame where anabolic signalling is shown to be increased in healthy younger muscle, but fails to increase in older muscle. Additionally, whey protein supplementation is able to induce greater

proliferation of SC during the recovery period (96). Consequently, protein based nutritional interventions throughout the regenerative period where anabolic and satellite cell mechanisms are activated (i.e. 0-24 h post-exercise) might induce a greater growth environment during muscle recovery and potentially greater muscle adaptation.

Previously, the application of ECC exercise in ageing has been questioned due to the stigma that exercise-induced muscle damaged can lead to irreparable damage (193). Herein, following ECC exercise functional recovery was achieved which suggests that, at least from a functional point of view, ECC exercise is safe to perform in older adults. Furthermore, ECC exercise induced anabolic signalling and SC activation, which was not present following CON exercise which may indicate ECC exercise creates a greater anabolic and adaptive environment than CON exercise, which might promote greater muscular adaptation and explain greater gains in mass and function (283), although this remains speculative. This has important applications for the development of effective interventions aimed at optimising ageing muscle molecular regenerative responses. For example, ECC exercise could offer a more effective exercise training based intervention in ageing muscle for promoting muscle hypertrophy versus CON exercise training. Further work could investigate optimal ECC exercise training programmes i.e. the effect of intensity and duration, to fully maximise the exercise-induced anabolic effects.

Current understanding of the precise molecular processes regulating age-related muscle regeneration processes are limited due to the use of very targeted analytical techniques, which only allow the investigation of one or a few target mRNA or proteins (i.e. western blotting and RT-qPCR). In an attempt to define more global molecular networks of muscle

regeneration, transcriptomic analysis has been implemented (219, 259). Studies utilising next generation sequencing, which is capable of detecting all mRNA, are sparse likely due to the expense and complex data analysis required (329). Utilising state-of-the-art RNA sequencing data within this thesis found ageing *per se* was associated with a down-regulation of genes involved in blood vessel development, plasma membrane and cell-cell junction expression. These age-related changes may explain the reduced capillarisation (59, 67) and compromised quantities of some cell adhesion proteins critical for force transfer and structural integrity (144, 274, 278). As such, these enriched genes could potentially be contributors to the age-related loss of muscle mass and function. Although, during ageing compromised capillarisation is not always observed (115, 335) and not all structural proteins are reduced in quantity, in fact some increase (144, 274, 278). As such, these data provide important information which could inform on interventional strategies aimed at offsetting age-related muscle mass and functional decline. For example, exercise, nutritional and/or therapeutical interventions could be designed around enhancing blood vessel development during ageing. That being said, data has shown that restoring vascular responses in ageing was not associated with improved MPS responses (257), however improved vascularisation could improve other aspects of regeneration such as SC responsiveness (157).

Surprisingly, following ECC versus CON exercise, the up and down-regulated transcriptional profiles were virtually identical, independent of age. This suggests that divergent contraction-types elicit similar transcriptional responses, at least 5 h after unaccustomed exercise in healthy humans. Perhaps the transcriptomic profiles are similar because participants were unaccustomed to exercise and thus elicited a similar non-specific post-exercise transcriptional response, which may become more refined over time (213). Previously, it was shown that in response to RE in the untrained state, the muscle protein synthetic response was non-specific

increasing both myofibrillar and mitochondrial MPS, but in response to 10 weeks of RET this MPS response was refined, such that only myofibrillar MPS increased which explains the gains in muscle mass seen with RET (349). This highlights that generic proteins required for structural and metabolic adaptations are increased in the unaccustomed exercise state and that this specificity of response can become more refined with RET, which might hold true at the gene level. Additionally, it is plausible that contraction-specific regulation of the transcriptome was missed due to the RNA sequencing of only one post-exercise time-point. The 5 h post-exercise time-point was chosen to be investigated herein since the nadir of force was 5 h post-ECC and repressed catabolic signalling and peak anabolic signalling occurred 5 h post-ECC and CON exercise, indicative of peak transcriptional activity at this time. Since chronic adaptations to ECC versus CON exercise are different (i.e. ECC exercise training produces greater gains in muscle mass and strength (283)) disparate transcriptional activity must occur at some point. Thus, further research is warranted which investigates the temporal transcriptional responses to ECC versus CON exercise to uncover at what point contraction-specific changes in transcription are apparent, and what gene ontology terms are enriched. Furthermore, by investigating the temporality of the transcriptome response over a more comprehensive time-course will provide insight into which genes are involved in i) functional restoration, ii) longer-term remodelling and iii) both functional restoration and longer-term remodelling.

