CONCURRENT EXERCISE:

FROM TRAINING TO TRANSCRIPTOME

By

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STATEMENT OF AUTHORSHIP AND SOURCES

This thesis contains no material that has been extracted in whole or in part from a thesis that I have submitted towards the award of any other degree or diploma in any other tertiary institution.

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ABSTRACT

The principle of training specificity dictates that adaptations to exercise training are specific to the mode, frequency, and duration of exercise performed, and result in distinct and divergent skeletal muscle phenotypes. Strength-based training promotes skeletal muscle hypertrophy and maximal force-generating capacity while endurance-based training improves skeletal muscle oxidative capacity and cardiorespiratory fitness. Previous research has suggested the capacity of skeletal muscle to adapt to strength and endurance training when performed simultaneously (i.e., concurrent exercise training) appears to be limited and results in blunted resistance-based adaptations compared to resistance training alone – a phenomenon referred to as the 'interference effect.'

The molecular basis of skeletal muscle adaptation to exercise training involves the propagation of numerous mechanical and chemical stimuli through signalling cascades that ultimately results in an increase in an array of exercise-induced proteins and increases in maximal enzyme activities. The nature of these alterations is specific to the frequency, intensity, volume, and type of metabolic demands placed upon the muscle during exercise. Given the divergent stimuli associated with endurance- and resistance-based exercise, it has been hypothesised that antagonistic molecular signals may underlie the adaptive interference observed with concurrent training. In order to circumvent this effect, strategies have focused on altering the proximity of training sessions (i.e., same day versus alternate day training) and training variables (i.e., frequency, volume, mode). Additionally, optimising post-exercise nutrition (i.e., dietary protein) has been proposed as a potential variable that may promote anabolic signalling and prevent the interference effect.

To determine whether these training strategies in association with a high protein diet (2 g•kg^{-1}•d^{-1}) can attenuate the 'interference effect,' 32 recreationally active males (age: 25±5 y; body mass index: 24±3 kg•m^{-2}; mean ± standard deviation) performed 12 wk of either isolated resistance (RES; *n*=10) or endurance (END; *n*=10) training (3 sessions•wk^{-1}), or concurrent resistance and endurance (CET; *n*=12) training (6 sessions•wk^{-1}). Maximal strength, maximal aerobic capacity, peak power, body composition, and muscle architecture were assessed throughout the intervention. To explore molecular responses that may underpin any impaired adaptation after concurrent exercise training, satellite cells and myonuclei were assessed by immunohistochemistry from skeletal muscle biopsy samples. In addition, exploratory transcriptomics was performed from a subset of participants from each training condition.

The results from the investigations undertaken for this thesis demonstrate that – despite efforts to circumvent the 'interference effect' by implementing recommended strategies of alternate day training, minimising exercise volume, and increasing dietary protein intake – maximal anaerobic power development was attenuated following 12 wk of concurrent exercise training. Myofibre hypertrophy increased to the same magnitude in all training modalities without changes to satellite cell content, suggesting that satellite cell content does not limit the magnitude of hypertrophy achieved during concurrent training. Conversely, myonuclear content displayed strong associations with the degree of myofibre hypertrophy. Transcriptome-wide analysis revealed that concurrent exercise training augments gene sets related to plasma membrane structures while suppressing those related to regulation of messenger ribonucleic acid (mRNA) processing and protein degradation, which may contribute to the 'interference effect' in myofibre hypertrophy. Additionally, considerable overlap of gene sets enriched for

terms related to extracellular matrix remodelling were observed amongst concurrent exercise training and isolated endurance cycle training, which may underlie attenuations in maximal anaerobic power outputs observed following concurrent training. Collectively, these reveal that the current recommendations to maximise muscle hypertrophy with concurrent training do not result in augmented hypertrophic responses compared to single-mode training, and cannot be explained by satellite cell content or inhibition of anabolic gene programs. These findings underpin future investigations of molecular pathways that have not been considered in the context of concurrent training adaptations.

Keywords: Concurrent exercise training, resistance training, endurance training, skeletal muscle, satellite cells, myogenesis, transcriptome

“Strength does not come from winning. Your struggles develop your strengths. When you go through hardships and decide not to surrender, that is strength.”

Arnold Schwarzenegger, bodybuilder, actor, and philosopher (1947–)

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Baubak Shamim, John A. Hawley, and Donny M. Camera. Protein Availability and Satellite Cell Dynamics in Skeletal Muscle. Sports Medicine. *Sports Medicine*. 48(6):1329-1343. 2018

Baubak Shamim, Miguel S. Conceição, Marcus J. Callahan, and Donny M. Camera. Where do satellite cells orbit? An endomysium space odyssey. *Journal of Physiology*. 596(10):1791-1792. 2018

Ryan G. Timmins, **Baubak Shamim**, Paul Tofari, and Donny M. Camera. Differences in lower limb strength and structure after 12-weeks of resistance, endurance, and concurrent training. *International Journal of Sports Physiology and Performance.* [ahead of print, 2020 Mar 25] 1–8. doi:10.1123/ijspp.2019-0788. 2020.

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Baubak Shamim, Paulo R. Jannig, Igor Cervenka, Paul Tofari, Jorge L. Ruas, John A. Hawley, and Donny M. Camera. Skeletal Muscle Transcriptomic Responses to Chronic Concurrent Exercise Training**.** Cell Symposia: Exercise Metabolism**.** Abstract P092. Sitges, ESP. May 2019.

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LIST OF ABBREVIATIONS

%TE	Typical error as a coefficient of variation
1RM	One-repetition maximum
4E-BP1	eIF4E binding protein 1
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide-1-4-ribofuranoside
AMP	Adenosine monophosphate
AMPK	5’ adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BB	Barbell
BCAA	Branched-chain amino acid
BM	Body mass
BMI	Body mass index
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CaMK	Ca^{2+}/Calmodulin-dependent protein kinase
cAMP	3',5'-cyclic adenosine monophosphate
cDNA	complementary DNA
CET	Concurrent exercise training
CG	Close grip
CMJ	Countermovement jump
CRE	cAMP response element
CSA	Cross-sectional area
CT	Computer tomography
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DB	dumbbell
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DXA	Dual-energy X-ray absorptiometry
Ebox	Enhancer box
ECM	Extracellular matrix
EE	Energy expenditure
eEF2	Eukaryotic elongation factor 2
eIF4E	Eukaryotic initiation factor 4E
EMG	Electromyogram
END	Endurance training
FBS	Fetal bovine serum
FDR	False discovery rate
FFM	Fat free mass
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GO	Gene ontology
GS	Goat serum

GSEA	Gene set enrichment analysis
GTPase	Guanosine triphosphate hydrolase
h	Hours
HGF	Hepatocyte growth factor
HIIT	High-intensity interval training
HR	Heart rate
ICC	Intraclass correlation coefficients
IGF-1	Insulin-like growth factor-1
IL-6	Interleukin 6
IMTP	Isometric mid-thigh pull
IPA	Ingenuity Pathway Analysis
KO	Knockout
LBM	Lean body mass
LLM	Leg lean mass
LME	Linear mixed-effect
MAP	Maximum aerobic power
MDC_{95}	Minimum detectable change at a 95% confidence interval
MEF2	Myocyte enhancing factor 2
MICT	Moderate-intensity continuous training
min	Minutes
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger ribonucleic acid
MT	Muscle thickness
mtDNA	Mitochondrial DNA
mTOR	Mechanistic/mammalian target of rapamycin
mTORC1	Mechanistic/mammalian target of rapamycin complex 1
mTORC2	Mechanistic/mammalian target of rapamycin complex 2
MV	Muscle volume
MVC	Maximum voluntary contraction
MYC	v-myc avian myelocytomatosis viral oncogene homolog
MyoD	Myoblast determination protein 1
MyoG	Myogenin
NCAM	Neural cell adhesion molecule
NFAT	Nuclear factor of activated T-cells
NGS	Next-generation sequencing
NRF1	Nuclear respiratory factor 1
NRF2	Nuclear respiratory factor 2
p38 MAPK	p38 mitogen-activated protein kinase
p70S6K1	70-kDa ribosomal protein S6 kinase 1
PAL	Physical activity level
Pax7	Paired box protein Pax-7
PBS	Phosphate-buffered saline
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PKB	Protein kinase B

PRAS40	Proline-rich Akt substrate of 40 kDa
Prox1	Prospero-related homeobox-1
raptor	Regulatory-associated protein of mTOR
RDL	Romanian deadlift
RES	Resistance training
Rheb	Ras homolog enriched in brain
RIN	RNA Integrity Number
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
RPE	Rating of perceived exertion
rRNA	Ribosomal RNA
SAM	Significance analysis of microarrays
SD	Standard deviation
Ser	Serine
SIRT1	Sirtuin 1
SJ	Squat jump
SL	Straight-legged
TBC	Tre2-Bub2-Cdc16
TBC1D7	TBC 1 domain family, member 7
TE	Typical error
TEI	Total energy intake
Tfam	Mitochondrial transcription factor A
TGF-β	Transformation growth factor-β
Thr	Threonine
TIF-1A	Transcription initiation factor-1A
TL	Thigh length
TOR	Target of rapamycin
TSC1	Tuberous sclerosis 1
TSC1/2	Tuberous sclerosis complex 1/2
TSC2	Tuberous sclerosis 2
UBF	Upstream binding factor
ULM	Upper body lean mass
USF-1	Upstream stimulatory factor 1
VL	Vastus lateralis
VO_2	Oxygen uptake
VO_{2peak}	Peak oxygen uptake
WG	Wide grip
wk	Weeks
WT	Wild type
y	Years

CHAPTER 1

INTRODUCTION AND OVERVIEW

This chapter has been adapted from the following published review:

Baubak Shamim, John A. Hawley, and Donny M. Camera. Protein Availability and Satellite Cell Dynamics in Skeletal Muscle. Sports Medicine. *Sports Medicine*. 2018 Jun;48(6):1329-1343.

1.1 Introduction

Skeletal muscle is a remarkably plastic tissue that is capable of altering its morphological and metabolic characteristics in response to perturbations to cellular homeostasis. The phenotypic flexibility of skeletal muscle observed in response to exercise training involves the transduction of a multitude of mechanical and chemical stimuli through cell signalling cascades that alter gene expression, protein content, and enzyme activity (Egan & Zierath, 2013). The nature and magnitude of these changes are specific to the frequency, intensity, volume, and type of metabolic demands placed upon the muscle during exercise (Baar, 2014*a*).

Exercise can broadly be classified as either 'strength-based' resistance training or 'aerobic-based' endurance training. Resistance-type exercise (e.g., weightlifting) promotes skeletal muscle hypertrophy, and increases strength, and anaerobic power (McDonagh & Davies, 1984*a*). Conversely, endurance-type exercise (e.g., cycling) promotes an oxidative phenotype through mitochondrial biogenesis, increasing maximal oxygen uptake and improving metabolic flexibility (Holloszy & Coyle, 1984). Though these adaptations are distinct, the fundamental basis of the adaptive process is similar between the different modes of exercise. Skeletal muscle contractions during a single bout of exercise stimulate signalling cascades that function to activate or inhibit numerous proteins through post-translational modifications. These signals are transmitted to the nucleus and converge upon transcription factors to initiate the rapid replication of specific deoxyribonucleic acid (DNA) gene sequences, enabling the transcription of messenger ribonucleic acids (mRNA). In concert, signals are relayed to ribosomes and translational regulatory proteins to facilitate translation of mRNAs into polypeptides to create new proteins (Perry *et al.*, 2010). Thus, when exercise bouts are repeated over time, the cumulative transcriptional and translational responses to each

bout of exercise results in functional adaptations that defend against future disturbances to homeostasis, ultimately improving physical capacity.

Many sports require a combination of strength and endurance adaptations to improve athletic performance (Nader, 2006). Furthermore, a combination of resistance-based and endurance-based exercise training is prescribed as a strategy to combat numerous diseases and improve longevity (Ruiz *et al.*, 2008; Pedersen & Saltin, 2015). As such, training programs often incorporate both modes of exercise, which is referred to as concurrent training. Concurrent training amplifies endurance capacity (Hickson *et al.*, 1988), but reduces the ability to develop muscular strength (Hickson, 1980) (**Figure 1.1**). The inhibition of resistance-based adaptations resulting from concurrent training has been termed the 'interference effect' and has been a topic of considerable debate (Nader, 2006; Wilson *et al.*, 2012; Hamilton & Philp, 2013; Baar, 2014*a*; Fyfe *et al.*, 2014; Perez-Schindler *et al.*, 2015; Varela-Sanz *et al.*, 2016; Murach & Bagley, 2016; Coffey & Hawley, 2017; Doma *et al.*, 2017; Fyfe & Loenneke, 2017; Eddens *et al.*, 2018; Berryman *et al.*, 2018; Hughes *et al.*, 2018).

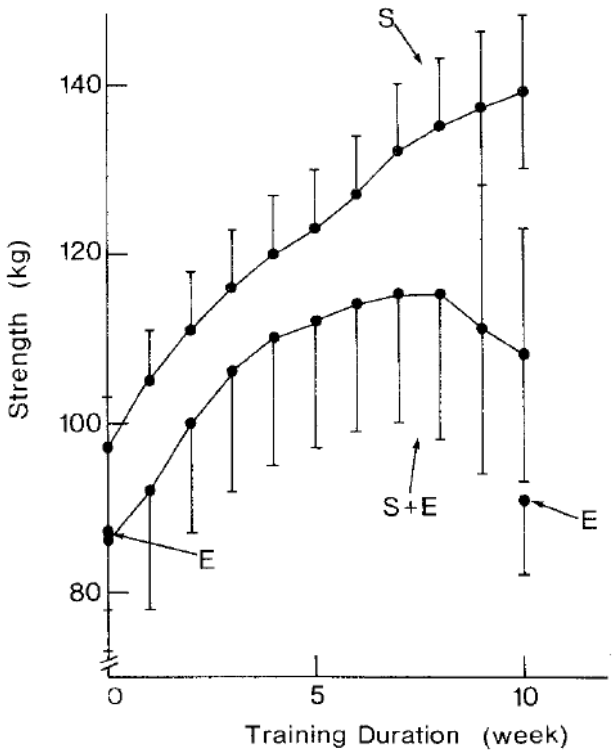

Figure 1.1 The concurrent training 'interference effect' to strength gains (reprinted from (Hickson, 1980), with permission). The figure depicts weekly change to one-repetition maximum squat in participants over 10 wk of resistance training alone (S), endurance training alone (E), or concurrent resistance and endurance training (S+E).

Since the seminal findings of Dr. Robert Hickson published in 1980 (Hickson, 1980), the results of numerous studies have demonstrated an interference in the development of hypertrophy, strength, and power with concurrent training compared to when resistance training is undertaken in isolation (Craig *et al.*, 1991; Hennessy & Watson, 1994; Kraemer *et al.*, 1995; Dolezal & Potteiger, 1998; Bell *et al.*, 2000; Häkkinen *et al.*, 2003; Wilson *et al.*, 2012; Mikkola *et al.*, 2012; Fyfe *et al.*, 2016*a*; Terzis *et al.*, 2016; Tomiya *et al.*, 2017; Fyfe *et al.*, 2018). Others, however, have failed to replicate these findings (Sale *et al.*, 1990; de Souza *et al.*, 2013; Lundberg *et al.*, 2013, 2014; Kazior *et al.*, 2016; Laird *et al.*, 2016; Ferrari *et al.*, 2016; Villareal *et al.*, 2017; Petré *et al.*, 2018). Still, the mechanism(s) underlying the 'interference effect' remain to be elucidated.

1.2 Adaptations to resistance exercise training

Resistance exercise is characterised by intermittent, near-maximal contractions against an external load that presents significant tensile challenges to the working muscle and surrounding structures, with the aim of improving cross-sectional area (CSA), tissue tolerance to load, and maximal force output (McDonagh & Davies, 1984*a*). It was assumed that the initial increases in strength following progressive resistance exercise were predominantly due to motor learning (i.e., muscle activation level) (Lorme & Watkins, 1951), with muscle hypertrophy occurring only after 3-5 weeks (wk) of training (Moritani & deVries, 1979). However, recent evidence has suggested that morphological changes also contribute to early resistance exercise-induced strength gains (Brook *et al.*, 2015). Using a unilateral resistance exercise training program, previously untrained young, healthy males undertook six wk of resistance training. Following only three wk of training, increases in muscle thickness, pennation angle, and fascicle length of the *vastus lateralis* were evident along with improvements in one-repetition maximum (1RM) and isometric maximum voluntary contraction (MVC) (Brook *et al.*, 2015). Accordingly, strength gains in response to resistance exercise training are a product of adaptations to both morphological and neural factors.

1.2.1 Molecular basis of resistance exercise training adaptations

In response to resistance exercise, rates of muscle protein synthesis (MPS) are elevated above basal levels for up to 48 h (Phillips *et al.*, 1997). When resistance exercise-induced elevations in MPS exceed muscle protein breakdown (MPB) over several weeks/months, there is an accretion of myofibrillar proteins resulting in muscle hypertrophy (Moore *et al.*, 2009*b*; Wilkinson *et al.*, 2014). Initiation of MPS begins

with heightened translational signalling, which is predominantly controlled by activation of the serine (Ser)/threonine (Thr) protein kinase, mechanistic/mammalian target of rapamycin, mTOR (Bodine *et al.*, 2001). mTOR exists within two complexes: complex 1 (mTORC1) and complex 2 (mTORC2). Though both complexes share the catalytic mTOR subunit, only mTORC1 contains the regulatory-associated protein of mTOR (raptor), which identifies substrates for mTORC1 by binding to target of rapamycin (TOR) signalling motifs on downstream targets such as the translational mediators, 70-kDa ribosomal protein S6 kinase 1 (p70S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) (Schalm & Blenis, 2002). These proteins mediate the assembly of the translation preinitiation complex in order to foster efficient protein synthesis (Holz *et al.*, 2005). Additionally, mTORC1 contains the inhibitory proline-rich Akt substrate of 40 kDa (PRAS40), which is dissociated from raptor through direct Akt/protein kinase B (PKB)-mediated phosphorylation (Sancak *et al.*, 2007). These features allow mTORC1 to integrate inputs from intra- and extracellular cues, such as growth factors, amino acids, energy levels, stress, and oxygen, to orchestrate protein synthesis or autophagy (Laplante & Sabatini, 2012) (**Figure 1.2**).

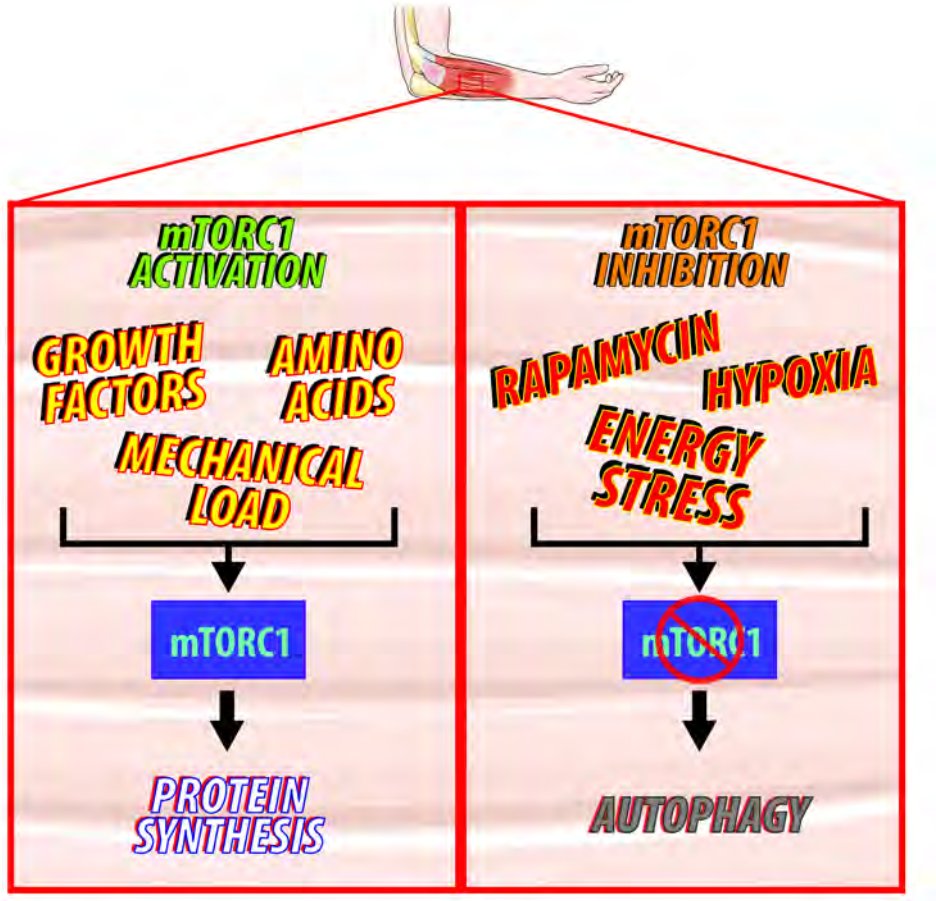

Figure 1.2 Intra- and extracellular inputs converging upon mechanistic/mammalian target of rapamycin complex 1 (mTORC1) that result in activation or inhibition.

Many of the signals that affect mTORC1 function converge on the upstream regulator tuberous sclerosis complex 1/2 (TSC1/2), which consists of the heterodimer tuberous sclerosis 1 (TSC1) and tuberous sclerosis 2 (TSC2), and Tre2-Bub2-Cdc16 (TBC) 1 domain family, member 7 (TBC1D7) subunit (Dibble *et al.*, 2012) (**Figure 1.3**). Mechanical loading in the form of resistance exercise stimulates phosphorylation of TSC2, resulting in mTORC1 activation (Jacobs *et al.*, 2013). Notably, the degree of load-induced phosphorylation of the downstream mTORC1 substrates p70S6K1 (Baar & Esser, 1999; Terzis *et al.*, 2008) and 4E-BP1 (Mitchell *et al.*, 2014) is closely correlated to skeletal muscle hypertrophy.

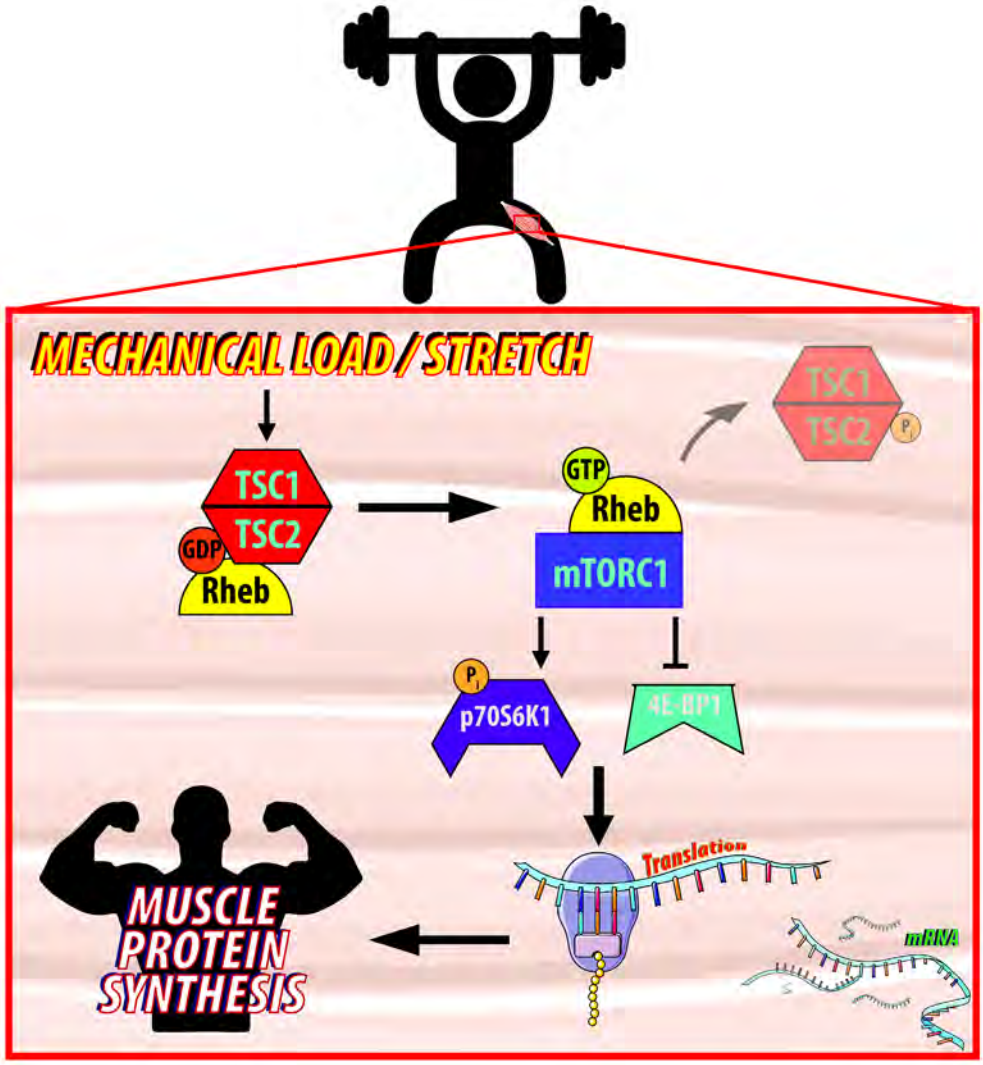

Figure 1.3 Mechanical load-induced mechanistic/mammalian target of rapamycin complex 1 (mTORC1) activation in skeletal muscle. The upstream regulator tuberous sclerosis TSC2 negatively regulates mTORC1 by acting as a guanosine triphosphate hydrolase (GTPase)-activating protein (GAP) for the Ras homolog enriched in brain (Rheb) GTPase. This converts Rheb into its inactive guanosine diphosphate (GDP)-bound state, and prevents it binding to the mTOR kinase domain and activating the complex (Inoki *et al.*, 2003*a*; Tee *et al.*, 2003). Conversely, mechanical loading, in the form of resistance exercise stimulates phosphorylation of TSC2, resulting in movement of TSC2 away from Rheb allowing mTORC1 activation (Jacobs *et al.*, 2013). Subsequently, protein translation is initiated by the downstream mTORC1 substrates, p70S6K1 (Baar & Esser, 1999; Terzis *et al.*, 2008) and 4E-BP1 (Mitchell *et al.*, 2014), resulting in increased muscle protein synthesis. Arrows denote signalling event. P_i denotes phosphorylation.

Administration of the macrolide drug, rapamycin, abolishes acute increases in MPS following resistance exercise (Drummond *et al.*, 2009), indicating that the initial surge in translational activity following exercise is mTORC1-dependent. However, several mTORC1-independent mechanisms can also stimulate MPS (West *et al.*, 2016) (**Figure 1.4**). In this regard, changes in myofibre CSA following eight wk of training are positively correlated with increased expression of ribosomal RNA (rRNA) and activation of ribosome biogenesis regulatory factors (e.g. UBF and transcription initiation factor-1A (TIF-1A)) (Figueiredo *et al.*, 2015). In support of this premise, it has been demonstrated that the magnitude of hypertrophy following four wk of resistance training is related to c-Myc protein content (Stec *et al.*, 2016). Collectively, it appears that translational capacity, in addition to translational activity, is an important mediator of resistance-training induced hypertrophy. Nevertheless, skeletal muscle hypertrophy is an intricately regulated process and requires several multifaceted events in order to occur. Thus, the degree of hypertrophy over a training regime cannot simply be interpreted by acute post-exercise elevations in MPS or intramuscular signalling alone (Mitchell *et al.*, 2014; Nader *et al.*, 2014; Fyfe *et al.*, 2016*b*). Accordingly, using advanced high-throughput techniques, such as RNA sequencing, to delineate additional mechanisms contributing to MPS may be necessary to appreciate the complexity of factors regulating exercise-induced hypertrophy.

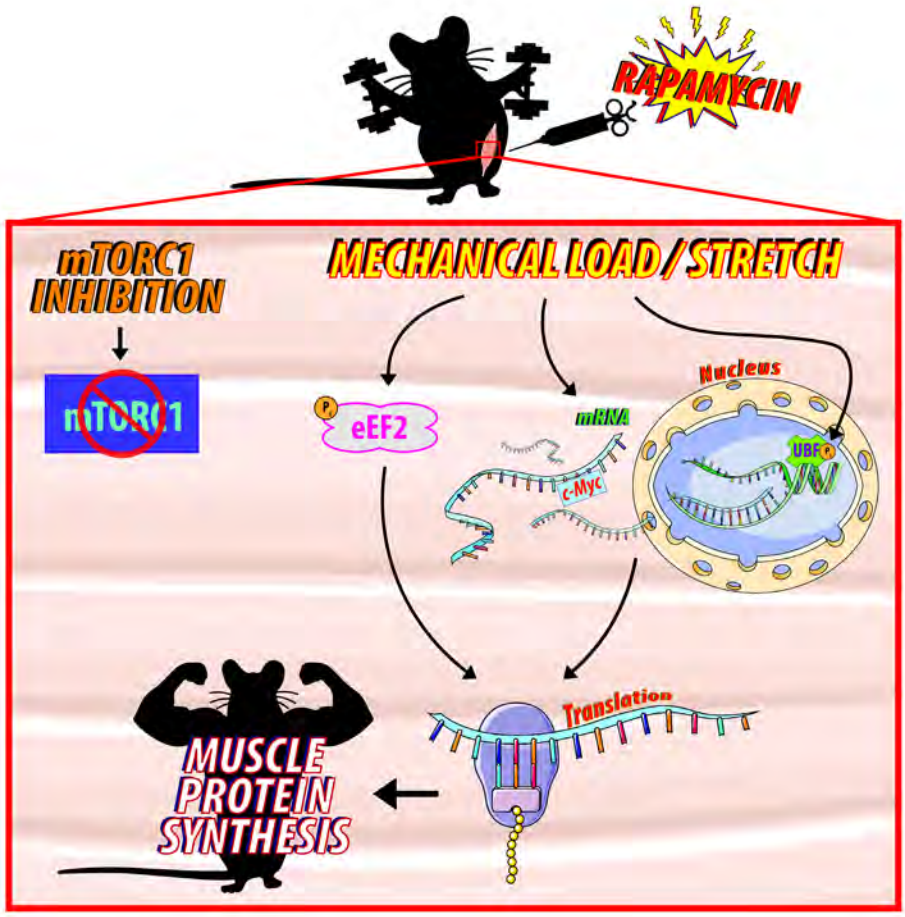

Figure 1.4 Activation of muscle protein synthesis (MPS) through mechanistic/mammalian target of rapamycin complex 1 (mTORC1)-independent pathways in skeletal muscle. Several mTORC1-independent mechanisms, such as activation of translation elongation family member, eukaryotic elongation factor 2 (eEF2) and regulators of ribosome biogenesis (e.g. upstream binding factor (UBF) and c-Myc) can also stimulate MPS following mechanical loading in skeletal muscle (West *et al.*, 2016). Arrows denote signalling event. P_i denotes phosphorylation.

1.2.2 Neuromuscular basis of resistance exercise training adaptations

While it is well recognised that lean body mass (LBM) and skeletal muscle CSA positively correlate with maximal strength (Maughan *et al.*, 1983), strength is not predictive of muscle CSA (Maughan *et al.*, 1983). This seemingly paradoxical relationship is likely due to the many neural factors that are involved in the development of strength gains in response to resistance exercise training, such as appropriate motor

unit recruitment (Sale, 1987, 1988) and movement specificity (Dankel *et al.*, 2017). Based on Henneman's size principle of motor unit recruitment, motor neurons, and the myofibres they innervate, are sequentially recruited according to size and the amount of force generation required (Henneman *et al.*, 1965). Thus, performing an isometric contraction at a submaximal intensity (e.g., 20% of MVC) will recruit less muscle than a near maximal contraction (e.g., 80% of MVC) (Alkner *et al.*, 2000). However, when performing numerous repetitive submaximal contractions to fatigue, such as those performed over multiple sets of moderate intensity resistance exercise, additional larger, higher-threshold motor units are recruited to support force production (Fuglevand *et al.*, 1993; Conwit *et al.*, 2000). In this manner, near maximal motor unit recruitment can be achieved when using submaximal loads (Fallentin *et al.*, 1993).

Although training to volitional failure with lower percentages of 1RM loads may incrementally recruit additional motor units and elicit similar degrees of muscle hypertrophy as higher percentages of 1RM, superior improvements in strength and rate of torque development have been observed when training with higher percentages of 1RM (Mitchell *et al.*, 2012; Ogasawara *et al.*, 2013; Schoenfeld *et al.*, 2015; Jenkins *et al.*, 2015, 2016*b*, 2016*a*). Furthermore, equivalent changes in 1RM strength and surface electromyogram (EMG) amplitude have been observed following eight wk of low volume, high load (~1RM) compared to high volume, moderate load (~8-12RM) resistance training (Mattocks *et al.*, 2017). These changes occurred in the absence of muscle hypertrophy, indicating that peak motor unit recruitment and training-induced changes in muscle size alone are not required for increases in muscle strength. However, the rate of torque development appears to correlate with and explain up to ~80% of the variance in MVC (Andersen & Aagaard, 2006), suggesting that

adaptations to time-dependent contractile properties may effect maximal strength output more than changes in muscle CSA.

In summary, resistance-based exercise activates mTORC1-dependent and independent signalling pathways that increase rates of MPS. When resistance exercise training is repeated over weeks/months, muscle hypertrophy occurs as a result of a net-positive balance between rates of MPS and MPB, which is largely attributable to the accrual of myofibrillar proteins within the sarcomere. Manipulating training variables such as total work volume (Terzis *et al.*, 2010; Burd *et al.*, 2010*b*, 2010*a*) and between-set rest interval (McKendry *et al.*, 2016) alters the magnitude of downstream mTORC1 signalling, which may promote hypertrophic responses. Additionally, resistance training promotes strength gains, which rely not only on muscle hypertrophy, but also neural adaptations that coordinate the rate of motor unit recruitment to generate force. Thus, while repeated contractions against submaximal loads can develop substantial strength, training with loads closer to an individual's 1RM appears to produce greater strength gains and may be necessary to maximise neural adaptations. Therefore, when designing resistance exercise training programs, training variables such as intensity, volume, time under tension, and inter-set rest interval must be strategically, and specifically, manipulated to maximise muscle strength and hypertrophy.

1.3 Adaptations to endurance exercise training

Endurance exercise is typically characterised by prolonged, continuous or repeated bouts of submaximal contractions that present significant cellular perturbations within the working muscle, with the overall aim of improving substrate delivery, economy of motion, oxygen uptake (VO_2) and lactate threshold (Holloszy & Booth, 1976; Holloszy & Coyle, 1984). These adaptations are primarily a product of a

preferential post-exercise increase in mitochondrial protein synthesis (Wilkinson *et al.*, 2008), and appear to be regulated, in large part, by the transcriptional coactivator, peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) (Wu *et al.*, 1999). Immediately after endurance exercise, PGC-1α is activated and binds to nuclear respiratory factor 1 (NRF-1) and 2 (NRF-2) to facilitate mitochondrial biogenesis (Baar *et al.*, 2002; Pilegaard *et al.*, 2003), and further stimulate its own transcription through an autoregulatory loop (Handschin *et al.*, 2003).

1.3.1 Molecular basis of endurance exercise training adaptations

PGC-1α exists in several isoforms, which are transcriptionally regulated through multiple tissue-specific promoter regions (Chinsomboon *et al.*, 2009). Whereas the canonical promoter is ubiquitously active and gives rise to PGC-1α1, a distinct alternative promoter in skeletal muscle gives rise to PGC-1α2 and is largely activated after endurance exercise in rodents (Baar *et al.*, 2002; Chinsomboon *et al.*, 2009) and humans (Norrbom *et al.*, 2011). Briefly, the alternative promoter consists of a 3',5'-cyclic adenosine monophosphate (cAMP) response element (CRE), two myocyte enhancing factor 2 (MEF2) binding sites (Akimoto *et al.*, 2004), and an enhancer box (Ebox) (Irrcher *et al.*, 2009), which allows for transcriptional control of PGC-1α2 by a number of exercise-induced stimuli including energy stress, calcium, and reactive oxygen species (ROS) (Baar, 2014*b*) (**Figure 1.5**).

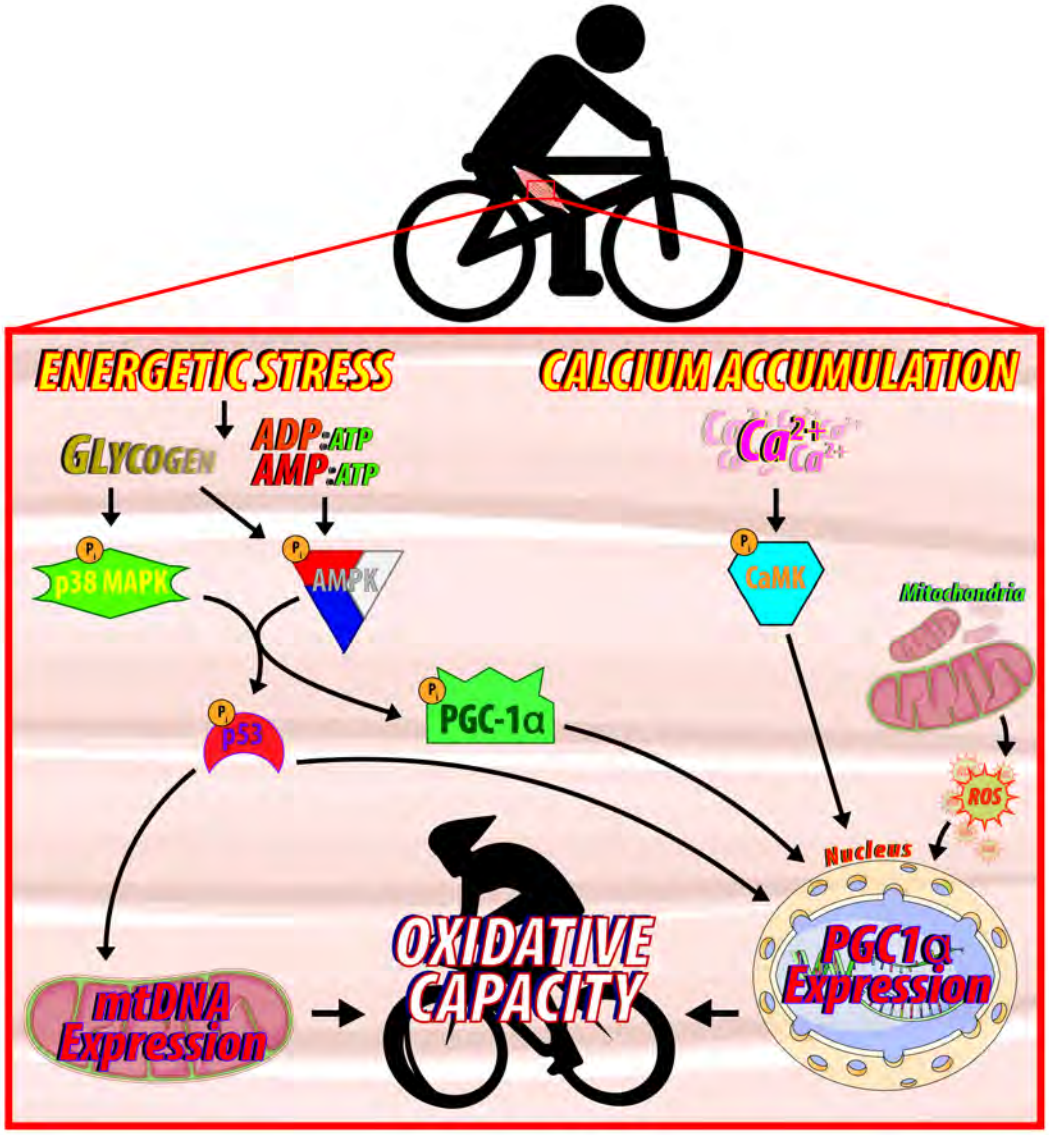
ENERGETIC STRESS
CALCIUM ACCUMULATION
GLYCOGEN
ADP:ATP
AMP:ATP
Ca2+
p38 MAPK
AMPK
CaMK
Mitochondria
p53
PGC-1α
ROS
Nucleus
OXIDATIVE CAPACITY
mtDNA Expression
PGC1α Expression

Figure 1.5 (p. 14) Endurance exercise-induced signalling cascades leading to enhanced oxidative capacity in skeletal muscle. During endurance exercise, the high frequency of muscular contractions results in fluxes in intracellular calcium ion (Ca^{2+}) concentrations, which activates Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII). When activated, CaMKII signalling activates MEF2 and CRE to promote proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) 2 (PGC-1α2) gene expression and subsequently mitochondrial biogenesis (Wu *et al.*, 2002). The accumulation of reactive oxygen species (ROS) produced by mitochondria and other metabolic enzymes during endurance exercise can also increase PGC-1α2 gene expression (Irrcher *et al.*, 2009). As adenosine triphosphate (ATP) is broken down to facilitate the metabolic demands of endurance exercise, a decrease in the energy charge of the cell (ADP/AMP:ATP) activates the 5' adenosine monophosphate-activated protein kinase (AMPK) (Oakhill *et al.*, 2011). When activated, AMPK directly phosphorylates PGC-1α thereby increasing PGC-1α-dependent induction of the PGC-1α promoter (Jäger *et al.*, 2007). As the intensity of endurance exercise increases, glycogen utilisation increases, which stimulates further PGC-1α phosphorylation through AMPK (Philp *et al.*, 2013) and p38 mitogen-activated protein kinase (p38 MAPK) (Puigserver *et al.*, 2001). The tumour suppressor protein p53 also regulates mitochondrial function and biogenesis by increasing mitochondrial transcription factor A (Tfam) mRNA and protein expression as well as mitochondrial DNA (mtDNA) content independently of PGC-1α (Park *et al.*, 2009). Arrows denote signalling event. P_i denotes phosphorylation.

In skeletal muscle, Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) is rapidly activated by fluxes in intracellular calcium ion (Ca^{2+}) concentrations in an intensity-dependent manner and this activation is largely sustained during continuous exercise (Rose *et al.*, 2006). When activated, CaMKII signalling activates MEF2 and CRE to promote PGC-1α2 gene expression and mitochondrial biogenesis (Wu *et al.*, 2002). In addition, ROS accumulation during endurance exercise stimulates binding of the transcription factor upstream stimulatory factor 1 (USF-1) to the Ebox site within the alternate promoter to increase PGC-1α2 gene expression (Irrcher *et al.*, 2009).

Posttranslational regulation of PGC-1α2 activity is modulated by phosphorylation and acetylation, through interactions with 5' adenosine monophosphate-activated protein kinase (AMPK) (Jäger *et al.*, 2007), p38 mitogen-activated protein kinase (p38 MAPK) (Puigserver *et al.*, 2001), and acetyltransferases Endurance exercise also reduces the interaction between PGC-1α and the histone acetyltransferase GCN5, resulting in deacetylation of PGC-1α further promoting mitochondrial biogenesis (Philp *et al.*, 2011).

Previous reports indicate that $p53^{Ser15}$ is a downstream substrate of both AMPK (Jones *et al.*, 2005) and p38 (She *et al.*, 2001). Acute muscle contractions in mice mimicking endurance exercise stimulates p53 phosphorylation in concert with increased activation of AMPK and p38 (Saleem *et al.*, 2009). Following endurance exercise in p53 knockout (KO) mice, mitochondrial transcription factor A (Tfam) mRNA and protein expression as well as mitochondrial DNA (mtDNA) content was significantly lower compared to exercised wild type (WT) mice (Park *et al.*, 2009). Notably, the KO mice displayed no difference in the expression of PGC-1α, NRF-1, or NRF-2 compared to WT mice (Park *et al.*, 2009), suggesting the actions of p53 on mitochondrial biogenesis are independent of PGC-1α, and rely on modulating Tfam to

regulate mtDNA transcription. In turn, the increased transcription of mtDNA-encoded proteins may assist in coordinating structural changes, such as those arising during fusion and fission, which alter mitochondrial morphology and quality (Youle & van der Bliek, 2012). However, KO of p53 does not inhibit mitochondrial biogenesis following ~6-8 wk of voluntary wheel running in mice (Saleem *et al.*, 2009), indicating that p53 signalling likely works in parallel with other mechanisms.

Endurance exercise-induced mitochondrial biogenesis is affected by the intensity of exercise (Burgomaster *et al.*, 2005, 2008; Gibala *et al.*, 2006; Egan *et al.*, 2010), as well as nutrient availability during exercise (Psilander *et al.*, 2012; Lane *et al.*, 2015). While performing moderate-intensity, continuous training (MICT) results in improvements in oxidative capacity (Holloszy & Booth, 1976; Holloszy & Coyle, 1984), high-intensity interval training (HIIT) appears to result in similar adaptations as MICT when the same (Bartlett *et al.*, 2012; Fyfe *et al.*, 2016*a*) or less (Gibala *et al.*, 2006; Burgomaster *et al.*, 2008) absolute volume of work is performed. Furthermore, performing six sessions of HIIT over 2 wk elicits a greater increase in mitochondrial content compared to MICT when work matched (MacInnis *et al.*, 2016). As the molecular pathways associated with aerobic adaptations are sensitive to energy stress, exercising at higher intensities induces large metabolic perturbations that activate signalling cascades that converge on PGC-1α to facilitate mitochondrial biogenesis (Fiorenza *et al.*, 2018).

In summary, endurance exercise improves oxidative capacity through enhanced mitochondrial biogenesis. At the molecular level, a number of cellular pathways exist which converge on PGC-1α and p53 to promote mitochondrial biogenesis. Given that mitochondrial associated pathways are sensitive to cellular energy stress, manipulating the intensity of endurance exercise can influence the degree to which these pathways

are activated, along with the magnitude of subsequent gene expression. Accordingly, when designing endurance training programs, factors such as intensity and duration of training sessions need to be taken into consideration to ensure a sufficient metabolic stress is introduced to maximally stimulate mitochondrial biogenesis.

1.4 Adaptations to concurrent exercise training

In 1980, Dr. Robert Hickson examined the specificity of training adaptations to concurrent training versus isolated resistance and endurance training. After 10 wk of concurrent, heavy resistance and high-intensity endurance training, improvements in 1RM strength were attenuated in individuals undertaking concurrent training versus those undertaking only resistance training (Hickson, 1980). From these findings, It was concluded that endurance exercise undertaken in the same training regimen as resistance exercise 'interferes' with maximal gains in strength compared to resistance training alone (Hickson, 1980). This phenomenon has since been termed the 'interference effect' of concurrent training and has been a topic of considerable debate. While the disparate findings mentioned previously may be attributed to divergent variables between studies such as the volume, frequency, and intensity of training, as well as the duration of interventions, training status of participants, and modes of exercise being employed (Coffey & Hawley, 2017), the underlying mechanism(s) of the 'interference effect' have, to date, remained elusive.

1.4.1 The 'molecular interference' theory

It has recently been hypothesised that the interference effect is a product of antagonistic molecular signalling events that manifest from divergent exercise stimuli (Nader, 2006; Hawley, 2009; Hamilton & Philp, 2013; Baar, 2014*a*; Perez-Schindler *et al.*, 2015; Coffey & Hawley, 2017). The basis for this theory revolves around the interaction between two key intracellular signalling mediators: mTORC1 and AMPK (**Figure 1.6**).