In older adult's post-CON exercise, these data showed that there was a unique differential gene expression profile enriched for the up-regulation of blood vessel development and cell adhesion and down-regulation of mitochondrial respiration. Since ageing *per se* was associated with down-regulated blood vessel development, the post-CON exercise up-regulation of blood vessel development might be a compensatory response in order to restore

blood vessel development homeostasis. Further, enriched cell adhesion transcripts may be increased to counteract age-related decreases in cell adhesion proteins (144, 274, 278) or on the contrary may contribute to the accumulation of adhesion proteins, which can be a pathophysiological compensatory increase to maintain structural integrity during ageing (18, 119). The down-regulation of mitochondrial respiration transcripts may underlie the exercise-specific responses seen following RET in older adults (349). When post-CON and post–ECC exercise differentially expressed genes between young and older adults were overlaid, post-CON exercise ageing muscle displayed the unique up-regulation of genes enriched for terms associated with cell adhesion, extracellular organisation and blood vessel development and the unique down-regulation genes enriched for terms relating to the metabolism and/or catabolism of amino acids, lipids, carboxylic acid and DNA. Together, these data seem to generally suggest that CON exercise in older adults results in an up-regulation of structurally-related terms, and a down-regulation in metabolism-related terms. Although speculative, impaired metabolism post-CON exercise may contribute to the often observed age-related blunting of muscle mass adaptations in response to RET (37). Furthermore, the up-regulated structural genes may contribute to the accumulation of excess extracellular matrix and adhesion proteins, which increase as a compensatory mechanisms to maintain structural integrity during ageing (18, 119).

In addition to suggested future work mentioned above, other avenues for future work are to; i) determine whether the molecular regenerative responses reported herein are attenuated following a second bout of exercise i.e. repeated bout effect, so that exercise interventions can be optimised, ii) investigate targeted and more global skeletal muscle regenerative mechanisms, as compliments to each other, over a period of exercise training. This will provide a more comprehensive understanding of the mechanisms which mediate longer term

adaptation/maladaptation during ageing and iii) more precisely define the temporal response of anabolic signalling responses during the early regenerative stage i.e. every hour from initiation until cessation, since herein only 0 and 5 h post-exercise time points were investigated. This will allow the optimisation of exercise and nutritional interventions aimed at potentiating the growth environment.

To summarise, the data within this thesis found anabolic and catabolic signalling and inflammatory responses were essential global (i.e. responded to both exercise types) mechanisms involved in the regeneration/ adaptation of skeletal muscle to an acute exercise in younger healthy adults. ECC exercise caused greater anabolic signalling and lead to an inflammatory-related transcriptional response, potentially indicative of greater muscular adaptation capacity compared to CON exercise. In older adults, these molecular responses in general (i.e. both exercise types) were blunted, which might underlie the blunted age-related adaptations to chronic exercise training. Interestingly, ECC exercise caused a SC response in the older adults which might have been due to the global intrinsic mechanisms (i.e. anabolic, catabolic and inflammatory responses) being suboptimal, thus requiring additional mechanisms to support regeneration/ adaptation. Transcriptomic analysis revealed down-regulated genes enriched for blood vessel development, plasma and cell-cell junctions in older adults, which may be contributors to the age-related loss of muscle mass and function. Post-ECC and CON exercise, similar transcriptional profiles were present which might represent an early phase non-specific remodelling response, which becomes refined over several exercise bouts. Finally, transcriptomic data seem to generally suggest that CON exercise in older adults results in an up-regulation of structurally-related terms, and a down-regulation in metabolism-related terms. Impaired metabolism post-CON exercise may contribute to the often observed age-related blunting of muscle mass adaptations in response

to RET (37) and may up-regulate structural genes to increase extracellular matrix and adhesion proteins as a compensatory mechanisms to maintain structural integrity (18, 119).

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8 Conference Proceedings and Awards

Conference proceedings: Posters

Deane, C. S., Phillips, B. E., Boereboom, C., Lund, J., Williams, J., Abdulla, H., Szewczyk, N. J., Atherton, P. J. & Etheridge, T. (2016) Effects of ageing upon muscle functional and inflammatory responses to eccentric exercise. Physiology 2016, The Physiological Society, Dublin.

Deane, C. S., Atherton, P. J., Szewczyk, N. J., Etheridge, T. & Phillips, B. E. (2015) The impact of eccentric and concentric exercise on muscle function in young and older men. Physiology 2015, The Physiological Society, Cardiff.

Awards

2016	**The Physiological Society** - Travel Grant £500 – Physiology 2016
2015	**The Physiological Society** - Travel Grant £410 – Foreign lab visit
2014	**Santander** - £2,500 Scholarship
2013	**Santander** - £1,000 Scholarship