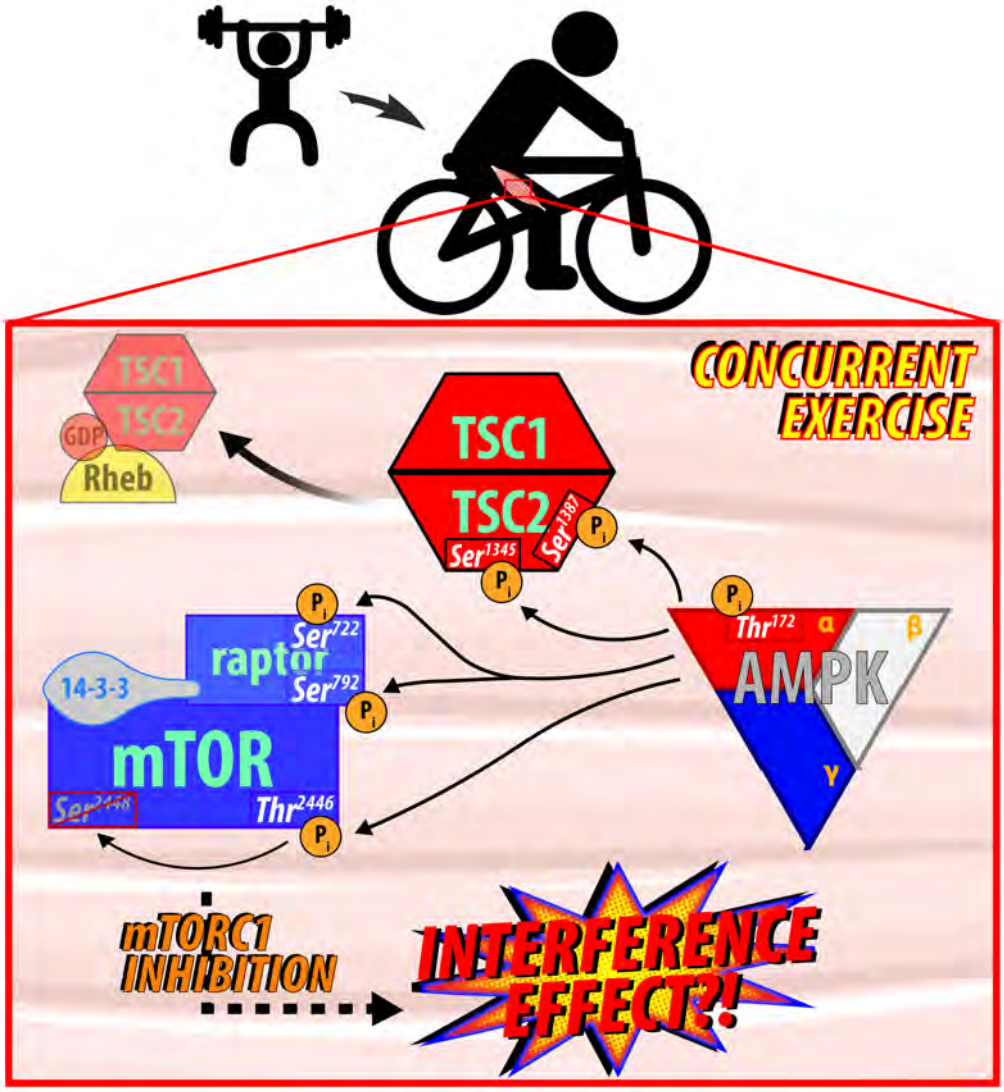

Figure 1.6 Proposed mechanism by which the activity of mechanistic/mammalian target of rapamycin complex 1 (mTORC1) is suppressed by 5' adenosine monophosphate-activated protein kinase (AMPK) and tuberous sclerosis complex 1/2 (TSC1/2), resulting in the 'interference effect'. In response to energy stress or hypoxia, AMPK phosphorylates tuberous sclerosis complex 2 (TSC2) and enhances its GTPase-activating protein (GAP) activity upon Rheb, causing mTORC1 inhibition (Inoki *et al.*, 2003*b*). Additionally, AMPK is able to directly phosphorylate raptor^{Ser722} and raptor^{Ser792} residues, which induces 14-3-3 protein binding and allosteric inhibition of mTORC1 (Gwinn *et al.*, 2008). Consequently, AMPK activation suppresses translational initiation and muscle protein synthesis (MPS) (Bolster *et al.*, 2002). Accordingly, phosphorylation of AMPK^{Thr172} is negatively correlated with overload-induced myofibre hypertrophy (Thomson & Gordon, 2005) and may underlie the 'interference effect'. Arrows denote signalling event. P_i denotes phosphorylation.

To identify the specific signalling events induced by resistance and endurance training, Atherton and colleagues (2005) electrically stimulated excised rat muscles at high (60 × 3 s, 100 Hz) or low frequencies (3 h, 10 Hz) to mimic resistance and endurance exercise, respectively. The results demonstrated that stimulations resembling resistance exercise exclusively induced activation of the protein kinase B (PKB)-TSC2-mTOR signalling cascade and its downstream translational regulators. Conversely, stimulations mimicking endurance exercise selectively activated AMPK-PGC-1α signalling and suppressed downstream regulators of translation initiation and elongation. Based on these observations, the authors proposed the existence of an 'AMPK-PKB switch' that acts as a mechanism to mediate the specificity of training adaptations (Atherton *et al.*, 2005). Indeed, *in vivo* treatment with the AMPK activator 5-aminoimidazole-4-carboxamide-1-4-ribofuranoside (AICAR) prior to resistance exercise attenuates downstream mTORC1 signalling to p70S6K1, 4E-BP1, and eEF2 in rodent muscle (Thomson *et al.*, 2008), suggesting simultaneous activation of the AMPK and mTORC1 signalling cascades is incompatible.

Since the phosphorylation of mTORC1 substrates following resistance exercise is associated with skeletal muscle hypertrophy (Baar & Esser, 1999; Terzis *et al.*, 2008) and AMPK is activated in response to endurance exercise (Winder & Hardie, 1996), it seems plausible to suggest that the reductions in hypertrophy, and associated strength adaptations observed after concurrent training may be explained through a mechanism involving interactions between mTORC1 and AMPK. However, attenuations in translation initiation signalling (using p70S6K1^{Thr389} as a proxy) have been observed when high-intensity cycling is performed either prior to, or following resistance exercise (Coffey *et al.*, 2009*a*) despite no differences in AMPK^{Thr172}, TSC2^{Thr1462}, and mTOR^{Ser2448} phosphorylation. While AMPK^{Thr172} phosphorylation is correlated to

AMPKα2 catalytic subunit activity (Park *et al.*, 2002), it does not appear to be an accurate surrogate for AMPK kinase activity (Chen *et al.*, 2003). Additionally, $TSC2^{Thr1462}$ is a downstream target of Akt (Manning *et al.*, 2002) whereas $TSC2^{Ser1345}$ and $TSC2^{Ser1387}$ are AMPK phosphorylation motifs (Inoki *et al.*, 2003*b*). Furthermore, AMPK can also directly phosphorylate $mTOR^{Thr2446}$ preventing Akt/PKB phosphorylation of $mTOR^{Ser2448}$ (Cheng *et al.*, 2004). Given the similar phosphorylation pattern of mTORC1 and AMPK regardless of contraction mode (Coffey *et al.*, 2009*a*), these observations suggest that acute signalling responses, alone, can not fully explain the 'interference effect'.

In contrast to the observations of Coffey and colleagues (2009*a*), resistance exercise alone has been shown to simultaneously induces both AMPKα2 activity and $mTOR^{Ser2448}$ phosphorylation, without inhibiting MPS 1 h post-exercise (Dreyer *et al.*, 2006). Similarly, a single bout of high-intensity cycling results in phosphorylation of $mTOR^{Ser2448}$ and downstream signalling to regulators of translation initiation, p70S6K1 and 4E-BP1, and elongation, eEF2, despite $TSC2^{Ser1387}$ phosphorylation and an absence of Akt^{Ser473} phosphorylation (Apró *et al.*, 2015). When the cycling was followed immediately by resistance exercise, no inhibitory effects of AMPKα2 activity were observed on mTORC1 signalling, p70S6K1 kinase activity, or MPS compared to resistance exercise performed in isolation (Apró *et al.*, 2015). Thus, it appears that exercise-induced AMPK activity alone does not prohibit activation of mTORC1, suggesting that other molecular signals contribute to the inhibition of anabolic signalling following concurrent exercise. While the underlying molecular pathways mediating the specificity of training adaptations are undoubtedly complex, further work is required to evaluate a wider scope of potential molecular mechanisms involved in the 'interference effect'.

1.4.2 The 'acute fatigue' hypothesis

An alternative hypothesis to explain the 'interference effect' focuses on residual muscular fatigue from endurance exercise performed prior to resistance exercise, referred to as the 'acute fatigue hypothesis' (Craig *et al.*, 1991). Due to residual fatigue from endurance exercise, the amount and intensity of work that can be performed in a subsequent resistance exercise session is decreased, resulting in compromised strength adaptations (Craig *et al.*, 1991; Leveritt & Abernethy, 1999) (**Figure 1.7**).

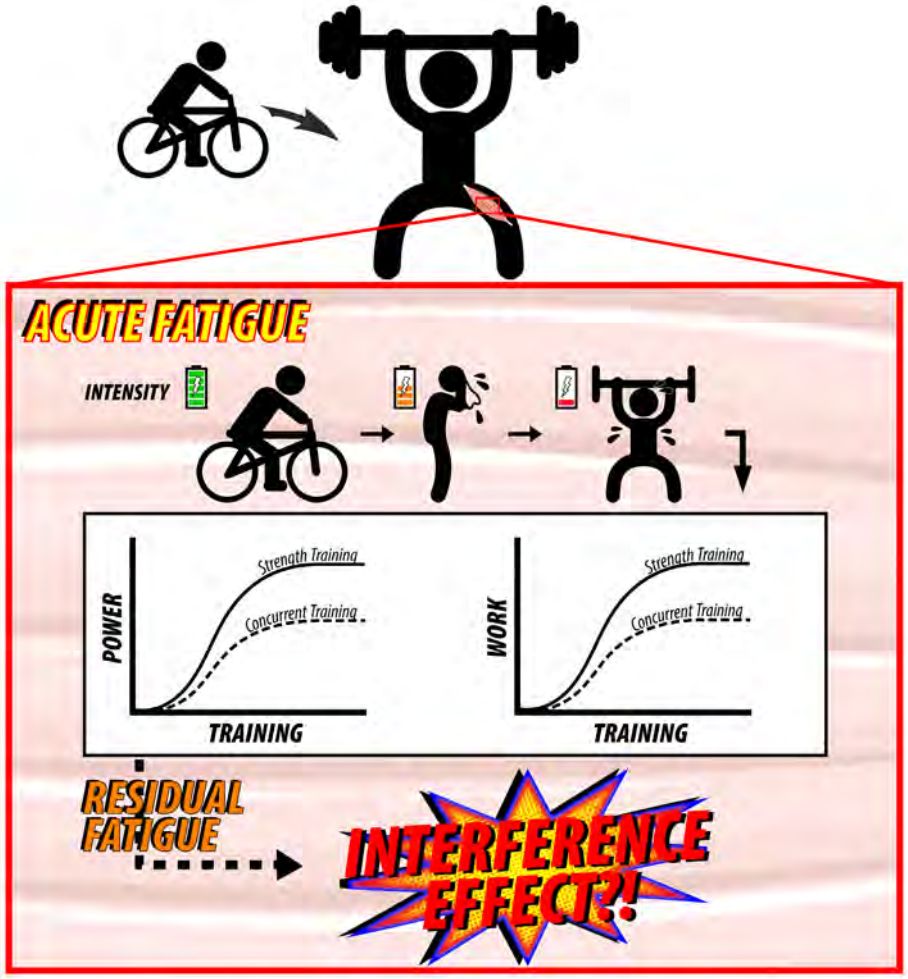

Figure 1.7 Proposed mechanism by which residual fatigue accumulated during concurrent training causes resistance training to be performed at suboptimal power and work levels, resulting in the 'interference effect'.

Previous work has demonstrated that 10 wk of concurrent training results in similar increases in lean body mass (LBM), but not strength, as resistance training alone (Craig *et al.*, 1991). Despite observing similar increases in LBM, the authors proposed that residual fatigue from the additional volume of work performed during the endurance component of concurrent training may compromise the work completed during strength training, resulting in less stimulus to the working muscles and reduced strength gains. Likewise, residual fatigue also appears to compromise peak power output following concurrent training. Using a five wk unilateral training program, Lundberg and colleagues (2014) demonstrated that endurance exercise immediately preceding a bout of resistance exercise compromises peak power. In this model, one leg completed 45 min of single-legged cycling, followed by 15 min of rest and a bout of resistance exercise comprising four sets of seven maximal knee extensions. The contralateral leg was subjected only to an identical bout of resistance exercise. In the leg that completed the preceding bout of endurance cycling, peak power during resistance exercise was ~20% lower (Lundberg *et al.*, 2014). Despite the leg that undertook concurrent training exhibiting greater changes in magnetic resonance imaging-derived muscle volume and similar increases in MVC compared to the resistance training only leg, peak concentric torque was compromised (Lundberg *et al.*, 2014).

These findings suggest that training at lower power outputs may affect adaptations to peak power production. In this regard, a ~40 min bout of endurance exercise reduces the total work performed during a subsequent bout of resistance exercise for up to 8 h (Sporer & Wenger, 2003). However, isokinetic, isometric, and isotonic strength does not appear to be affected with recovery periods greater than 8 h (Leveritt *et al.*, 2000).

Further investigation into the duration of recovery required between concurrent training sessions required to maximise adaptations over seven wk of training suggests that a minimum of 6 h is needed to achieve similar gains in strength as resistance training alone, but up to 24 h is required to achieve similar responses in torque production (Robineau *et al.*, 2016). Notably, torque production after severely damaging muscle contractions appears to be diminished for up to 96 h post-exercise, despite measures of strength recovering by 48 h (Gibala *et al.*, 1995). Moreover, myofibrillar disruption to the Z-disc is present for up to 48 h after contraction-induced muscle damage (Gibala *et al.*, 1995). The authors also observed signs of fibre regeneration associated with areas of myofibrillar damage (Gibala *et al.*, 1995), including 'satellite-like' cells - myogenic precursor cells which assist in myofibre regeneration (Brack & Rando, 2012).

1.5 The contribution of satellite sells to skeletal muscle hypertrophy

The regenerative capacity of skeletal muscle is dependent on an undifferentiated niche of myogenic specific precursor cells, referred to as satellite cells. In adult skeletal muscle, satellite cells exist in a quiescent state and are located between the sarcolemma and basal lamina (Mauro, 1961). Classically, they are activated in response to muscle damage, such as mechanical stress caused by exercise (Crameri *et al.*, 2004; Dreyer *et al.*, 2006; Babcock *et al.*, 2012; Joanisse *et al.*, 2013; Snijders *et al.*, 2014*a*; Farup *et al.*, 2014*a*). Once activated, satellite cells proliferate and differentiate in order to contribute to the repair of existing muscle fibres through the formation of new myonuclei, a process known as myogenesis (Blaauw & Reggiani, 2014). In turn, the addition of new myonuclei increases the transcriptional capacity of the fibre to support further hypertrophy (**Figure 1.8**).

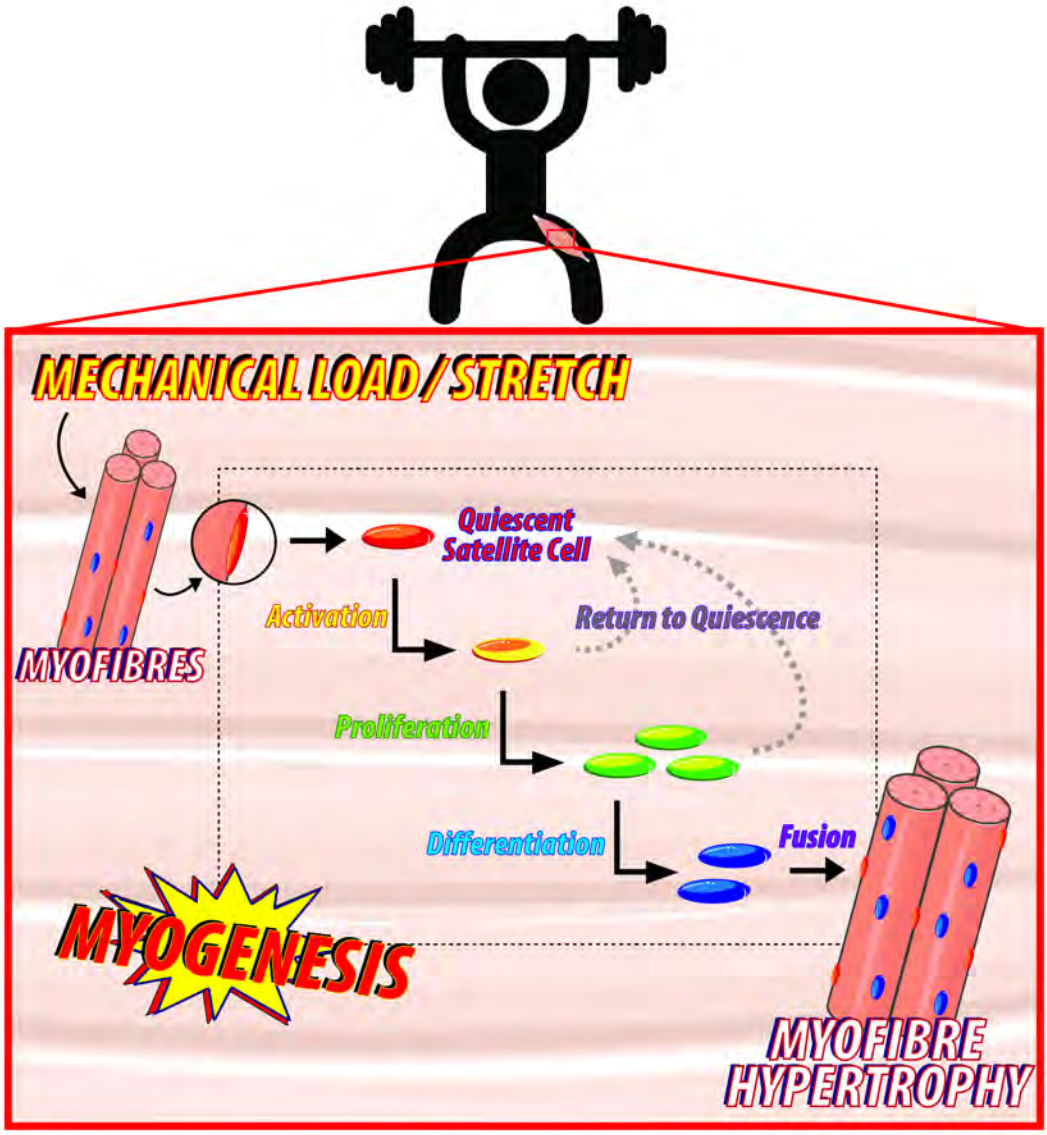

Figure 1.8 Schematic of myogenesis assisted hypertrophy in response to exercise-induced mechanical stress in myofibres. In adult skeletal muscle, satellite cells exist in a quiescent state at rest. Following mechanical stress (such as exercise), satellite cells transition into an active state. Upon activation, satellite cells can either proliferate to increase the contents of the satellite cell pool, or return to quiescence. Following proliferation, satellite cells may continue along the path of myogenic commitment and differentiate into myoblasts that will fuse with an existing myofibre, or return to quiescence to replenish the satellite cell pool. The generation and addition of myonuclei, known as myogenesis, is a process that is believed to help support myofibre hypertrophy in response to exercise.

Evidence for the requirement for satellite cells in supporting overload hypertrophy is equivocal. McCarthy and colleagues (McCarthy *et al.*, 2011) demonstrated that in a novel mouse strain developed to deplete >90% of satellite cells, short-term (two wk) mechanical overload-induced hypertrophy was not blunted compared to wild type mice, suggesting satellite cells are not required for load-induced hypertrophy. In contrast, results from other investigations show that satellite cell depletion effectively attenuates muscle fibre hypertrophy over both short (two wk) (Egner *et al.*, 2016) and long-term (eight wk) (Fry *et al.*, 2014*a*) overload. While the notion that satellite cells are required to facilitate muscle growth responses is a topic of considerable debate (Petrella *et al.*, 2006, 2008; Verdijk *et al.*, 2009, 2014; Bellamy *et al.*, 2014; Dirks *et al.*, 2017; Reidy *et al.*, 2017*b*; McCarthy *et al.*, 2017; Karlsen *et al.*, 2015; Murach *et al.*, 2017), current evidence indicates that the presence and activation of satellite cells are obligatory for supporting training-induced adaptations.

1.5.1 Satellite cell activity in response to exercise

In human skeletal muscle, the activation of satellite cells following resistance exercise is well established (Kadi *et al.*, 2004; Olsen *et al.*, 2006; Verdijk *et al.*, 2007; Petrella *et al.*, 2008; Verdijk *et al.*, 2009; Mackey *et al.*, 2011; Snijders *et al.*, 2012; Babcock *et al.*, 2012; Snijders *et al.*, 2014*b*, 2014*a*; Fry *et al.*, 2014*b*; Bellamy *et al.*, 2014; Farup *et al.*, 2014*a*; Snijders *et al.*, 2016; Nederveen *et al.*, 2017; Reidy *et al.*, 2017*b*). Though less predominant, unaccustomed aerobic training can result in muscle hypertrophy, which is accompanied by increases in both satellite cell and myonuclear content (Fry *et al.*, 2014*b*; McKenzie *et al.*, 2016). Furthermore, recent evidence suggests a contribution of satellite cells to muscle fibre remodelling in the absence of hypertrophy following endurance training (Joanisse *et al.*, 2013, 2015). Notably,

satellite cell proliferation has been shown to be impaired following acute concurrent exercise in young healthy men (Babcock *et al.*, 2012). In turn, it has been proposed that the potential for myofibre hypertrophy in response to chronic concurrent exercise training may be limited by satellite cell content (Babcock *et al.*, 2012).

The activation of satellite cells is influenced by the delivery of growth factors to muscle such as insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), and the myokine interleukin 6 (IL-6) (McKay *et al.*, 2008, 2009; Nederveen *et al.*, 2016, 2017). Changes to the concentrations of circulating cytokines or growth factors can induce satellite cell activation (Conboy *et al.*, 2005; Merritt *et al.*, 2013; Corrick *et al.*, 2015; Rodgers *et al.*, 2017). However, information on the effect of nutrient delivery, specifically amino acids from dietary protein consumption, on satellite cell activation, is lacking. This is surprising considering the numerous studies demonstrating the stimulatory effects of protein ingestion on muscle hypertrophy with exercise (Cermak *et al.*, 2012; Morton *et al.*, 2018) and the purported roles of satellite cells to promote muscle hypertrophy. Given *in vitro* findings showing that leucine availability can promote myocyte proliferation and differentiation (Averous *et al.*, 2012; Chen *et al.*, 2013; Dai *et al.*, 2015; Duan *et al.*, 2017), dietary protein ingestion in conjunction with exercise may provide an additional stimulus to rescue satellite cell proliferation following concurrent exercise and promote myofibre hypertrophy.

1.5.2 The impact of protein ingestion on satellite cell responses to exercise

Dietary protein availability is critical for providing amino acids to facilitate skeletal muscle repair and regeneration during recovery from exercise. Accordingly, adequate protein needs to be consumed to facilitate the synthesis of new proteins during the immediate (2-3 h) post-exercise recovery period, which provides the basis for both resistance and endurance training-induced adaptations in skeletal muscle (Moore *et al.*, 2009*b*; Breen *et al.*, 2011; Camera *et al.*, 2015). Moreover, the addition of new satellite cell-derived nuclei through exercise-induced myonuclear turnover is essential to the continued contribution of genetic information for protein synthesis (Burd & De Lisio, 2017). Several interrelated factors including the dose (Moore *et al.*, 2009*a*), type (Tang *et al.*, 2009), timing (Res *et al.*, 2012) and distribution (Morton *et al.*, 2015; Areta *et al.*, 2013) of protein ingestion directly impact the anabolic effects of post-exercise protein ingestion. An in-depth discussion of these factors is beyond the scope of this review and readers are referred to several comprehensive reviews on this topic (Phillips & van Loon, 2011; Moore *et al.*, 2014; Phillips, 2016).

Recently, Rodgers and colleagues (Rodgers *et al.*, 2014) demonstrated that the leucine sensitive mTORC1 controls the transition of satellite cells between a quiescent and an initial ‘alert’ phase of the cell cycle in mice. This finding is noteworthy as subsequent investigations have demonstrated that mTORC1 signalling is rapidly activated during skeletal muscle regeneration (Jash *et al.*, 2014) and is not only required for the adaptive transition of cell cycle phases, but obligatory for satellite cell proliferation, differentiation, and overall skeletal muscle regeneration (Han *et al.*, 2008; Zhang *et al.*, 2015). Given the ability of leucine to both activate mTORC1 directly (Sancak *et al.*, 2008) and promote proliferation and differentiation *in vitro* through an mTORC1-MyoD cascade (Dai *et al.*, 2015), protein ingestion in conjunction with an

appropriate exercise stimulus may provide an additional signal to promote satellite cell activation *in vivo* (**Figure 1.9**). However, few studies to date have investigated the interaction between protein supplementation and satellite cell activity in human skeletal muscle. Specifically, whether increased protein availability during a chronic concurrent training program can rescue the inhibition of satellite cell activity previously observed after a single bout is unknown. Thus, it seems that factors contributing to the time-course of myofibre repair may deserve further consideration to fully understand the 'interference effect'.

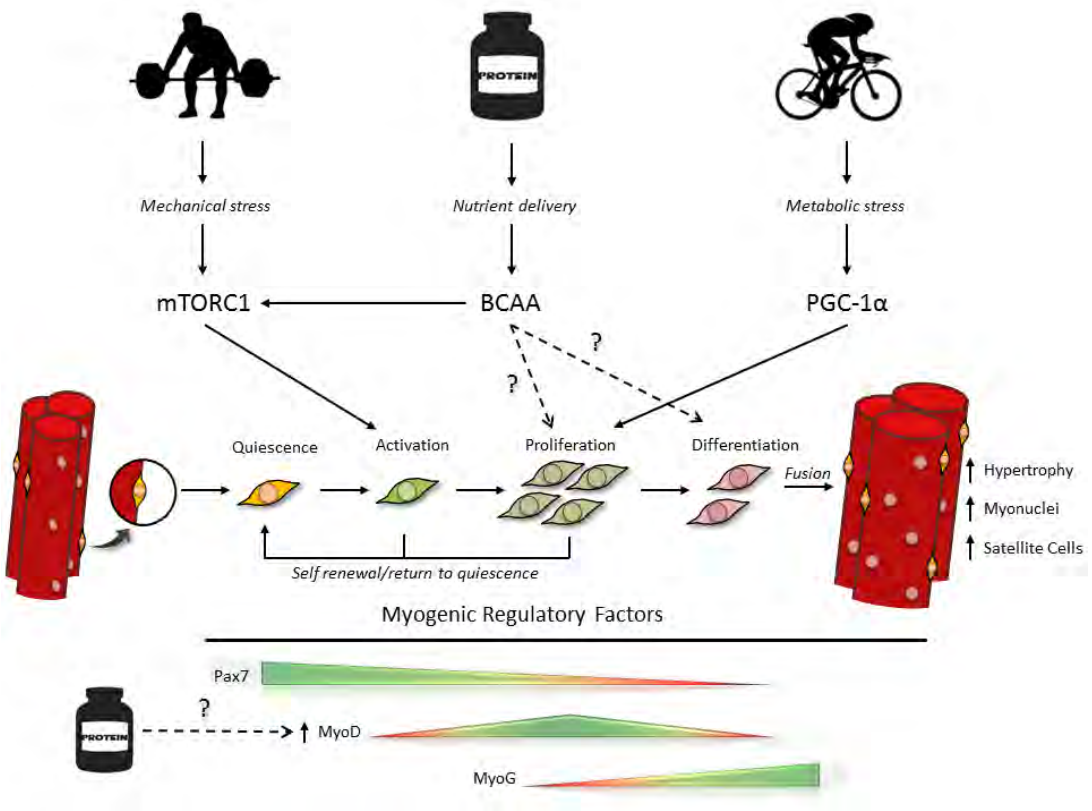

Figure 1.9 Schematic of the potential mechanistic underpinning for satellite cell stimulation by resistance exercise, endurance exercise, and protein ingestion, as well as the expression pattern of associated transcription factors based on evidence presented from *in vitro* and murine models. Following a bout of resistance exercise, mechanical stress activates the mechanistic target of rapamycin complex 1 (mTORC1), which, in turn, promotes the transition of satellite cells from a quiescent state into an active state. Upon activation, satellite cells can either continue along the path of myogenic commitment to proliferate into myoblasts, or return to quiescence and self–renew to maintain the satellite cell pool. Metabolic stress caused by endurance exercise stimulates the activity of the transcriptional coactivator peroxisome proliferator–activated receptor–γ coactivator 1α (PGC–1α), which can promote the proliferation of satellite cells. Protein/branch chained amino acid (BCAA) supplementation may enhance both proliferation and differentiation of satellite cells. Though the mechanisms are not fully understood, potential pathways of satellite cell modulation through protein/BCAA supplementation have been included as dashed arrows. Myogenic regulatory factor expressions are present in higher levels (green) through specific stages and become suppressed (red) as the myogenic process advances as depicted by the shift from green to red in representative expression bars. Solid black arrows indicate increases/activation of downstream target proteins/ processes.

1.5.3 A multi-perspective approach to prevent the 'interference effect'

Given the complexity of the mechanisms contributing to myofibre repair and underlying adaptations to both endurance and resistance exercise training, it seems appropriate that a multi-perspective approach is taken in an attempt to avoid the 'interference effect'. Theoretical, literature-based practical recommendations to prevent or reduce the interference to resistance-based adaptations have been formulated around several concurrent training variables (Murach & Bagley, 2016) in order to minimise fatigue and any potential antagonistic cell signalling that may influence the underlying mechanistic basis for such an effect (Baar, 2014*a*). However, exploration of a broader range of cell signalling pathways through next-generation sequencing technology may reveal previously undetected gene expression networks and molecular transducers involved in the 'interference effect'.

1.6 –Omics-based approaches for profiling skeletal muscle responses to exercise

Advances in biotechnology and computational biology have led to a new era of integrative, high-throughput molecular biology. Global approaches of measuring families of cellular molecules, such as nucleic acids, proteins, and intermediary metabolites, have been termed '-omic' technologies. With these constantly evolving –omics-based tools, the ability to characterise and assess most, if not all, of the members of a family of molecules or biochemical pathways in a single analysis has resulted in a dramatic increase in knowledge about the molecular responses and cross-talk that occur following a given stimulus. Through advanced computational methods, known as bioinformatics, large-scale biological data sets can be used to generate 'molecular maps' of complex physiological responses by assimilating known molecular interaction networks from *in vivo* and *in vitro* data to create predictive *in silico* models. This pursuit

to provide comprehensive computational models of biological systems has led to the establishment of multiple international consortia providing publicly available –omics data repositories and annotations of functional classification schemes, allowing direct, comparable descriptions of homologous traits across phylogenic classes.

Since mapping the euchromatic sequence of the human genome (International Human Genome Sequencing Consortium, 2004), several technological innovations in high-throughput nucleic acid sequencing have rapidly increased the rate and accessibility of sequencing workflows. Next-generation sequencing (NGS) technology has prevailed as a massively paralleled 'sequencing-by-synthesis' approach. Early 'sequencing-after-synthesis' technology required fragmentation of DNA segments that were separately sequenced and assembled thereafter. For example, Sanger sequencing required selective incorporation of 2',3' dideoxynucleotides chain-terminating inhibitors of DNA polymerase during replication to create DNA fragments before sequencing (Sanger *et al.*, 1977). Similarly, whole genome shotgun sequencing required DNA to be broken randomly into numerous smaller DNA fragments, then sequencing these small portions of the genome and overlapping ends of target DNA segments to assemble a continuous sequence *a posteriori* (Staden, 1979). In contrast to 'sequencing-after-synthesis' technology, NGS allows the automated sequencing of multiple fragments of the entire genome simultaneously (Margulies *et al.*, 2005). Briefly, NGS entails monitoring real-time *de novo* DNA biosynthesis whereby a DNA-template, bound to a 'barcoded' adaptor bead is loaded into a microwell on a microfabricated reactor flow cell ('chip'). Next, sequential and repetitive emulsions of the four nucleotide bases, adenine, guanine, cytosine, and thymine are run over the chip, and DNA complementary to template strands are synthesised by polymerase chain reaction. As polymerisation occurs, a detector senses either light-emitting reactions in

the presence of fluorescently labelled nucleotides, or changes in pH by the release of hydrogen ions during the formation of covalent bonds as a deoxyribonucleoside triphosphate (dNTP) is incorporated into the DNA strand.

Recently, emphasis has shifted to better understanding the functional genome and considerable focus has been directed towards gene products, specifically ribonucleic acid (RNA) species. Prior to NGS, wide-scale gene expression studies relied on hybridisation-based microarrays that required an *a priori* knowledge of predetermined sequences. Transcriptomics, the analysis of RNA transcripts, has turned to NGS-based methods, such as RNA sequencing (RNA-Seq), to interrogate sequences with a non-biased approach. This gives researchers the ability to record alternative splicing events, post-transcriptional modifications, and non-coding transcripts. As RNA-Seq is based on NGS, it requires the reverse transcription of RNA to complementary DNA (cDNA) prior to sequencing. The arising sequence features can then be aligned to the sequence of a reference genome to reconstruct which genomic regions were being transcribed and annotate which genes are being expressed. In this manner, measuring genome-wide gene expression (i.e., mRNA) is possible and has become a popular tool in characterizing skeletal muscle responses to exercise (Timmons *et al.*, 2006, 2010; Keller *et al.*, 2010; Timmons, 2011; Rowlands *et al.*, 2011; Phillips *et al.*, 2013; Thalacker-Mercer *et al.*, 2013; Ghosh *et al.*, 2013; Lundberg *et al.*, 2016; Robinson *et al.*, 2017; Laker *et al.*, 2017; Timmons *et al.*, 2018*a*; Damas *et al.*, 2018*b*; Popov *et al.*, 2019; Agudelo *et al.*, 2019). An example workflow for implementing RNA-Seq technology into a clinical exercise investigation is shown in **Figure 1.10**.

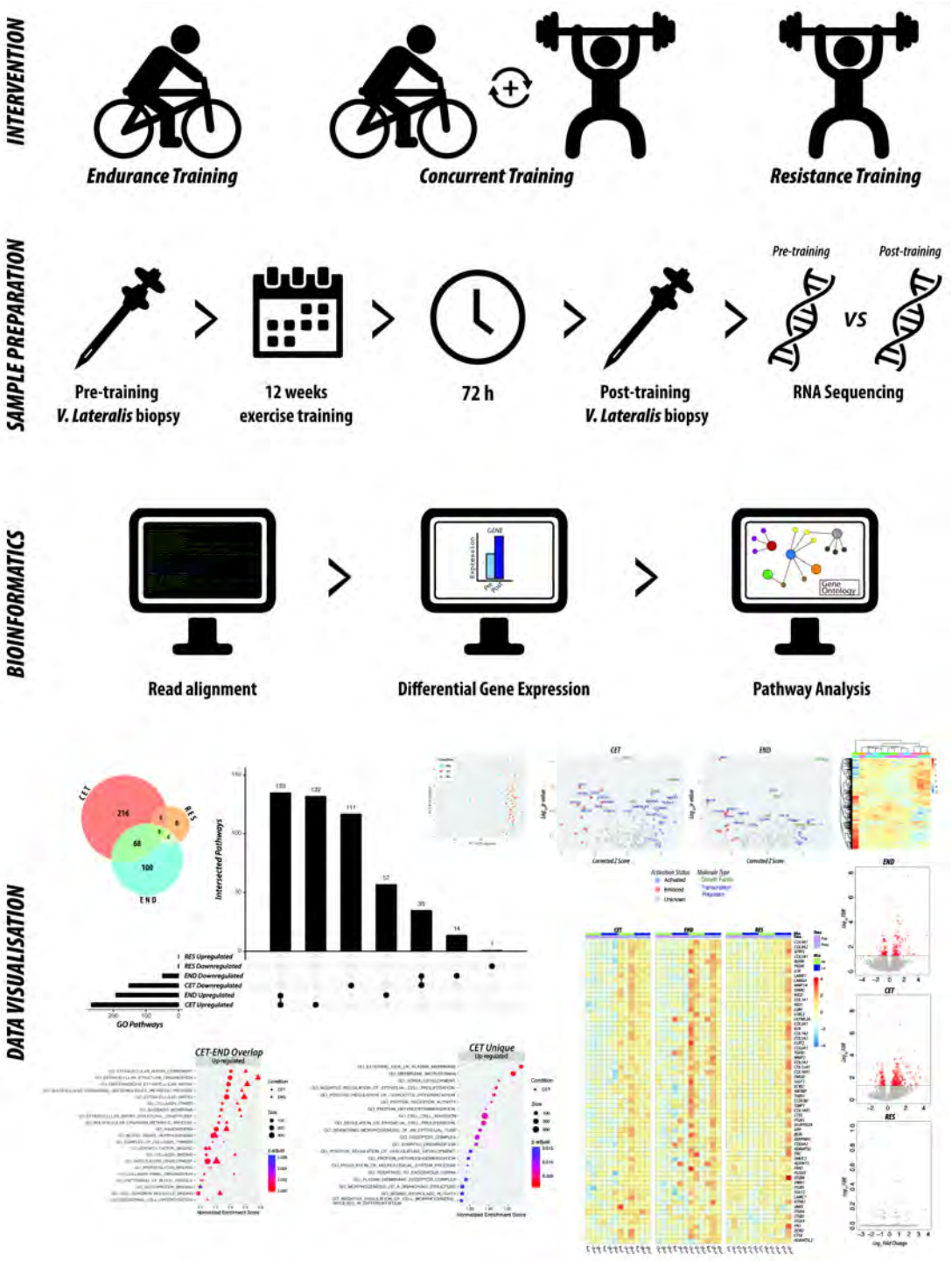

Figure 1.10 Workflow of study employing RNA sequencing technology.

Any observable phenotype is the final product of genes interacting with environmental/epigenetic stimuli that give rise to intermediate RNA species, which subsequently instigates an alteration to the level, localization, and activity of proteins. On this basis, phosphoproteomics – the quantitative study of global protein phosphorylation – has recently been employed to provide a direct representation of the signalling events mediating exercise adaptations (Hoffman *et al.*, 2015; Potts *et al.*, 2017). Technological demands and complexity aside, proteomics is not without limitations and interpretation of the results requires the careful consideration of several caveats. First, turnover of many cellular proteins occur in less than two days, and a large proportion of the proteome has half-lives relatively close to this timeframe (Cambridge *et al.*, 2011). Moreover, proteins with known or predicted phosphorylation sites have significantly higher rates of turnover compared to proteins without such sites (Cambridge *et al.*, 2011). Likewise, only a small amount of kinase phosphorylation is needed to stimulate protein synthesis (Crozier *et al.*, 2005). Thus, in the absence of direct evidence for physiological adaptations to repeated bouts of training, interpreting the degree to which acute proteome-wide phosphorylation events align with exercise training-induced phenotypes is limited (Hoffman *et al.*, 2015; Potts *et al.*, 2017).

Given observations that divergent exercise modes can produce similar AMPK and mTORC1 signalling patterns (Dreyer *et al.*, 2006; Camera *et al.*, 2010; Apró *et al.*, 2015), assigning their seemingly equivocal, acute activity to discrete phenotypes is likely to be too simplistic of an approach to appreciate training adaptations. Recent evidence demonstrates that altered transcription levels of genes associated with mitochondrial (Egan *et al.*, 2013), myogenic (Andersen & Gruschy-Knudsen, 2018), and structural (Karlsen *et al.*, 2019) proteins can be detected up to 96 h post-exercise. Despite poor correlations between steady-state protein and mRNA levels, a high

correlation exists between changes in transcript levels and protein in response to a stimulus, albeit with a proportionality compressed response at the level of the proteome compared to transcriptome (Bonaldi *et al.*, 2008). Thus, examination of the transcriptome may reveal previously overlooked clues about latent, functional biological processes that are more reflective of the phenotypes associated with structural and metabolic alterations accompanying training disciplines. For this reason, it has been suggested that examining contraction-induced gene expression networks may identify unique 'molecular signatures' underlying physiological adaptations to different modes of exercise training (Timmons, 2011).

1.6.1 Skeletal muscle transcriptomic responses to concurrent exercise

Both resistance (Damas *et al.*, 2018*b*) and endurance-based (Keller *et al.*, 2010) exercise have pronounced effects on transcriptome-wide gene expression, which may ultimately determine training adaptation (Timmons *et al.*, 2010). To date, only two studies have characterised skeletal muscle transcriptomic responses to concurrent exercise in humans (Lundberg *et al.*, 2016; Robinson *et al.*, 2017). The first investigation (Lundberg *et al.*, 2016) explored the effects of concurrent exercise on transcriptomic responses to an acute bout of exhaustive aerobic exercise followed immediately by resistance exercise. Using a unilateral leg model, ten moderately trained young (~26 y) men performed 45 min of isolated and dynamic knee extensions on a modified cycle-ergometer with one leg, followed by 15 min of rest, and then performed four sets of seven maximal knee extensions on an isoinertial flywheel ergometer in both legs. Biopsies from the *vastus lateralis* were obtained from both legs 3 h after resistance exercise, and gene expression profiles of the two exercise modes were quantified using microarray technology. Differentially expressed genes (DEG) were estimated by

pairwise significance analysis of microarrays (SAM) (Tusher *et al.*, 2001). Gene ontology (GO) enrichment for biological functions of DEG sets was assessed on the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Dennis *et al.*, 2003). Further Ingenuity Pathway Analysis (IPA) of predicted upstream regulators (Krämer *et al.*, 2014) was performed to determine regulatory molecules of gene expression networks.

Among the enriched biological functions identified by DAVID following concurrent exercise, the most highly up-regulated processes were related to carbohydrate metabolism, while the most down-regulated processes were related to angiogenesis, transmembrane receptor signalling, and skeletal muscle tissue development (Lundberg *et al.*, 2016). Notably, IPA upstream regulator analysis revealed greater activation of mTOR and v-myc avian myelocytomatosis viral oncogene homolog (MYC), a transcription factor involved in cell proliferation, with a concomitant increase in DEG related to AMPK following concurrent exercise. However, there are several challenges when translating molecular responses observed during the initial period after acute exercise to distinct long-term training adaptations. First, there is a clear disconnect between transcriptomic changes following acute exercise and subsequent adaptations to chronic training (Phillips *et al.*, 2013). Similarly, the acute biopsy sampling time point may produce nonspecific transcriptional responses that are not reflective of differing physiological loads (Timmons, 2011). Nevertheless, the findings of Lundberg and colleagues (2016) illustrate that acute concurrent exercise stimulates a transcriptional profile initiating the expression of genes that are generally thought to have antagonistic roles.

The second investigation (Robinson *et al.*, 2017) utilised a parallel groups design to assess transcriptomic responses to 12 wk of endurance, resistance, or

concurrent training in both young (~25 y) and older (~70 y) males and females. Briefly, participants in the endurance only group performed high-intensity interval training (> 90% peak oxygen uptake [VO_{2peak}]) on an electronically braked cycle ergometer 3 $d \bullet wk^{-1}$ and moderate intensity treadmill (70% VO_{2peak}) walking 2 $d \bullet wk^{-1}$. Participants in the resistance only group performed 60 min of lower body exercises 2 $d \bullet wk^{-1}$ and upper body exercises 2 $d \bullet wk^{-1}$. Participants in the concurrent training group performed 30 min of moderate intensity cycling (70% VO_{2peak}) 5 $d \bullet wk^{-1}$ followed by 30 min of resistance exercise on 4 $d \bullet wk^{-1}$ split between upper and lower body exercises each performed 2 $d \bullet wk^{-1}$. Strength (leg press 1RM), aerobic capacity (VO_{2peak}), and fat free mass (FFM) by dual-energy X-ray absorptiometry (DXA) were assessed pre- and post-intervention. Biopsies from the *vastus lateralis* were obtained pre-intervention and 72 h following the last exercise bout of the 12 wk program to determine gene expression profiles of the three training modes by RNA-Seq. DEGs were analysed by edgeR (Robinson *et al.*, 2010), GO enrichment determined by Gene Set Enrichment Analysis (GSEA) (Subramanian *et al.*, 2005), and predicted upstream regulators of DEG were determined by IPA.

Following the 12 wk training intervention, 1RM increased in the resistance and concurrent training groups, VO_{2peak} increased in the endurance and concurrent training groups, and FFM increased in all three training groups (Robinson *et al.*, 2017). GSEA and IPA revealed that gene sets and transcriptional regulators related to angiogenesis were up-regulated after all training protocols, but GO terms unique to concurrent training were not identified. Similarly, statistical contrasts between training condition and time were not made for 1RM, VO_{2peak}, or FFM, making it unclear if there were differences in the magnitude of change for these variables. In addition, the concurrent training group underwent a 12 wk sedentary period prior to the training intervention,

which may have affected subsequent training responses (Fisher *et al.*, 2017; Seaborne *et al.*, 2018). Furthermore, the endurance and resistance components of the concurrent training program were not matched to that of the endurance or resistance training groups, making direct comparisons between training modalities challenging.

While the findings of Lundberg and colleagues (2016) and Robinson and colleagues (2017) have provided an innovative approach to profiling transcriptome-wide molecular responses to concurrent exercise, several questions remain unanswered:

1. What are the effects of alternate day concurrent training when resistance and endurance training loads are matched to isolated resistance or endurance training?
2. Are there enriched gene sets that are unique to concurrent training that may explain the 'interference effect'?

1.7 Objectives and scope of the thesis

This thesis describes a series of investigations into the 'interference effect' from indices of exercise performance and skeletal muscle morphology to transcriptome-wide molecular responses following a period of chronic concurrent training. **Chapter 2** describes a study that determined whether modulating training variables and closely monitoring nutrient intake, based on current, literature-based recommendations, can attenuate the 'interference effect'. Specifically, the study was designed to test the hypothesis that implementing longer recovery periods (i.e., 6-24 h) between exercise sessions, minimising endurance frequency to ≤ 3 d•wk^{-1}, integrating cycling rather than running as the endurance exercise mode (to minimise muscle damage) and incorporating a high protein diet over 12 wk of concurrent training would result in unimpaired adaptations to a) maximal strength, hypertrophy, and power, compared to

resistance training and b) maximal aerobic capacity compared to endurance training. **Chapter 3** describes a study which determined whether changes to satellite cell content following 12 wk of concurrent training underlie the 'interference effect'. Myofibre hypertrophy and indices of myogenesis were measured to test the hypothesis that limited hypertrophic potential with concurrent training can be explained by increases in satellite cell content being the same as isolated endurance and resistance training, despite performing a greater volume of work. **Chapter 4** describes an exploratory study in which alterations to gene expression patterns were determined following 12 wk of concurrent training. Whole-transcriptome gene expression was measured in an attempt to unravel potential gene sets that underpin limitations in exercise adaptations following concurrent training.

CHAPTER 2

ADAPTATIONS TO CONCURRENT TRAINING IN COMBINATION WITH HIGH PROTEIN AVAILABILITY: A COMPARATIVE TRIAL IN HEALTHY, RECREATIONALLY ACTIVE MEN

This chapter has been adapted from the following published article:

Baubak Shamim, Brooke L. Devlin, Ryan G. Timmins, Paul Tofari, Connor Lee Dow, Vernon G. Coffey, John A. Hawley, and Donny M. Camera. Adaptations to Concurrent Training in Combination with High Protein Availability: A Comparative Trial in Healthy, Recreationally Active Men. *Sports Medicine*. 2018 Dec;48(12):2869-2883.

2.1 Abstract

This study implemented a high protein diet (2 g•kg^{-1}•d^{-1}) throughout 12-wk of concurrent exercise training to determine whether interferences to adaptation in muscle hypertrophy, strength, and power could be attenuated compared to resistance training alone. Thirty-two recreationally active males (age: 25±5 y, body mass index: 24±3 kg•m^{-2}; mean±SD) performed 12-wk of either isolated resistance (RES; *n*=10) or endurance (END; *n*=10) training (3 sessions•w^{-1}), or concurrent resistance and endurance (CET; *n*=12) training (6 sessions•w^{-1}). Maximal strength (1RM), body composition, and power were assessed pre- and post-intervention. Leg press 1RM increased ~24 ± 13% and ~33 ± 16% in CET and RES from PRE-to-POST (*P*<0.001), with no difference between groups. Total lean mass increased ~4% in both CET and RES from PRE-to-POST (*P*<0.001). Ultrasound estimated *vastus lateralis* volume increased ~15% in CET and ~11% in RES from PRE-to-POST (*P*<0.001), with no difference between groups. Wingate peak power relative to body mass displayed a trend (*P* = 0.053) to be greater in RES (12.5±1.6 W•kg^{-1}) than both CET (10.8±1.7 W•kg^{-1}) and END (10.9±1.8 W•kg BM^{-1}) at POST. Absolute VO_{2peak} increased 6.9% in CET and 12% in END from PRE-to-POST (*P*<0.05), with no difference between groups. Despite high protein availability, select measures of anaerobic power-based adaptations, but not muscle strength or hypertrophy, appear susceptible to "interference effects" with CET and should be closely monitored throughout training macro-cycles. This trial was registered with the Australian-New Zealand Clinical Trials Registry (ACTRN12617001229369).

2.2 Key Points

1. Little consideration has been given to the role of increased protein availability to facilitate anabolic adaptations to concurrent training.
2. Concurrent training combined with a high protein diet does not impair gains in maximal strength, countermovement jump, squat jump, VO_{2peak}, lean mass, or muscle architectural changes compared to resistance or endurance training alone.
3. Despite optimal protein intake strategies, select measures of anaerobic power are compromised during a concurrent training block and should be monitored carefully.

2.3 Introduction

The simultaneous development of strength, power, and endurance adaptations is an attribute required by many athletes, particularly those involved in team sports (Nader, 2006). Both muscular strength and cardiorespiratory fitness have been associated with lower declines in muscle function, chronic metabolic diseases, and all-cause mortality (Ruiz *et al.*, 2008; Pedersen & Saltin, 2015). Incorporating both resistance- and endurance-based exercise into training programs, termed concurrent training, is therefore common practice in both athletic (Baker, 2001; Argus *et al.*, 2010) and clinical populations (Atashak *et al.*, 2016; Bassi *et al.*, 2016; Robinson *et al.*, 2017). Further, World Health Organization global recommendations for physical activity for overall health and well-being in adults stipulate the performance of a combination of both resistance and endurance type exercises to improve cardiovascular and muscular fitness (World Health Organization, 2010).

The principle of training specificity dictates that adaptations to chronic training are specific to the mode of exercise performed and result in distinct and divergent skeletal muscle phenotypes (Hawley *et al.*, 2014). For example, endurance training improves skeletal muscle oxidative capacity and whole-body maximal oxygen uptake, leading to a more fatigue-resistant muscle (Holloszy & Coyle, 1984; Hawley, 2002). Conversely, strength training develops maximal force-generating capacity and skeletal muscle hypertrophy (McDonagh & Davies, 1984*b*). Given these vastly divergent adaptations, the simultaneous development of muscular endurance and strength/power with concurrent training presents a high degree of complexity in exercise prescription (Coffey & Hawley, 2017). Indeed, findings from multiple studies demonstrate ‘interference’ in the magnitude of increase in hypertrophy, strength and power with concurrent training compared to resistance training undertaken in isolation (Hickson,

1980; Craig *et al.*, 1991; Hennessy & Watson, 1994; Kraemer *et al.*, 1995; Dolezal & Potteiger, 1998; Bell *et al.*, 2000; Häkkinen *et al.*, 2003; Mikkola *et al.*, 2012; Fyfe *et al.*, 2016*a*, 2018), although these observations are not unequivocal (Sale *et al.*, 1990; de Souza *et al.*, 2013; Lundberg *et al.*, 2013, 2014).

Theoretical recommendations to prevent or reduce interference to strength adaptations have been formulated based on existing literature regarding concurrent training variables (Wilson *et al.*, 2012; Murach & Bagley, 2016), nutrition (Perez-Schindler *et al.*, 2015), and molecular biology (Hawley, 2009; Baar, 2014*a*; Coffey & Hawley, 2017). It has been suggested that maximal strength and hypertrophy with concurrent training can be attained through implementing longer recovery periods (i.e., 6-24 h) between exercise sessions, minimising endurance frequency to ≤3 days per week, integrating cycling rather than running as the endurance exercise mode (to minimise muscle damage) and incorporating post-exercise nutritional strategies (Murach & Bagley, 2016). With regard to nutrition, little consideration has been given to the role of increased protein availability to facilitate adaptations to concurrent training. It has previously been shown (Camera *et al.*, 2015) that protein ingestion following a single bout of concurrent exercise increased rates of muscle protein synthesis to similar levels observed when protein was ingested following resistance exercise (Moore *et al.*, 2009*b*). Considering the importance for dietary protein to promote muscle growth and remodelling (Cermak *et al.*, 2012; Morton *et al.*, 2018) increased protein availability around concurrent training has the potential to reduce the interference effect of endurance exercise on skeletal muscle hypertrophy. Accordingly, a high protein diet and other strategies were implemented to reduce the interference effect on maximal muscle strength, hypertrophy and power following 12 wk of concurrent training compared to resistance training alone. It was hypothesised that

concurrent training under these conditions would result in no differences to the degree of adaptations made to a) maximal strength, hypertrophy, and power, compared to resistance training and b) maximal aerobic capacity compared to endurance training.

2.4 Methods

2.4.1 Participants

Thirty-two young, healthy, recreationally active males (**Table 2.1**) who had not participated in a structured exercise program for ≥6 months preceding the study volunteered to participate. Participants were deemed healthy and eligible to participate based on their responses to a cardiovascular risk-factor questionnaire. The experimental procedures and risks associated with the study were explained to all participants prior to providing written informed consent. The study was approved by the Australian Catholic University Human Research Ethics Committee and was carried out in accordance with the standards set by the latest revision of the Declaration of Helsinki. This trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12617001229369).

Table 2.1 Participant characteristics.

	Training Group					
	CET (*n* = 12)		**RES (*n* = 10)**		**END (*n* = 10)**	
	PRE	*POST*	*PRE*	*POST*	*PRE*	*POST*
Age (y)	26 ± 4	-	24 ± 6	-	24 ± 5	-
Height (cm)	177 ± 7	-	182 ± 8	-	179 ± 7	-
Mass (kg)	76.4 ± 10.2	79.3 ± 9.7[a]	75.5 ± 10.3	78.8 ± 11[a]	79.5 ± 9.3	81.5 ± 8.9[a]
BMI ($kg \cdot m^{-2}$)	24.4 ± 2.9	25.3 ± 2.6[a]	22.8 ± 2.8	23.8 ± 2.9[a]	24.8 ± 3.1	25.5 ± 2.9[a]

Values are presented as means ± SD. a = $P < 0.05$ from PRE. Abbreviations: BMI, body mass index; CET, concurrent exercise training; RES, resistance training; END, endurance training.

2.4.2 Experimental design

An overview of the study protocol is shown in **Figure 2.1**. The study employed a parallel groups design where participants were stratified according to lean body mass (LBM) and allocated to either a resistance only (RES; $n = 10$), endurance only (END; $n = 10$), or concurrent resistance and endurance exercise training (CET; $n = 12$) group for 12-wk. For the duration of the intervention, all participants consumed a high protein diet (2 $g \bullet kg^{-1} \bullet d^{-1}$). Participants first completed three preliminary testing days: on the first visit, body composition was assessed by whole-body dual-energy X-ray absorptiometry (DXA) and B-mode ultrasound to measure *vastus lateralis* (VL) architecture; on the second visit, participants performed tests for maximal aerobic capacity (VO_{2peak}) and anaerobic power (Wingate) as well as familiarisation of strength and jump performance measurements; on the third visit, participants completed an isometric mid-thigh pull, countermovement and squat jump, followed by 1-repetition maximum (1RM) testing. At this visit, participants also met with the study dietitian for an initial consultation to discuss food preferences as well as target protein and energy intakes prior to commencing the training intervention. Measurements of 1RM and VO_{2peak} were repeated at the end of week 6 to adjust training loads. At the end of week 12, participants were re-tested for VO_{2peak}, Wingate, 1RM, isometric strength, and

power in the same order as baseline. All testing and training sessions were completed in the strength and performance lab under direct supervision of the same member of the research team.

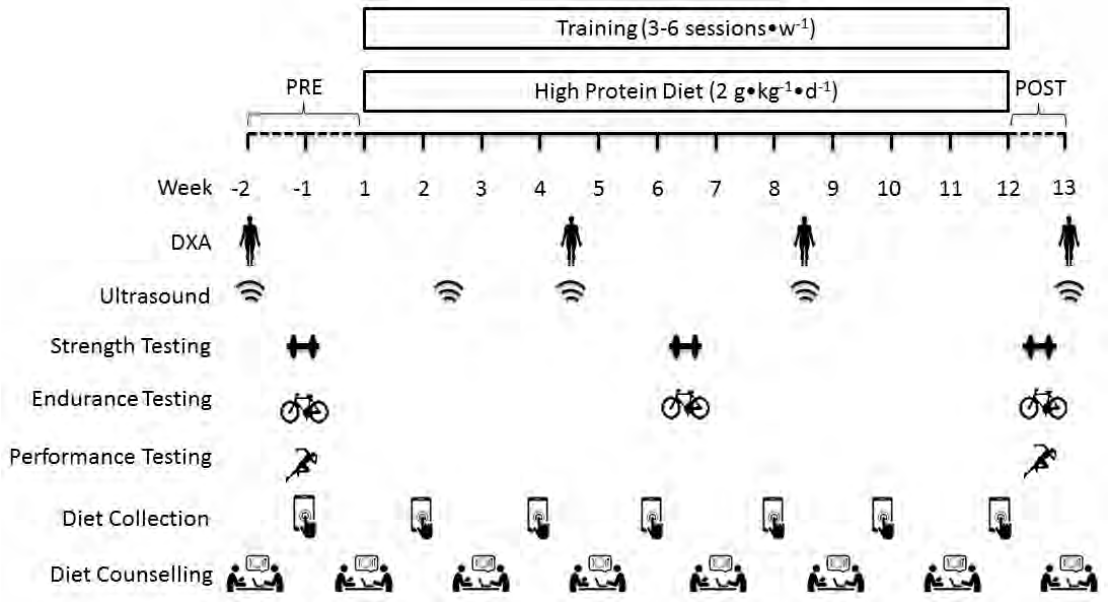

Figure 2.1 Schematic overview of study timeline.

2.4.3 Exercise training

For the duration of the intervention, participants in the RES and END group performed three non-consecutive days of training each week. Participants in the CET group trained 6 d•wk^{-1} and performed identical resistance and endurance programs on alternating days as those in the RES and END groups, respectively. This training pattern was implemented in the CET group based on current recommendations to perform resistance and endurance exercise on alternating days to maximise the potential for lower-body strength development (Eddens *et al.*, 2018; Murlasits *et al.*, 2018) and lengthen recovery time between sessions to minimise any potential interference between training modalities (Baar, 2014*a*; Murach & Bagley, 2016). All training programs were periodised to progressively modify the volume and intensity of training

in order to provide an appropriate overload stimulus. Specific details of each training regime are described subsequently. Participants were encouraged to complete the designated training programs in their entirety with financial incentives provided for all three groups for largest pre- to post-intervention increases in 1RM (CET and RES) and VO_{2peak} (CET and END) (Torrens *et al.*, 2016).

2.4.4 VO_{2peak} testing

VO_{2peak} was determined during an incremental test to volitional fatigue on a Lode cycle ergometer (Excalibur sport, Lode, The Netherlands) (Hawley & Noakes, 1992). Throughout the maximal test, participants breathed through a mouthpiece attached to a metabolic cart (TrueOne® 2400, Parvomedics, USA) to determine O_2 consumption. Maximum aerobic power (MAP) was determined as previously described (Hawley & Noakes, 1992) and was assessed prior to training, at the end of week 6, and upon completion of the 12-wk training intervention. The MAP from pre-training and week 6 were used to prescribe loads for the endurance training.

2.4.5 Strength testing

Maximal strength was determined through 1RM for plate-loaded 45^{o} incline leg press, bilateral knee extension, and bench press. Participants were demonstrated proper lifting technique prior to engaging in 1RM testing. Briefly, participants warmed up at a self-selected load for each movement until reaching a rating of perceived exertion (RPE) of ~8, using a Borg Category Ratio 10 scale (Borg & Borg, 2013), for a single repetition. Thereafter, a series of single repetitions were attempted, with 5 min recovery, until the maximal load possible for one repetition with full range of motion was determined. For the leg press, full range of motion was established as beginning

with the knees in full extension (0^{o}), performing 90^{o} of knee flexion, and returning to full knee extension. For the knee extension, full range of motion was established as beginning with the knees in 90^{o} of flexion and extending to full extension. For bench press, full range of motion was established as beginning with the arms in full elbow extension, lowering the barbell to the position of the chest until momentum has been terminated, and returning to full elbow extension. Participants were instructed to maintain contact of the head, shoulders, and buttocks with the bench and feet planted on the ground throughout the entire movement. The 1RM's from pre-training and week 6 were used to prescribe training loads for the resistance-training program.

Maximal lower-body isometric strength (N and $N \bullet kg^{-1}$) was measured prior to, and upon completion of the 12-wk intervention using an isometric mid-thigh pull (IMTP) as previously described (Tofari *et al.*, 2017). All data was collected on a force plate sampling at 600 Hz (400 Series Force Plate, Fitness Technologies, Australia) and analysed using proprietary software (Ballistic Measurement System, Fitness Technology, Australia).

2.4.6 Power testing

Performance tests were conducted prior to, and upon completion of the 12-wk intervention to determine maximal anaerobic power output. Detailed descriptions of each measurement can be found in **Appendix A**.

2.4.7 Body composition

Total lean mass, as well as leg and upper-body lean mass, and fat mass were estimated by DXA (GE Lunar iDXA Pro, GE Healthcare; software: Encore 2009,

version 16) pre-intervention, after weeks 4 and 8 of exercise training, and post-intervention following best practice guidelines (Nana *et al.*, 2015).

2.4.8 Architectural assessment of vastus lateralis

Segmental muscle thickness, pennation angle, fascicle length and volume changes of the VL were assessed utilising B-mode ultrasound at baseline, after weeks 2, 4, 8, and post-intervention (**Appendix A**).

2.4.9 Resistance training

Resistance training consisted of whole body exercises with a focus on the leg press, knee extension and bench press movements, with these exercises performed at an intensity of ~60-98% of 1RM. All exercises were separated by a 3-min between-set recovery period. If the participant was unable to achieve the prescribed number of repetitions, the weight was lowered by ~5-10% for the following set to uphold the repetition scheme. All sessions were preceded by a standardised warm up for the lower- or upper-body, respective of the training session. Progressive overload was applied by periodically manipulating the number of sets, repetitions, and relative intensity of load throughout the 12-wk program. A detailed outline of the resistance-training program can be found in **Appendix A (Table A.1)**.

2.4.10 Endurance training

Endurance cycle training was performed on Lode cycle ergometers and consisted of a mixture of a hill simulation ride of varying intensity (25-110% of MAP), moderate-intensity continuous training at 50% MAP, moderate-intensity interval

training at 70% MAP, and high-intensity interval training at 100% MAP. Moderate-intensity intervals were separated by a 60 second recovery period at ~40% MAP, to establish a 2.5:1 or 5:1 work-to-rest ratio. High-intensity intervals were separated by 20-60 s recovery periods, completed at ~40% MAP, to establish a 1:5, 1:2, or 1:1 work-to-rest ratio. All cycling sessions were preceded by 3–5 min of cycling at ≤50 W. Heart rate (HR), energy expenditure (EE), and RPE were collected at the end of each cycling stage. Progressive overload was applied by manipulating the number of intervals and relative intensity of load throughout the 12-wk program. A detailed outline of the endurance-training program can be found in **Appendix A (Tables A.2A-A.2B)**.

2.4.11 Diet

A free-living, high-protein (2 g•kg^{-1}•d^{-1}) eating plan was implemented over the 12-wk intervention. Participants attended consultations with an Accredited Practicing Dietitian on a fortnightly basis and were provided with guidelines to reach protein and energy targets, including the distribution of protein intake throughout the day across 4-6 meals (Areta *et al.*, 2013; Loenneke *et al.*, 2016) and the consumption of ~20-30 g of protein prior to sleep to maximise potential for muscle protein synthesis (Moore *et al.*, 2009*a*; Snijders *et al.*, 2015*b*; Trommelen *et al.*, 2018). All participants were provided with ~34 g of whey protein (Pure Warrior 100% WPI, Swisse™, Australia) to be consumed upon cessation of every training session (Macnaughton *et al.*, 2016) and given a whey protein supplement (Whey Protein Concentrate, Bulk Nutrients, Australia) to consume as needed throughout the 12-wk intervention.

Food records were kept daily by participants throughout the 12-wk intervention using mobile phone applications Easy Diet Diary (Xyris Software Pty Ltd, Australia, for participants with iPhones®, Apple Inc., USA; n = 20) and MyFitnessPal

(MyFitnessPal Inc., USA, for participants with Android-based devices, Google Inc., USA; n = 12). All dietary intake data was analysed using FoodWorks 8© (Xyris Software Pty Ltd, Australia) to ensure the same food database was used for all analysis. Diet records were analysed for energy ($kJ \cdot kg^{-1}$), protein, carbohydrate, and fat ($g \cdot kg^{-1}$ for all macronutrients) to provide a daily average for the entire 12-wk intervention. Complete dietary methods are detailed within the **Appendix A**.

2.4.12 Statistical analysis

An *a priori* power calculation (G*Power Version 3.1) using a F-test, repeated measures, within-between interaction ANOVA revealed 30 participants were needed to detect a medium effect (Cohen's f = 0.25) with a significance level of $\alpha = 0.05$ and 80% power for change in lean body mass as measured by DXA (Faul *et al.*, 2009). Baseline characteristics and mean training variables (RPE, HR, time to complete set, training time, rest interval, and between session rest) were analysed by one-way ANOVA (group). Strength, performance, VO_{2peak}, body composition, training volume, and diet data were analysed by two-way ANOVA (group x time) with repeated measures. Where ANOVA revealed significance, $P \leq 0.05$, a Student-Newman-Keuls *post hoc* test was conducted for pairwise multiple comparisons (SigmaPlot 12, Systat Software Inc., USA). When normality (Shapiro-Wilk) was violated, a nonparametric Kruskal-Wallis test was performed to determine differences between conditions where statistical significance differed from ANOVA (CMJ height and distal VL muscle thickness; SPSS v25, IBM, USA). All data are expressed as mean ± SD.

2.5 Results

2.5.1 Participant characteristics

There were no differences between groups in baseline characteristics for height, BM, BMI, or age (**Table 2.1**). There was a main effect for time for change in BM ($P < 0.001$). BM increased from PRE to POST by 3.9% in CET, 4.3% in RES, and 2.7% in END ($P < 0.001$). There was a main effect for time for change in BMI ($P < 0.001$). BMI increased from PRE to POST by 3.9% in CET, 4.4% in RES, and 2.7% in END ($P < 0.001$; **Table 2.1**).

2.5.2 Body composition

There was a main effect for time for change in total LBM ($P < 0.001$). Total LBM increased from PRE to POST by 3.8% in CET, 3.8% in RES, and 2.9% in END ($P < 0.001$). There was a main effect for time for change in LLM ($P < 0.001$). LLM increased from PRE to POST by 5.4% in CET, 6% in RES, and 5.2% in END. There was a main effect for time for change in ULM ($P < 0.001$). ULM increased from PRE to POST by 2.9% in CET and 2.8% in RES ($P < 0.01$). Additionally, a main effect for time was observed for changes in fat mass ($P = 0.009$). Fat mass increased from PRE to POST by 9.5% in RES ($P = 0.037$; **Table 2.2**).

Table 2.2 Change in body composition throughout the 12 week training intervention as measured by dual-energy X-ray absorptiometry (DXA).

	Time			
Measure	**PRE**	**WK4**	**WK8**	**POST**
Total Lean Mass (kg)				
CET	58.4 ± 6.34	59.8 ± 6.48[a]	60.6 ± 6.68[ab]	60.6 ± 6.46[ab]
RES	59.6 ± 6.71	60.9 ± 6.48[a]	61.6 ± 6.63[a]	61.9 ± 6.6[ab]
END	58.9 ± 5.45	60.0 ± 5.74[a]	60.0 ± 5.2[a]	60.6 ± 5.02[a]
Leg Lean Mass (kg)				
CET	20.7 ± 2.78	21.5 ± 2.79[a]	21.8 ± 2.78[a]	21.8 ± 2.72[a]
RES	20.6 ± 2.36	21.5 ± 2.29[a]	21.5 ± 2.34[a]	21.8 ± 2.17[a]
END	20.8 ± 2.28	21.6 ± 2.47[a]	21.5 ± 2.27[a]	21.9 ± 2.38[ac]
Upper Lean Mass (kg)				
CET	34.8 ± 3.68	34.9 ± 3.76[a]	35.4 ± 3.97[a]	35.3 ± 3.82[a]
RES	35.5 ± 4.34	35.9 ± 4.27	36.5 ± 4.39[a]	36.5 ± 4.48[a]
END	34.6 ± 3.37	34.9 ± 3.58	35.1 ± 3.24	35.2 ± 2.97
Fat Mass (kg)				
CET	15.4 ± 6.67	15.3 ± 6.61	15.7 ± 6.37	16.2 ± 5.76
RES	13.2 ± 5.84	13.6 ± 5.79	13.8 ± 5.95	14.3 ± 6.17[a]
END	17.9 ± 6.36	18.2 ± 6.24	18.2 ± 6.19	18.4 ± 6.06

Values are presented as means ± SD. a = $P < 0.05$ from PRE. b = $P < 0.05$ from WK4. c = $P < 0.05$ from WK8. Abbreviations: CET, concurrent exercise training; RES, resistance training; END, endurance training.

2.5.3 Vastus lateralis architecture

There was a main effect for time ($P < 0.001$) and a trend for a group by time interaction ($P = 0.051$) for change in proximal VL muscle thickness. Proximal VL muscle thickness increased from PRE to POST by 14.9% in CET, 15.7% in RES, and 5.8% with END ($P < 0.01$). Proximal VL thickness at POST was greater in CET and RES compared to END ($P < 0.05$). There was an interaction for group by time for change in midpoint VL muscle thickness ($P < 0.001$). Midpoint VL muscle thickness increased from PRE to POST by 17.5% in CET, 13.7% in RES, and 9.8% in END ($P < 0.001$). Midpoint VL thickness at POST was greater in CET and RES compared to END

($P < 0.05$). Distal VL muscle thickness did not change ($P = 0.054$; **Appendix B, Table B.1**).

There was a main effect for time for change in proximal VL pennation angle ($P < 0.001$). Proximal VL pennation angle increased from PRE to POST by 17.2% in CET, 15.8% in RES, and 15.4% in END ($P < 0.001$). There was a main effect for time for change in midpoint VL pennation angle ($P < 0.001$). Midpoint VL pennation angle increased from PRE to POST by 12.4% in CET, 12.2% in RES, and 13.9% in END ($P < 0.001$). There was a main effect for time for change in distal VL pennation angle ($P < 0.001$). Distal VL pennation angle increased from PRE to POST by 12.3% in CET, 19% in RES, and 13.5% in END ($P \leq 0.005$; **Appendix B, Table B.1**). There was a main effect for group for change in proximal VL fascicle length ($P < 0.001$). Proximal VL fascicle length decreased from PRE to POST by 6.3% in END ($P = 0.024$). Proximal VL fascicle length was significantly greater at POST in CET (9.3 ± 0.8 cm) compared to END (8.5 ± 0.6 cm; $P = 0.036$). There was a main effect for group for change in midpoint VL fascicle length ($P = 0.004$). Midpoint VL fascicle length was greater at PRE in CET (9 ± 0.9 cm) and RES (8.9 ± 0.8 cm) compared to END (8.3 ± 0.9 cm; $P < 0.05$). Midpoint VL fascicle length was also greater at POST in CET (9.5 ± 0.5 cm) and RES (9.1 ± 0.5 cm) compared to END (8.1 ± 0.6 cm; $P < 0.01$). There was a main effect for time for change in distal VL fascicle length ($P = 0.031$). Distal VL fascicle length increased from PRE to POST by 10.4% in CET ($P = 0.024$; **Appendix B, Table B.1**).

There was a main effect for time ($P < 0.001$) and a trend ($P = 0.051$) for a group by time interaction for changes in approximated VL muscle volume. Estimated VL muscle volume increased from PRE to POST by 15.3% in CET, 11.4% in RES, and 7.8% in END ($P < 0.001$; **Appendix B, Table B.1**).

2.5.4 Strength

There was an interaction for group by time for change in absolute ($P < 0.001$) and relative to BM ($P < 0.001$) leg press 1RM. Absolute leg press 1RM increased in CET by 16.4% from PRE to WK6, 6.4% from WK6 to POST, and 23.7% from PRE to POST ($P < 0.01$). For RES, leg press 1RM increased 21.2% from PRE to WK6, 9.9% from WK6 to POST, and 33.4% from PRE to POST ($P \leq 0.001$; **Figure 2.2A**). Relative leg press 1RM was greater at POST in both CET (3.9 ± 0.6 kg•kg BM^{-1}) and RES (3.9 ± 0.5 kg•kg BM^{-1}) compared to END (3.2 ± 0.6 kg•kg BM^{-1}; $P = 0.05$; **Figure 2.2B**).

There was an interaction for group by time for change in absolute ($P < 0.001$) and relative to BM ($P < 0.001$) knee extension 1RM. Absolute knee extension 1RM increased in CET by 24.7% from PRE to WK6, 18.7% from WK6 to POST, and 48.7% from PRE to POST ($P < 0.001$). For RES, knee extension 1RM increased 32.2% from PRE to WK6, 12.7% from WK6 to POST, and 49.4% from PRE to POST ($P \leq 0.001$). Knee extension 1RM was also greater at POST in both CET (159 ± 29 kg) and RES (157 ± 25 kg) compared to END (126 ± 21 kg; $P < 0.05$). Knee extension 1RM increased 12.5% from PRE to POST in END ($P = 0.024$; **Figure 2.2C**); however, relative to BM, knee extension 1RM remained unchanged from PRE to POST in END ($P = 0.122$; **Figure 2.2D**).

There was an interaction for group by time for change in absolute ($P < 0.001$) and relative to BM ($P < 0.001$) bench press 1RM. Absolute bench press 1RM increased in CET by 5.6% from PRE to WK6, 4.6% from WK6 to POST, and 10.4% from PRE to POST ($P < 0.05$). For RES, bench press 1RM increased 6% from PRE to WK6, 4.9% from WK6 to POST, and 11.3% from PRE to POST ($P < 0.01$; **Figure 2.2E**). Relative bench press 1RM for CET trended towards an increase at WK6 ($P = 0.055$), and increased from both PRE and WK6 by POST ($P < 0.05$; **Figure 2.2F**).

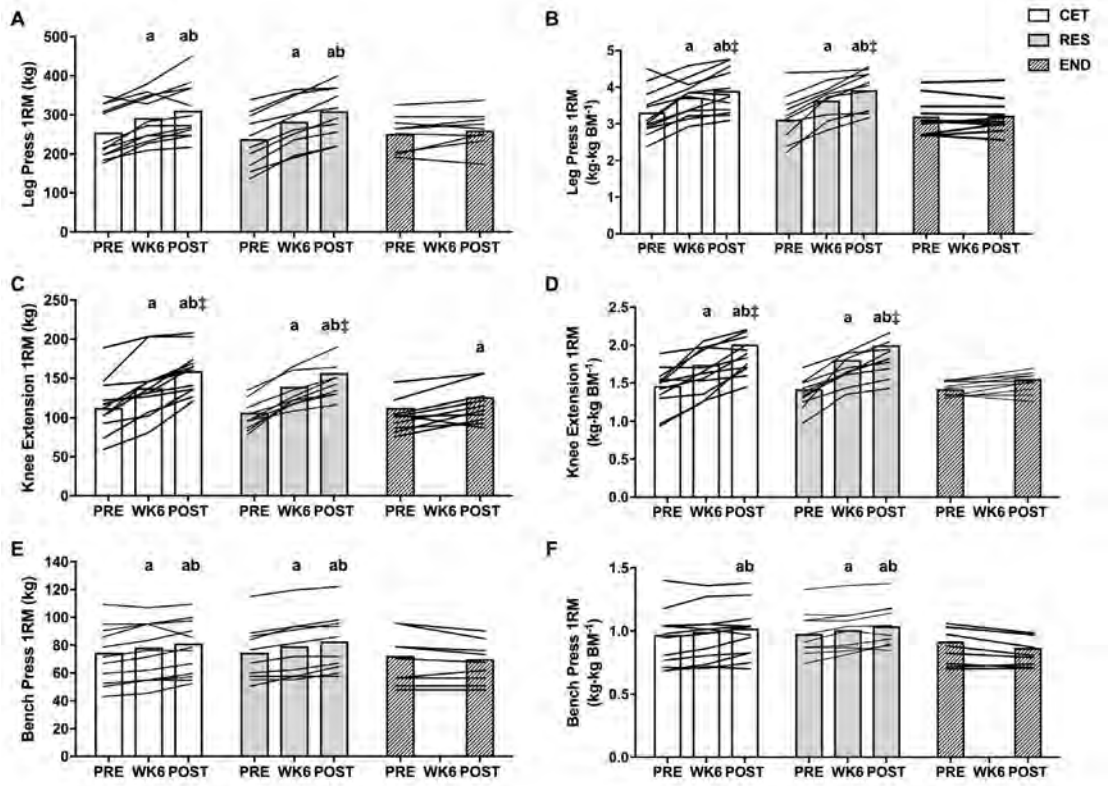

Figure 2.2 Changes to 1-repetition maximum (1RM) strength throughout the 12 week intervention for absolute (panels A-C) and relative (panels D-F) leg press, knee extension, and bench press (END, n = 9). Values are presented as individual data with group mean. a = P < 0.05 from PRE. b = P < 0.05 from WK6. ǂ = P < 0.05 from END at time point. Abbreviations: CET, concurrent exercise training; RES, resistance training; END, endurance training.

There was an interaction for group by time for change in IMTP peak force (P = 0.045). IMTP peak force increased from PRE to POST by 10.1% in CET and 9.6% in RES (P < 0.01*;* **Appendix B, Figure B.1**). There was main effect for time for change in IMTP peak force relative to BM (P = 0.045). Relative IMTP increased from PRE to POST by 6.8% in CET and 6% in RES (P < 0.05; **Appendix B, Figure B.1**).

2.5.5 Power testing

There was an interaction for group by time for change in CMJ peak velocity (P = 0.021). CMJ peak velocity increased from PRE to POST by 3% in CET and 2.3% in RES (P < 0.05; **Figure 2.3A**). CMJ peak velocity at POST was greater in RES (2.95 ± 0.17 m•s^{-1}) compared to END (2.68 ± 0.27 m•s^{-1}; P = 0.027). CMJ height did not change (P = 0.089; **Figure 2.3B**). There was an interaction for group by time for change in CMJ peak power (P = 0.047). CMJ peak power increased from PRE to POST by 5.6% in CET and 7% in RES (P < 0.05; **Figure 2.3C**). There was an interaction for group by time for change in CMJ peak power relative to BM (P = 0.047); however, *post hoc* analysis revealed no changes to CMJ relative peak power across all groups (**Figure 2.3D**).

There was a main effect for time for changes in SJ peak velocity (P = 0.006). SJ peak velocity increased from PRE to POST by 2.9% in CET and 3.8% in RES (P < 0.05; **Figure 2.3E**). SJ peak velocity at POST was greater in RES (2.78 ± 0.23 m•s^{-1}) compared to END (2.51 ± 0.23 m•s^{-1}; P = 0.037). There was an interaction for group by time for change in SJ height (P = 0.047). SJ height increased from PRE to POST by 6.6% in CET and 7.6% in RES (P < 0.05; **Figure 2.3F**). SJ height at POST was greater in CET (37.3 ± 4.86 cm) and RES (39.6 ± 6.77 cm) compared to END (32.2 ± 6.09 cm; P < 0.05). There was an interaction for group by time for change in SJ peak power (P = 0.005). SJ peak power increased from PRE to POST by 6.4% in CET and 11.4% in RES (P < 0.01; **Figure 2.3G**). There was an interaction for group by time for change in SJ peak power relative to BM (P = 0.012). SJ relative peak power increased from PRE to POST by 3.6% in CET and 7.7% in RES (P < 0.05; **Figure 2.3H**). SJ relative peak power was greater at POST in RES (53.4 ± 7.16 W•kg BM^{-1}) compared to END (45.7 ± 6.9 W•kg BM^{-1}; P = 0.047).

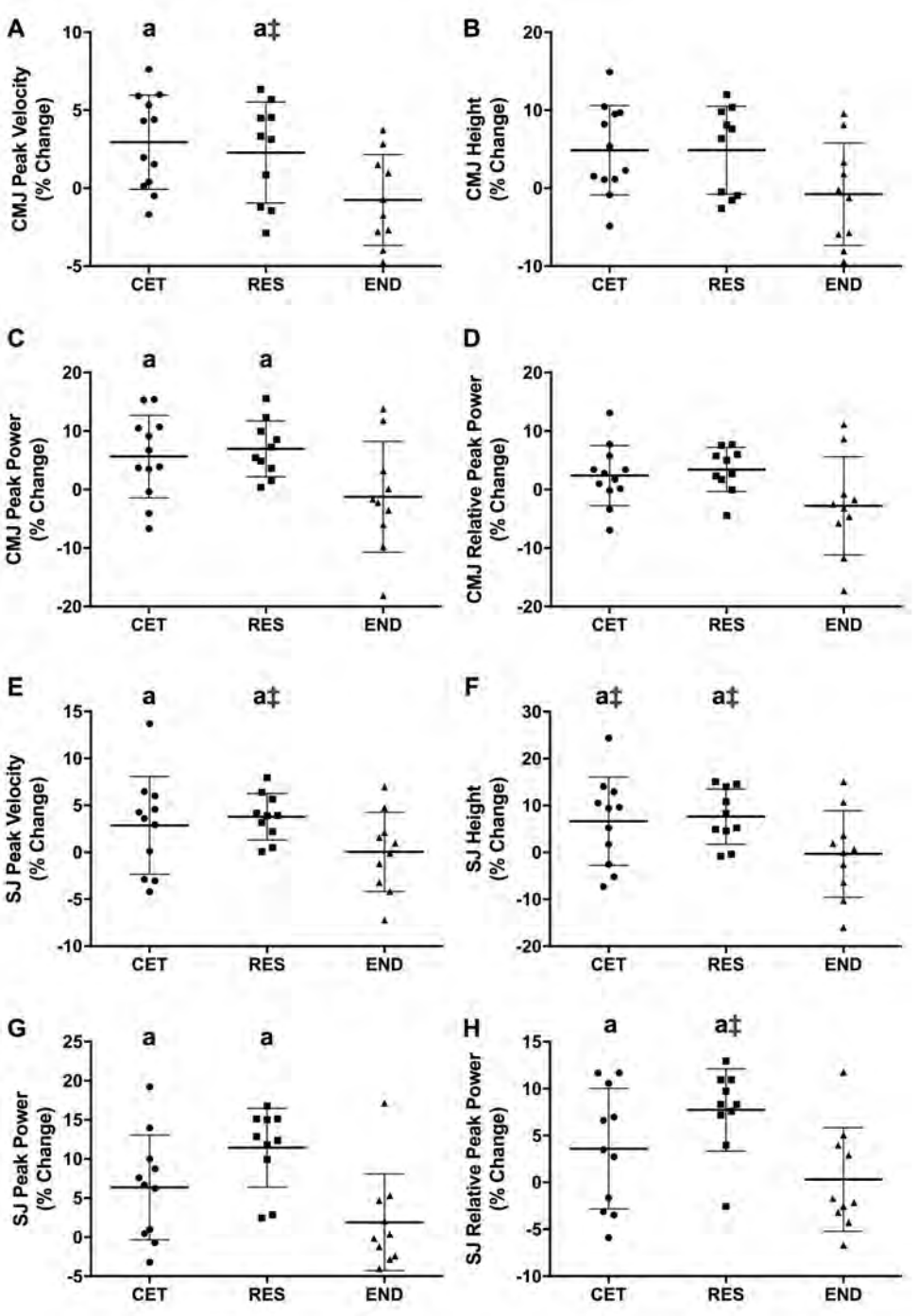

Figure 2.3 Change to countermovement jump (CMJ) and squat jump (SJ). Per cent changes from PRE to POST for CMJ are presented for A) peak velocity, B) height, C) peak power, and D) relative peak power. Per cent changes from PRE to POST for SJ are presented for E) peak velocity, F) height, G) peak power, and H) relative peak power. Values are presented as individual data with group mean ± SD. a = $P < 0.05$ from PRE. ǂ = $P < 0.05$ from END at POST. Abbreviations: CET, concurrent exercise training; RES, resistance training; END, endurance training.

2.5.6 VO_{2peak}

There was an interaction for group by time for change in absolute ($P < 0.001$) and relative to BM ($P < 0.001$) VO_{2peak}. Absolute VO_{2peak} ($L \cdot min^{-1}$) increased in CET by 9.1% from PRE to WK6, and 6.9% from PRE to POST ($P < 0.05$). For END, absolute VO_{2peak} increased 10.6% from PRE to WK6, and 12% from PRE to POST ($P < 0.001$); however, there was no difference between CET and END at POST ($P = 0.208$**; Figure 2.4A**). Relative VO_{2peak} in CET increased by 6.9% from PRE to WK6 ($P = 0.029$), but did not change from PRE to POST ($P = 0.272$). For RES, relative VO_{2peak} decreased 4.8% from PRE to POST ($P = 0.016$). In contrast, relative VO_{2peak} increased in END by 9.1% from PRE to WK6, and 9.8% from PRE to POST ($P < 0.005$); however, there was no difference between CET and END at POST ($P = 0.415$; **Figure 2.4B**).

There was an interaction for group by time for change in absolute ($P < 0.001$) and relative to BM ($P < 0.001$) MAP. Absolute MAP increased in CET by 5.3% from PRE to WK6, 8.5% from WK6 to POST, and 14% from PRE to POST ($P < 0.05$). For RES, absolute MAP decreased by 4.5% from PRE to POST ($P = 0.015$). For END, absolute MAP increased 13.6% from PRE to WK6, and 16.4% from PRE to POST ($P < 0.001$); however, there was no difference between CET and END at POST ($P = 0.605$; **Figure 2.4C**). Relative MAP increased in CET by 7.1% from WK6 to POST ($P = 0.002$), and 9.8% from PRE to POST ($P < 0.001$). For RES, relative MAP decreased by 8.4% from PRE to POST ($P < 0.001$). For END, relative MAP increased by 11.2% from PRE to WK6 ($P < 0.001$), and 13.5% from PRE to POST ($P < 0.001$); however, there was no difference between CET and END at POST ($P = 0.830$; **Figure 2.4D**).

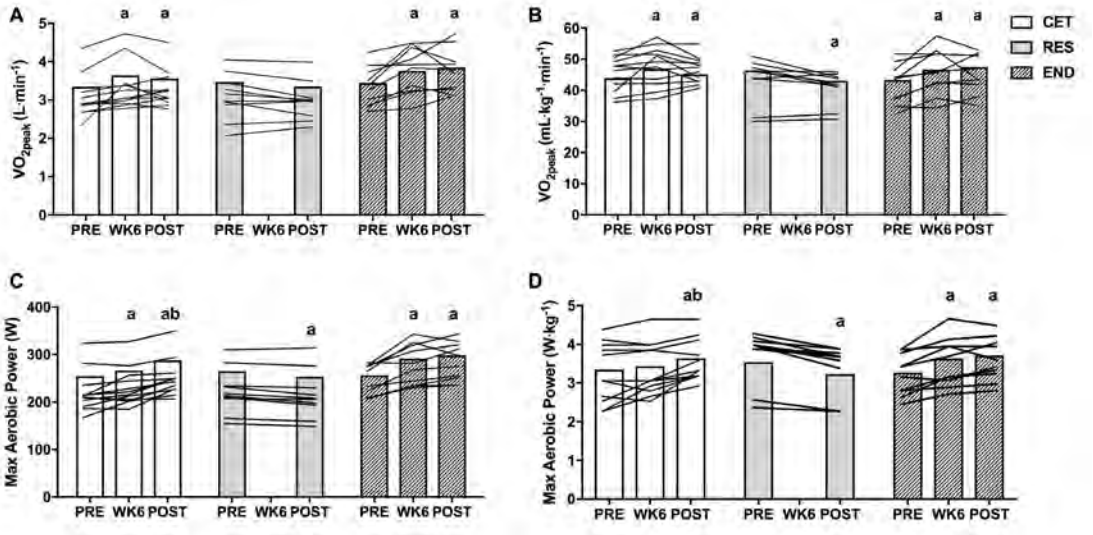

Figure 2.4 Changes to A) absolute and B) relative peak aerobic capacity (VO_{2peak}) as well as C) absolute and D) relative maximum aerobic power throughout the 12-week intervention. Values are presented as individual data with group mean. a = P < 0.05 from PRE. b = *P* < 0.05 from WK6. Abbreviations: CET, concurrent exercise training; RES, resistance training; END, endurance training.

2.5.7 Wingate indices

There was main effect for time (*P* < 0.001), but not group (*P* = 0.487) for training-induced change in Wingate peak power. Wingate peak power increased from PRE to POST by 14% in RES and 7.2% in END (*P* < 0.05) while there was no change in CET (*P* = 0.115; **Figure 2.5A**). A main effect for time (*P* = 0.001) and a trend for group (*P* = 0.053) was observed for change in Wingate peak power when expressed relative to BM. Wingate relative peak power increased from PRE to POST by 9.8% in RES (*P* = 0.002). Wingate relative peak power at POST was greater in RES (12.5 ± 1.6 W•kg BM^{-1}; *P* < 0.05) compared to both CET (10.8 ± 0.8 W•kg BM^{-1}) and END (10.9 ± 1.8 W•kg BM^{-1}; **Figure 2.5B**).

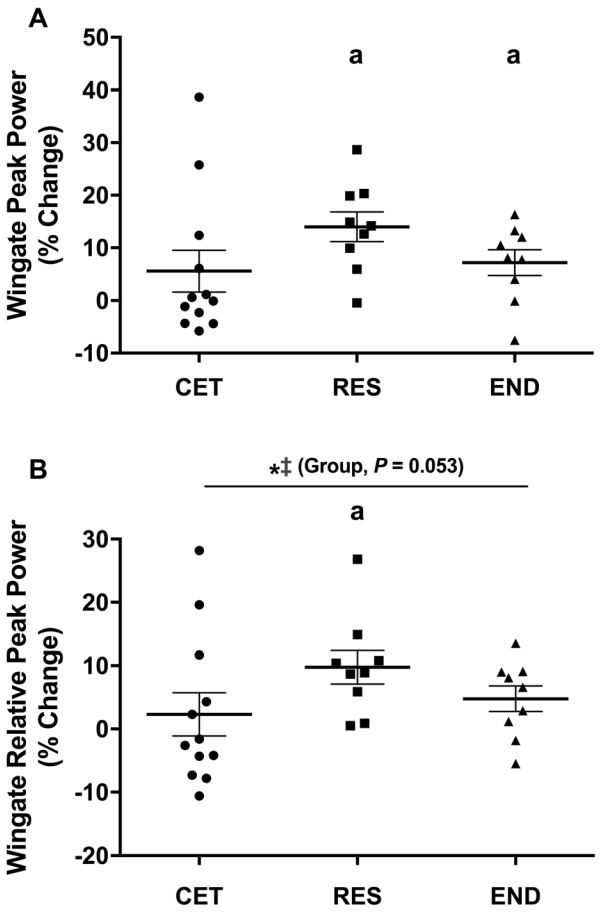

Figure 2.5 Change to A) absolute and B) relative peak power as measured during the Wingate test. Values are presented as per cent change from PRE to POST and presented as individual data with group mean ± SD (RES, *n* = 9; END, *n* = 9). a = *P* < 0.05 from PRE. ǂ = *P* < 0.05 from END at POST. * = *P* < 0.05 from CET at POST. Abbreviations: CET, concurrent exercise training; RES, resistance training; END, endurance training.

2.5.8 Training volume and variables

There was main effect for time for change in resistance training volume (*P* < 0.001); however *post hoc* analysis revealed no difference) between CET and RES across the training intervention (*P* = 0.385; **Appendix B, Table B.2**). Similarly, one-way ANOVA revealed no differences between CET and RES for average time to complete set (*P* = 0.564), between-set rest interval (*P* = 0.915), or RPE (*P* = 0.838; **Appendix B, Figure B.2**). There was main effect for time for change in endurance training volume (*P* < 0.001); however, *post hoc* analysis revealed no difference between

CET and END across the training intervention (P = 0.708; **Appendix B, Table B.2**). One-way ANOVA revealed no difference in average training hours (P = 0.488) or HR (P = 0.222) between CET and END across the training intervention. However, average RPE was 10% higher in CET compared to END (P < 0.001). Recovery time between sessions (**Appendix B, Figure B.2**) was significantly less in CET (23.6 h; P < 0.001) compared to both RES (47.7 h) and END (48 h).

2.5.9 Diet

There was main effect for time (P = 0.005) and group (P = 0.026) for change in energy intake. Energy intake was significantly greater at baseline in RES (~11,300 kJ) compared to END (~8,780 kJ; P = 0.007). Average daily energy intake during training increased from baseline by 12.5% in CET and 20.1% in END (P < 0.05). There was no difference in energy intake across conditions during the training intervention (CET = ~11,400 kJ; RES = ~11,700 kJ; END = ~10,600 kJ; P = 0.348). There was main effect for time (P < 0.001) and group (P = 0.046) for change in protein intake. Protein intake was significantly greater at baseline in CET (1.6 g•kg^{-1}•d^{-1};) and RES (1.7 g•kg^{-1}•d^{-1}) compared to END (1.3 g•kg^{-1}•d^{-1}: P < 0.05). Average daily protein intake during training increased from baseline by 40.6% in CET, 26.3% in RES, and 61.7% in END (P < 0.005). Carbohydrate intake was greater at baseline in RES (4.1 g•kg^{-1}•d^{-1}; P = 0.044) than END (3.1 g•kg^{-1}•d^{-1}); however, no effect for group (P = 0.072), time (P = 0.6), or group by time (P = 0.116) was observed. There was a main effect for group for fat intake (P = 0.004). Fat intake was significantly greater at baseline in RES (1.5 g•kg^{-1}•d^{-1}; P < 0.05) compared to both CET (1.1 g•kg^{-1}•d^{-1}) and END (1 g•kg^{-1}•d^{-1}; **Table 2.3**).

Table 2.3 Average dietary intake at baseline and daily throughout the 12 wk training intervention.

	Time	
	Baseline	**Training**
Energy ($kJ \cdot d^{-1}$)		
CET	10200 ± 2360	11400 ± 1490[a]
RES	11300 ± 1780[ǂ]	11700 ± 1360
END	8780 ± 1900	10600 ± 1630[a]
Protein ($g \cdot kg^{-1} \cdot d^{-1}$)		
CET	1.6 ± 0.51[ǂ]	2.2 ± 0.17[a]
RES	1.7 ± 0.47[ǂ]	2.1 ± 0.17[a]
END	1.3 ± 0.48	2.0 ± 0.13[a]
Carbohydrate ($g \cdot kg^{-1} \cdot d^{-1}$)		
CET	3.5 ± 0.81	3.8 ± 1.00
RES	4.1 ± 1.04[ǂ]	3.6 ± 0.65
END	3.1 ± 0.88	3.0 ± 0.63
Fat ($g \cdot kg^{-1} \cdot d^{-1}$)		
CET	1.1 ± 0.28	1.2 ± 0.34
RES	1.5 ± 0.4*[ǂ]	1.5 ± 0.25
END	1.0 ± 0.32	1.2 ± 0.3

Values are presented as means ± SD. a = $P < 0.05$ from Baseline. ǂ = $P < 0.05$ from END at time point. * = $P < 0.05$ from CET at time point. Abbreviations: CET, concurrent exercise training; RES, resistance training; END, endurance training.

2.6 Discussion

This is the first investigation to compare the effects of long-term (i.e., 12 wk) concurrent training in combination with a high protein diet on a broad range of adaptations in skeletal muscle. The data show that concurrent resistance and endurance training when performed 3 $d \bullet wk^{-1}$ on alternate days, in combination with a high protein availability, does not impair gains in maximal strength, lean mass, or aerobic capacity compared to resistance training alone. In contrast, concurrent training may attenuate specific lower-body developments to maximal anaerobic power output compared to resistance training alone and should be closely monitored. These findings provide novel information for practitioners for prescribing evidence-based recommendations for

concurrent training strategies capable of maximising strength, hypertrophy, and aerobic adaptation responses.

The concurrent training 'interference effect' in strength and power adaptations was first observed by Hickson (Hickson, 1980). Since that seminal study, numerous investigations (Kraemer *et al.*, 1995; Dolezal & Potteiger, 1998; Bell *et al.*, 2000; Häkkinen *et al.*, 2003; Mikkola *et al.*, 2012; Fyfe *et al.*, 2016*a*) have confirmed observations of compromised strength gains when strength and endurance training are undertaken concurrently. In contrast, others (McCarthy *et al.*, 2002; Balabinis *et al.*, 2003; Ahtiainen *et al.*, 2009; Hendrickson *et al.*, 2010; Lundberg *et al.*, 2013; Laird *et al.*, 2016; Tomiya *et al.*, 2017) have reported little or no impairments to strength when undertaking concurrent training. Such disparities may be attributed a number of factors including volume, intensity, and frequency of sessions, as well as training status of participants, modes of exercise being employed, and duration of intervention (Coffey & Hawley, 2017). Indeed, the duration of many studies is less than the 8-wk time point at which the interference effect was first observed (Balabinis *et al.*, 2003; Lundberg *et al.*, 2013). It has been proposed that maximal muscle growth with concurrent training can be achieved by implementing appropriate recovery periods (i.e., 6-24 h) between exercise sessions, incorporating post-exercise nutritional strategies, minimising endurance/aerobic exercise to 2-3 $d \bullet wk^{-1}$, and integrating cycling compared to running as the endurance exercise mode (Baar, 2014*a*; Perez-Schindler *et al.*, 2015; Murach & Bagley, 2016). To address some of these issues and determine whether they might reduce the interference effect, a comprehensive study protocol in which, for the first time in a concurrent training paradigm, the aforementioned recommendations along with a 'high' protein diet were incorporated over a 12-wk training intervention to

determine whether interferences in muscle strength, power and hypertrophy could be offset by following these guidelines.

The first major finding of this work was that muscle strength and hypertrophy with CET were not compromised compared to RES alone. The lack of 'interference' effect was likely due to several interrelated factors implemented in the training intervention. Firstly, the CET group performed resistance and endurance sessions on alternate days to allow a minimum of ~24 h recovery between bouts. Both proximity (Craig *et al.*, 1991; Sporer & Wenger, 2003; Robineau *et al.*, 2016) and order (Okamoto *et al.*, 2007; Cadore *et al.*, 2013; Pinto *et al.*, 2015) of endurance and resistance exercises performed during a concurrent training program can compromise muscle activation and force development, which can hinder the intensity and effort at which subsequent resistance exercise is performed, leading to reduced dynamic strength gains (Eddens *et al.*, 2018). Increasing the recovery time or performing individual modes of exercise on separate days altogether (Sale *et al.*, 1990; Bentley *et al.*, 1998, 2000; Leveritt *et al.*, 2000; Sporer & Wenger, 2003; Robineau *et al.*, 2016) alleviates residual fatigue and prevents impairments to force development. These findings provide supporting evidence of the importance within a concurrent program of performing divergent modes of exercise on alternate days to promote strength adaptations.

The volume of endurance exercise performed can also impact strength adaptations (Wernbom *et al.*, 2007). Findings from a meta-analysis of 21 studies revealed a positive association between duration (length of session) and frequency (days per week) of endurance exercise and the degree of interference to strength gains (Wilson *et al.*, 2012). However, concurrent training incorporating work-matched moderate-intensity continuous (MICT) or high-intensity interval training (HIT) attenuates lower-body strength by a similar magnitude (Fyfe *et al.*, 2016*a*), indicating

that training intensity may not mediate interferences to maximal strength. In the current study, endurance training consisted of a combination of MICT and HIT cycling, with sessions lasting, on average, ~30 min, for 3 d•wk^{-1}. This combination, which significantly increased VO_{2peak}, effectively circumvented any interference to strength development over 12 wk of concurrent training. In this regard, the increase in absolute VO_{2peak} observed with CET is in line with previous literature (Hickson, 1980; Hickson *et al.*, 1988; Häkkinen *et al.*, 2003; Chtara *et al.*, 2005; Rønnestad *et al.*, 2010; Mikkola *et al.*, 2012; Cadore *et al.*, 2013; Silva *et al.*, 2014; Fyfe *et al.*, 2016*a*). However, relative VO_{2peak} was only increased from baseline at WK6 in CET, while END demonstrated improvements from baseline at both WK6 and POST. As both CET and END performed the same volume of cycling, and increased BM similarly throughout the intervention, it is unclear why an increase in relative VO_{2peak} was not observed at POST in CET. Notably, CET displayed a higher average RPE during training, perhaps indicating a greater degree of residual fatigue. Nonetheless, both absolute and relative MAP increased from WK6 to POST in CET, which was not observed in END. Incorporating strength training into an endurance program can improve time to exhaustion and time trial performance (Hickson *et al.*, 1988; Chtara *et al.*, 2005; Mikkola *et al.*, 2012; Beattie *et al.*, 2014; Vikmoen *et al.*, 2017). In agreement, the increase in MAP from WK6 to POST with CET, but not END, highlights the benefit of incorporating resistance exercises to an endurance program for enhancing aerobic performance.

Given the disparities between training regimens and juxtaposition of between-mode recovery amongst studies, it is difficult to attribute the underlying cause of blunted hypertrophy previously observed with concurrent training (Kraemer *et al.*, 1995; Bell *et al.*, 2000; Rønnestad *et al.*, 2012*a*; Fyfe *et al.*, 2016*a*, 2018). One variable

that may partially explain diminished hypertrophy with concurrent training is post-exercise protein feeding. Skeletal muscle hypertrophy occurs as a result of repeated and cumulative increases in rates of muscle protein synthesis (MPS) after exercise and ingestion of dietary proteins (Moore *et al.*, 2012; Areta *et al.*, 2013; Camera *et al.*, 2015). Previous investigations have shown protein ingestion following a single bout of concurrent exercise increases acute rates of MPS, while simultaneously attenuating markers of muscle catabolism, compared to a placebo control (Camera *et al.*, 2015). Given the importance for dietary protein to enhance muscle growth and remodelling processes, insufficient protein intake around concurrent training sessions may not have maximally stimulated MPS, resulting in the attenuated muscle hypertrophy observed previously (Kraemer *et al.*, 1995; Bell *et al.*, 2000; Rønnestad *et al.*, 2012*a*; Fyfe *et al.*, 2016*a*, 2018). While such a hypothesis is attractive, it is acknowledged that without a placebo comparison, the degree to which protein supplementation facilitated lean mass increases observed in the current investigation can only be speculated. Furthermore, cycling performed in isolation has been shown to induce leg muscle hypertrophy (Konopka & Harber, 2014), so to what extent protein supplementation influenced the similar post-intervention increase in leg lean mass observed in END compared to CET and RES is unclear.

In contrast to muscle strength and hypertrophy responses, improvements to aspects of muscle power, determined by relative Wingate peak power output, showed a tendency to decrease with CET compared to RES. Previous studies report maximal power output may be more susceptible to impaired development with concurrent training (Kraemer *et al.*, 1995; Häkkinen *et al.*, 2003; Chtara *et al.*, 2008; Wilson *et al.*, 2012; Fyfe *et al.*, 2016*a*). Compromised power output after concurrent training may be due to impaired rate of force development (Dudley & Djamil, 1985; Häkkinen *et al.*,

2003; Rønnestad *et al.*, 2012*a*; Mikkola *et al.*, 2012; Fyfe *et al.*, 2016*a*) or changes to fibre type (Kazior *et al.*, 2016) and shortening velocity (Linari *et al.*, 2004). Force development relies on neural components (e.g., axonal conduction velocity) and myofibre size (Henneman, 1957; Bawa *et al.*, 1984), as well as structural properties (e.g., dystrophin) to transfer force across joints (Hughes *et al.*, 2017). During muscular contraction, force is transferred from the muscle to tendon both longitudinally (Huxley & Niedergerke, 1954) as a result of sarcomere shortening and laterally (Street, 1983) via the extracellular matrix (ECM). Resistance training increases collagen synthesis in the ECM and tendon (Miller *et al.*, 2005), which, over time, increases tendon cross-sectional area (Kongsgaard *et al.*, 2007; Rønnestad *et al.*, 2012*b*) and stiffness (Couppé *et al.*, 2008). Increases in tendon stiffness are associated with greater torque production and athletic performance (Watsford *et al.*, 2010). Notably, such adaptations to connective tissue appear to be impaired with concurrent training (Rønnestad *et al.*, 2012*b*), and may be a source of diminished capacity to generate force rapidly. Similarly, concurrent training can alter fibre type distribution (Kazior *et al.*, 2016), which may result in changes to power development, as optimal shortening velocity and stretch-dependent force differs between fibre types (Linari *et al.*, 2004), Given the similar architectural changes between CET and RES in the present study, it is possible that CET impaired resistance training-induced adaptations to connective tissue and fibre type distribution, resulting in compromised power outputs.

In contrast to changes in relative Wingate peak power, other measures of power such as the CMJ and SJ were not impaired with CET. This anomaly may be explained by differences in neuromuscular activation between tests. Unlike the single CMJ or SJ, the 30-s all-out Wingate requires coordination of repetitive high-force contractions of antagonistic muscles of the contralateral leg (Driss & Vandewalle, 2013). Given the

greater frequency and total volume of exercise, it is possible that Wingate performance may have been attenuated with CET as a function of accumulated fatigue and compromised neuromuscular coordination of repeated high-force contractions. As power producing-capacity is a hallmark of athletic performance (Watsford *et al.*, 2010), future studies incorporating electromyography on multiple muscle groups are needed to monitor fatigue and alterations to neural drive with concurrent training. It should also be noted that the current study may be underpowered to detect appreciable changes in power output as power calculations were based on lean mass change as the primary outcome measure.

Several limitations in the present study are acknowledged. First, without a placebo comparison, limited inferences can be made on whether similar increases in lean mass and strength were due to protein supplementation per se or other factors (i.e., between-session recovery, resistance training program, etc.). Future studies combining concurrent training with protein or placebo supplementation are needed to determine the capacity of protein to directly combat interferences to lean mass and strength. Second, alternate modes of endurance training (i.e., cycling versus running) were not compared. Given the need for sport specific conditioning, future investigations comparing the incorporation of cycling or running in a concurrent training program are needed to identify if both modalities can be equally compatible with strength training. Finally, it is acknowledged that concurrent training bouts cannot always be performed on alternating days; particularly with team sports which often train twice per day (Jones *et al.*, 2017). Future studies comparing shorter recovery (i.e., 6-8 h) between sessions in trained athletes are therefore required to optimise adaptations to the demands of same day concurrent training. Similarly, the higher training load associated with concurrent training may increase risk of overtraining and have detrimental impacts on performance

outcomes and rates of injury (Gabbett, 2016). It is presently unclear whether matching the weekly hours of training between concurrent and single-mode training (i.e., 3 h•wk^{-1}) can produce similar degrees of adaptation.

2.7 Conclusion

In conclusion, this is the first investigation to determine the effects of chronic concurrent training in combination with a high protein diet on adaptations to muscle strength, aerobic capacity, and maximal power output, as well as lean mass and architectural changes in skeletal muscle. These findings demonstrate that concurrent resistance and endurance training, each performed 3 d•wk^{-1}, in the face of a high protein diet, did not impair gains in maximal strength, CMJ, SJ, VO_{2peak}, lean mass or muscle architectural changes compared to resistance training alone. However, concurrent training does attenuate improvements to select aspects of lower-body maximal anaerobic power output compared to resistance training, demonstrating a susceptibility in adaptation responses in this paradigm despite recommended optimal protein intake strategies.

2.8 Practical Applications

These findings provide support for theoretical recommendations for practitioners prescribing concurrent training strategies capable of maximising strength, hypertrophy and aerobic adaptation responses. First, perform resistance training and endurance training on alternate days to provide sufficient recovery/rest between modes of exercise such that residual fatigue does not limit session intensity (Eddens *et al.*, 2018). Second, ensure an adequate intake and even distribution of high quality proteins throughout the day, with particular emphasis on intake around exercise (Loenneke *et*

al., 2016). Third, limiting endurance training (where possible) to ~30 min per session performed 3 d•wk^{-1} is sufficient to improve aerobic performance without compromising maximal dynamic strength (Wilson *et al.*, 2012).

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Author Contributions

B. Shamim, V.G. Coffey, J.A. Hawley, and D.M. Camera designed the study; B. Devlin performed all DXA scans, dietary consults, and dietary analyses; B. Shamim, R.G. Timmins, P. Tofari, and D.M. Camera conducted all exercise and performance testing; B. Shamim, C. Lee Dow, and D.M. Camera supervised all exercise training sessions; B. Shamim, R.G. Timmins, P. Tofari, and D.M. Camera performed all data and statistical analysis; B. Shamim and D.M. Camera wrote the manuscript; all authors approved the manuscript before submission.

Compliance with Ethical Standards

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Conflicts of Interest

Baubak Shamim, Brooke L. Devlin, Ryan G. Timmins, Paul Tofari, Connor Lee Dow, Vernon G. Coffey, John A. Hawley, and Donny M. Camera declare no conflicts of interest.

CHAPTER 3

MYOFIBRE HYPERTROPHY IN THE ABSENCE OF CHANGES TO SATELLITE CELL CONTENT FOLLOWING CONCURRENT EXERCISE TRAINING IN YOUNG HEALTHY MEN

3.1 Abstract

Increases in satellite cell content support myofibre hypertrophy and are influenced by exercise mode. Changes in satellite cell and myonuclear content were determined following a period of combined resistance and endurance exercise training (concurrent training, CET) in 32 recreationally active males (age: 25±5 y; body mass index: 24±3 kg•m^{-2}; mean±SD) who undertook 12-wk of either isolated (3 d•w^{-1}) resistance (RES; *n*=10), endurance (END; *n*=10), or alternate day (6 d•w^{-1}) concurrent (CET, *n*=12) training. *Vastus lateralis* muscle biopsies were obtained pre-intervention and after 2, 8, and 12 wk of training to determine fibre type-specific cross-sectional area (CSA), satellite cell content (Pax7^{+}DAPI^{+}), and myonuclei (DAPI^{+}) using immunofluorescence microscopy. After 12 wk, myofibre CSA increased in all training conditions in type II (P = 0.0149) and mixed fibres (P = 0.0102), with no difference between conditions. Satellite cell content remained unchanged in both type I and type II fibres after training. Significant correlations were observed between increases in fibre type-specific myonuclear content and CSA of Type I (r = 0.63, P < 0.0001), Type II (r = 0.69, P < 0.0001), and mixed fibres (r = 0.72, P < 0.0001). Resistance, endurance, and concurrent training induce similar myofibre hypertrophy in the absence of satellite cell and myonuclear pool expansion. These findings suggest that satellite cell content does not limit the magnitude of hypertrophy at least during the first 12 wk of concurrent training, and that individuals with more myonuclear content displayed greater myofibre hypertrophy.

3.2 Introduction

The combination of resistance- and endurance-based exercise training, or 'concurrent exercise training', is undertaken by individuals participating in a wide array of sports to improve muscular strength and aerobic capacity (Nader, 2006). It has recently been proposed that the potential for myofibre hypertrophy in response to chronic concurrent exercise training may be limited by satellite cell content (Babcock *et al.*, 2012). Satellite cells are myogenic precursor cells that reside between the sarcolemma and basal lamina (Mauro, 1961). In adult skeletal muscle, satellite cells exist in a quiescent state and are activated in response to various stimuli, such as exercise-induced mechanical stress and growth factors (Snijders *et al.*, 2015*a*). Once activated, satellite cells can differentiate to form new myonuclei and increase transcriptional capacity through a process known as myogenesis, or return to quiescence to replenish the satellite cell pool through self-renewal (Kadi *et al.*, 2005).

As myonuclei are post-mitotic, the addition of new myonuclei to support fibre adaptations is dependent on the differentiation of satellite cells. It has been hypothesised that a myonucleus has control over a finite amount of cytoplasm, referred to as the myonuclear domain (Cheek, 1985). In turn, accretion of myonuclei with exercise training is assumed to accommodate the increased demands for transcriptional activity and synthesis of new proteins to support hypertrophy (Cheek, 1985; Allen *et al.*, 1999) only when the myonuclear domain exceeds a threshold of ~2,250 μm^2 (Petrella *et al.*, 2006, 2008) or a ~26% increase in myofibre cross-sectional area occurs (Kadi *et al.*, 2004). Moreover, both satellite cell (Petrella *et al.*, 2008; Verdijk *et al.*, 2014; Moore *et al.*, 2018) and myonuclear (Petrella *et al.*, 2006, 2008) content have been shown to positively correlate with changes in myofibre cross-sectional area, suggesting an important relationship between myogenesis and myofibre hypertrophy.

Increases in satellite cell content are detectable for up to eight days and peak approximately three days post-exercise (Snijders *et al.*, 2015*a*). The degree of satellite cell activation and proliferation appears to be influenced by the mode of exercise performed. Evidence of satellite cell proliferation following resistance exercise is well documented (Crameri *et al.*, 2004, 2007; Babcock *et al.*, 2012; Snijders *et al.*, 2014*a*; Farup *et al.*, 2014; Nederveen *et al.*, 2015, 2017; Reidy *et al.*, 2017*a*; Damas *et al.*, 2018*a*; Pugh *et al.*, 2018). Conversely, the capacity for acute endurance exercise to expand the satellite cell pool is less apparent (Snijders *et al.*, 2011; Babcock *et al.*, 2012; Nederveen *et al.*, 2015), and may be dependent on exercise mode (Mackey *et al.*, 2007) or intensity (Nederveen *et al.*, 2015; McKenzie *et al.*, 2016). Nonetheless, augmentations in satellite cell content have been reported following prolonged endurance training both in the presence (Charifi *et al.*, 2003; Verney *et al.*, 2008; Murach *et al.*, 2016) and absence (Joanisse *et al.*, 2013) of myofibre hypertrophy.

Following a single bout of unilateral concurrent exercise, Babcock and colleagues (2012) demonstrated that satellite cell proliferation was impaired compared to resistance exercise performed alone in the contralateral leg in young, healthy males. Based on this observation, the authors hypothesised that concurrent exercise impairs satellite cell responses and may contribute to limitations in myofibre hypertrophy observed with chronic concurrent exercise training. However, baseline satellite cell content prior to exercise was elevated in the concurrent exercise leg compared to the resistance exercise only leg. Thus, the stable satellite cell content in response to concurrent exercise may have been indicative of a reduced need for proliferation rather than an inhibition of satellite cell activation *per se*. Recently, Pugh and colleagues (2018) demonstrated that a single bout of concurrent or resistance exercise result in comparable increases in satellite cell content in sedentary, overweight and obese,

middle-aged individuals, suggesting that concurrent exercise does not prevent satellite cell expansion if a high intensity endurance exercise stimulus is provided. Still, to date, no studies have directly investigated the effect of chronic concurrent exercise training on changes in satellite cell content compared to isolated resistance or endurance training.

As satellite cell content appears to be associated with changes in myofibre cross-sectional area (Petrella *et al.*, 2008; Verdijk *et al.*, 2014; Moore *et al.*, 2018), and performing a greater volume of work during concurrent exercise does not result in greater satellite cell proliferation compared to resistance exercise (Babcock *et al.*, 2012; Pugh *et al.*, 2018), the aim of the present investigation was to evaluate whether changes in fibre type-specific satellite cell abundance can explain limitations in the magnitude of hypertrophy achieved during concurrent training. Given the higher training volume completed over 12 wk of concurrent training resulted in similar lean mass gains as isolated resistance and endurance training despite implementing the recommended strategies to maximise hypertrophic potential (Shamim *et al.*, 2018*b*), it was hypothesised that concurrent training would result in similar increases to satellite cell content compared to isolated resistance or endurance training.

3.3 Methods

3.3.1 Experimental overview

Using a parallel-groups design, participants were stratified according to lean body mass and allocated to either a resistance only (RES; n = 10), endurance only (END; n = 10) or concurrent resistance and endurance exercise training (CET; n = 12) group for 12 wk. Measures of maximal strength, aerobic capacity, and anaerobic power, as well as body composition were performed pre- and post-intervention and have been

previously reported (Shamim *et al.*, 2018*b*; Timmins *et al.*, 2020). Resting skeletal muscle biopsies were taken from the *vastus lateralis* at baseline (pre-intervention), and after 2, 8, and 12 wk of training. The study was approved by the Australian Catholic University Human Research Ethics Committee and was carried out in accordance with the latest revision of the Declaration of Helsinki. This trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12617001229369).

3.3.2 Participants

Thirty-two young, healthy, recreationally active males (age: 25±5 y, body mass index: 24±3 $kg \cdot m^{-2}$; mean±SD) who had not participated in a structured exercise program for ≥6 months preceding the study volunteered to participate. Participants were deemed healthy and eligible to participate based on their responses to a cardiovascular risk-factor questionnaire. All experimental procedures and risks associated with the study were explained to participants prior to providing written informed consent.

3.3.3 Exercise training

A detailed outline of the training programs has been reported elsewhere (Shamim *et al.*, 2018*b*). Briefly, for the duration of the intervention, participants in the RES and END group performed three non-consecutive days of training each week. Participants in the CET group trained 6 $d \cdot wk^{-1}$ and performed identical resistance and endurance programs on alternating days as those in the RES and END groups, respectively. All training sessions were performed under the supervision of a member of the research team. Resistance training consisted of whole-body exercises with a focus on the leg press, knee extension and bench press movements, with these exercises performed at an intensity of ~60-98% of 1RM. If a participant was unable to achieve

the prescribed number of repetitions, the weight was lowered by ~5-10% for the following set to uphold the repetition scheme. Endurance cycle training was performed on Lode cycle ergometers and consisted of a mixture of a hill simulation ride of varying intensity (25-110% of maximum aerobic power (MAP), moderate-intensity continuous training at 50% MAP, moderate-intensity interval training at 70% MAP, and high-intensity interval training at 100% MAP. Moderate-intensity intervals were separated by a 60 second recovery period at ~40% MAP, to establish a 2.5:1 or 5:1 work-to-rest ratio. High-intensity intervals were separated by 20-60 s recovery periods, completed at ~40% MAP, to establish a 1:5, 1:2, or 1:1 work-to-rest ratio. All sessions were preceded by a standardised warm up for the respective training modality. Progressive overload was applied by periodically manipulating the number of sets and repetitions (resistance training), number of intervals (endurance training), and relative intensity of load the 12-wk program.

3.3.4 Diets

A free-living, high-protein (2 $g \bullet kg^{-1} \bullet d^{-1}$) eating plan was implemented over the 12-wk intervention. Participants completed daily food records and attended consultations with an Accredited Practicing Dietitian on a fortnightly basis to monitor protein and energy intakes. All participants consumed ~34 g of whey protein upon cessation of every training session to promote muscle protein synthesis (Macnaughton *et al.*, 2016). Diet records were analysed for energy ($kJ \bullet kg^{-1}$), protein, carbohydrate, and fat ($g \bullet kg^{-1}$ for all macronutrients) to provide a daily average for the entire 12-wk intervention. Daily averages of the these dietary parameters have been previously published (Shamim *et al.*, 2018*b*).

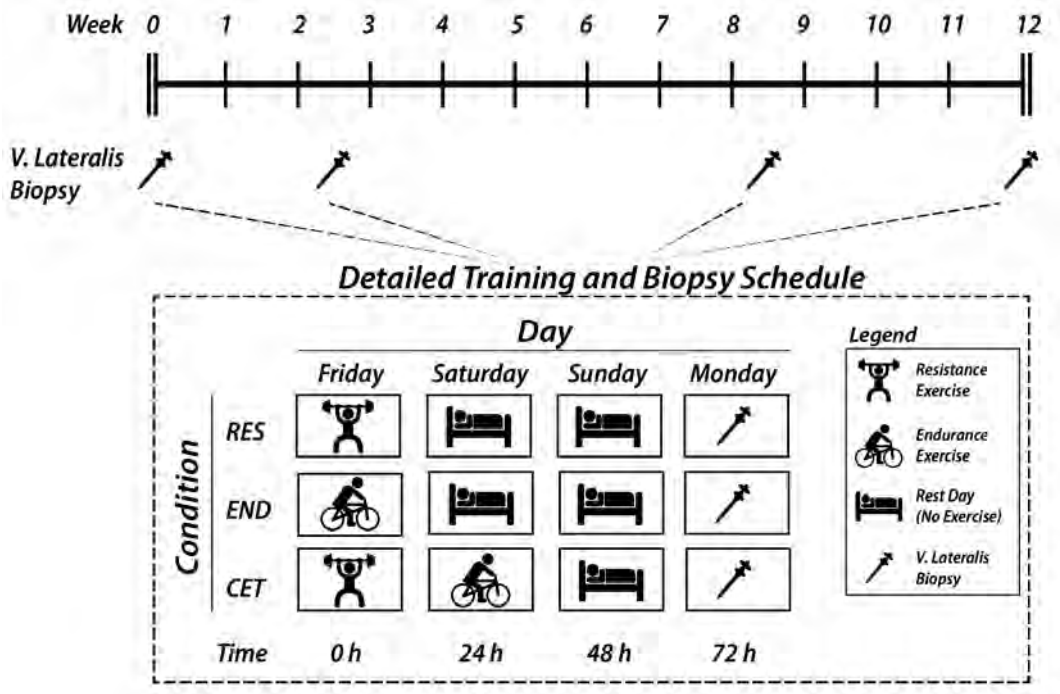

Figure 3.1 Schematic overview of study timeline and detailed vastus lateralis muscle biopsy sampling times following endurance (END), resistance (RES), or concurrent (CET) training.

3.3.5 Skeletal muscle biopsy

Resting skeletal muscle biopsies were taken after an overnight fast pre-intervention and 72 h after the last exercise session of weeks 2, 8, and 12 from the *vastus lateralis* using a Bergstrom needle modified for manual suction under local anaesthesia (2% Xylocaine). Biopsies in the CET condition were taken 72 h after resistance exercise to determine the effects of endurance exercise performed on alternate days on satellite cell expansion (**Figure 3.1**). Samples were immediately frozen in liquid nitrogen or embedded in optimum cutting temperature compound (Scigen) and frozen in liquid nitrogen-cooled isopentane. Samples were stored at -80^{o}C for subsequent analysis.

3.3.6 Immunohistochemistry

Muscle cross-sections (7 μm) were stained for cross-sectional area (CSA), fibre type, myonuclei, and satellite cells. Cross-sections were fixed in 2% formaldehyde, washed, blocked for 90 min (1X phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA), 5% fetal bovine serum (FBS), 0.2% Triton X-100, 2% goat serum (GS), and 0.02% NaN_3), and then incubated overnight at 4^{o}C in primary antibodies for Pax7 (1:4, DSHB) and laminin (1:250, ab11575, Abcam). Sections were washed, then incubated for 2 h in secondary antibodies for Alexa Fluor 488 (#A32731) and 594 (#A11032; Invitrogen). Sections were re-fixed, washed, blocked for 2 h (5% GS containing 0.01% Triton x-100 and 0.05% NaN_3), and then incubated in primary antibody for MHCI (1:4, A.4.951, DSHB) overnight at 4^{o}C. Sections were washed then incubated for 2 h in secondary antibody (Alexa Fluor 488, #A11029, Invitrogen), nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI, 1:20,000, Life Technologies), and cover slips affixed with ProLong™ Diamond Antifade Mountant (Invitrogen).

Staining for fibre CSA and fibre-typing was undertaken following fixation by blocking sections for 2 h (5% GS containing 0.01% Triton X-100 and 0.05% NaN_3), then incubating in primary antibodies for MHCI and laminin overnight at 4^{o}C. Sections were washed, incubated in appropriate secondary antibodies, washed again, and then affixed with cover slips. As an antibody for MHCIIa was not used, fibres that were negative for MHCI are referred to as MHCII, which includes MHCIIa and MHCIIx fibre types (Babcock *et al.*, 2012).

All antibodies were diluted in 1% BSA, and secondary antibodies diluted 1:500. Images were observed under an EVOS™ FL Auto 2 microscope (Invitrogen) at 20X (nuclei) and 10X (CSA) objectives. An average of 218 ± 30 (90 ± 31 Type I, 128 ± 35

Type II) and 230 ± 44 (98 ± 24 Type I, 132 ± 38 Type II) fibres were assessed per participant at each time point for CSA and satellite cell enumeration, respectively. Peripheral muscle fibres that displayed irregular edge staining patterns or disrupted cell membranes and longitudinal fibres (circularity < 0.6) were excluded from analyses. Images were analysed using ImageJ-Fiji.

3.3.7 Statistical analysis

Linear mixed-effect (LME) model, fit by restricted maximum likelihood estimate with random intercept for subject was used to test the effect of training condition on myofibre CSA, myofibre-type distribution, myonuclear number, and satellite cell content. Interactions for training condition × time were tested by the same LME. Where LME revealed significance, a Bonferroni *post hoc* test for pair-wise comparisons was performed. The relationships between baseline satellite cell content and change in fibre CSA as well as change in myonuclear content and change in fibre CSA were determined by calculating Pearson correlation coefficients (*r*). Statistical significance was set at $P < 0.05$. Data are presented as mean ± standard deviation. Statistical analysis was performed using R (v3.5.2).

3.4 Results

3.4.1 Fibre cross-sectional area and distribution

Following the 12 wk training intervention, there was a main effect of condition ($P = 0.0317$), but not time or condition by time for an increase in Type I fibre CSA. However, when corrected for multiple comparisons, *post hoc* analysis revealed no significant differences in Type I fibre CSA for condition (**Figure 3.2A**). Only a main

effect of time (P = 0.0474), but not condition or condition by time was observed for an increase in Type II fibre CSA after the different training modalities. *Post hoc* analysis revealed an increase in Type II fibre CSA at Wk12 compared to Pre (P = 0.0149; **Figure 3.2B**). Similarly, when the mean CSA of both Type I and II fibres was assessed, there was a main effect of time (P = 0.0487), but no main effect for condition or condition by time. *Post hoc* analysis revealed an increase in mixed fibres CSA at Wk12 compared to Pre (P = 0.0102; **Figure 3.2C**).

Fibre-type distribution was unaffected by the training intervention, and displayed no main effect of condition, time or condition by time (**Figure 3.3A-B**).

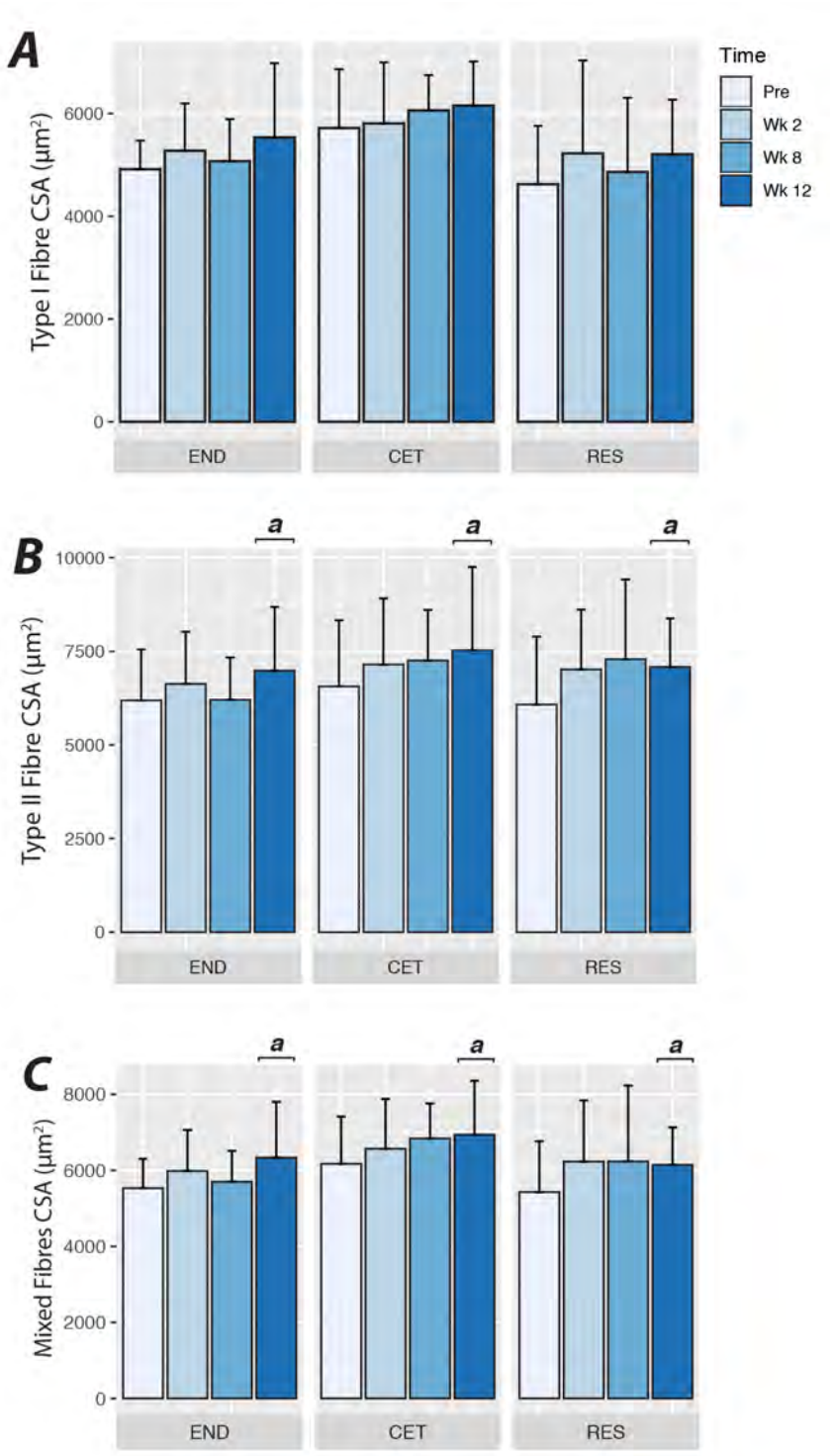

Figure 3.2 Changes to myosin heavy chain type I (A), myosin heavy chain type II (B), and mixed (C) myofibre cross-sectional area (CSA) in response to endurance (END; n = 10), resistance (RES; n = 10), or concurrent (CET; n = 12) training. *a* = significantly different from Pre time point ($P < 0.05$). Values are presented as mean ± standard deviation.

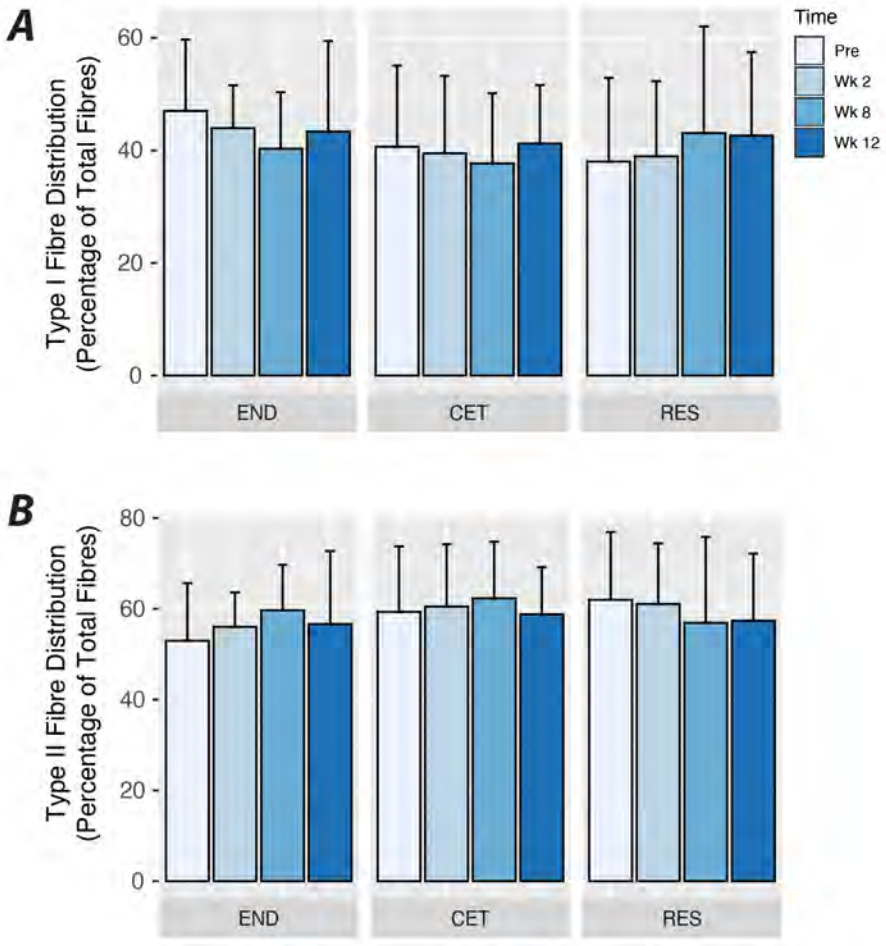

Figure 3.3 Changes to type I (A) and type II (B) fibre distribution in response to endurance (END; *n* = 10), resistance (RES; *n* = 10), or concurrent (CET; *n* = 12) training. Values are presented as mean ± standard deviation.

3.4.2 Satellite cell content

Satellite cells were determined as staining positive for both DAPI and Pax7 and having a location between the basal lamina and the plasma membrane of myofibres (see **Figure 3.4** for representative stain). In response to 12 wk of exercise training there was no main effect of condition, time or condition by time for change in Type I satellite cell content (**Figure 3.5A**). Similarly, there was no main effect of condition, time or condition by time for change in Type II satellite cell content (**Figure 3.5B**). There was no main effect of condition, time or condition by time for change in mixed fibre-type satellite cell content (**Figure 3.5C**).

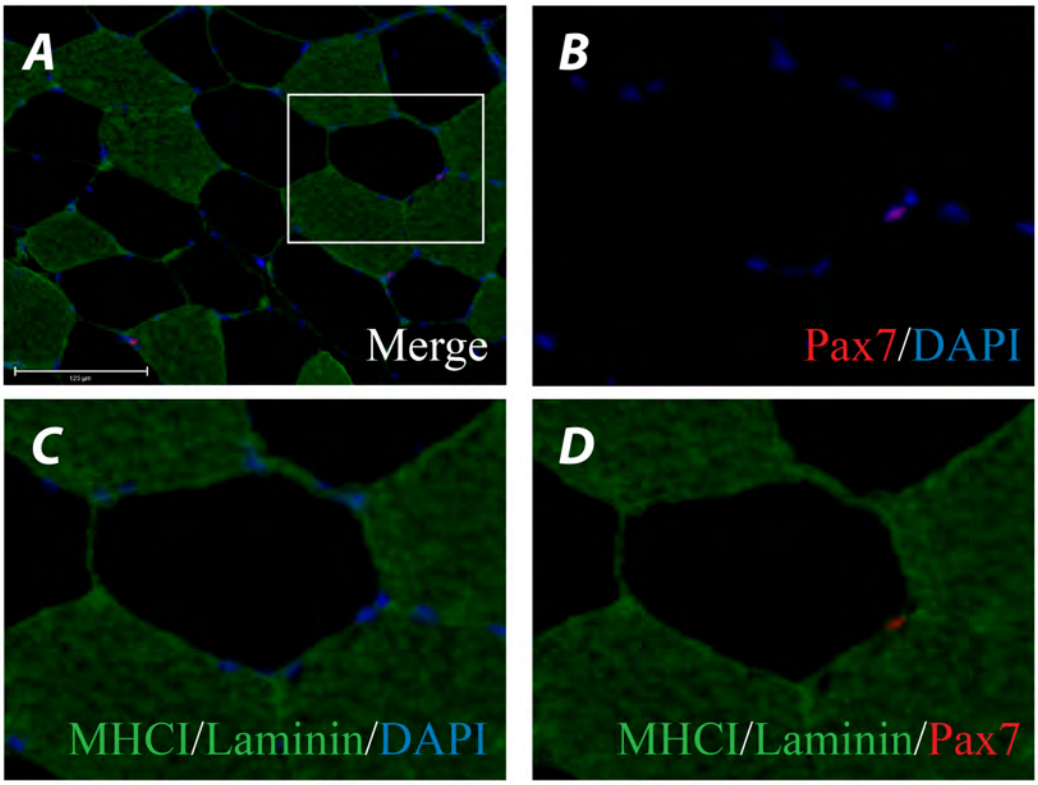

Figure 3.4 Representative image of a myosin heavy chain (MHC) I/Laminin/Pax7/DAPI stain of a muscle cross-section (A). Channel view of Pax7/DAPI (B), MHCI/Laminin/DAPI (C), and MHCI/Laminin/Pax7 (D).

3.4.3 Myonuclear content and domain

In response to training, there was a main effect of time (P = 0.044), but not condition or condition by time for an increase in Type I myonuclear content. Post hoc analysis revealed that myonuclear content was greater at Wk8 compared to Wk2 (P = 0.0031) and Wk12 (P = 0.0410; **Figure 3.5D**). Conversely, training did not alter Type II myonuclear content as no main effect of condition, time or condition by time was observed (**Figure 3.5E**). When the mean myonuclear content of both Type I and II fibres was assessed, a main effect of time (P = 0.0302), and condition by time (P = 0.0350), but not condition was observed. *Post hoc* analysis revealed mixed fibre myonuclear content was greater at Wk8 (2.25 ± 0.679 $DAPI^+$/Fibre) compared to Wk2 (1.85 ± 0.532 $DAPI^+$/Fibre; P = 0.0297; **Figure 3.5F**).

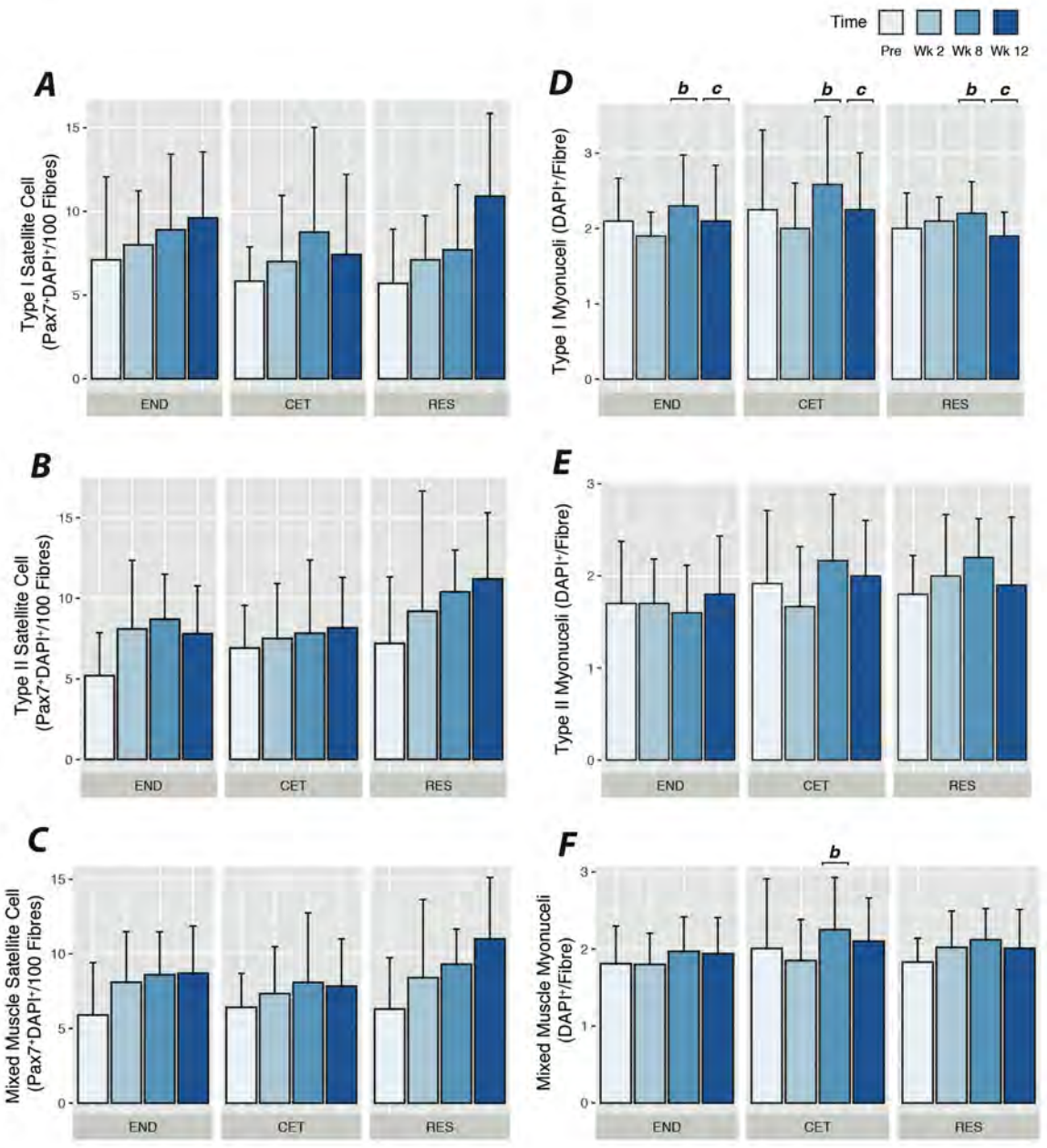

Figure 3.5 Fibre type-specific satellite cell (A-C) and myonuclear (D-F) expansion in response to endurance (END, n = 10), resistance (RES; n = 10), or concurrent (CET; n = 12) training. Change in myosin heavy chain type I (A), myosin heavy chain type II (B), and mixed (C) myofibre satellite cell content. Change in myosin heavy chain type I (D), myosin heavy chain type II (E), and mixed (F) myofibre myonuclear content. *b* = significantly different from week 2 time point ($P < 0.05$). *c* = significantly different from week 8 time point ($P < 0.05$). Values are presented as mean ± standard deviation.

Despite an increase in Type I myonuclear content, no main effect of condition, time or condition by time was seen for Type I myonuclear domain. Conversely, Type II myonuclear domain increased in response to training as a main effect of time (P = 0.0341), but not condition or condition by time. However, when corrected for multiple comparisons, *post hoc* analysis revealed no significant differences in Type II myonuclear domain with time. Likewise, when the mean myonuclear domain of both Type I and II fibres was assessed, no main effect of condition, time or condition by time was present (data not shown).

3.4.4 Pearson's correlation coefficients of muscle characteristics

There were significant correlations between increases in fibre type-specific myonuclear content and increases in fibre CSA for Type I (r = 0.63, P < 0.0001; **Figure 3.6A**), Type II (r = 0.69, P < 0.0001; **Figure 3.6B**), and mixed fibres (r = 0.72, P < 0.0001; **Figure 3.6C**). There was no relationship between pre-intervention fibre type-specific satellite cell content and increases in fibre CSA for Type I (r = -0.073, P = 0.69; **Figure 3.6D**), Type II (r = 0.048, P = 0.8; **Figure 3.6E**), or mixed fibres (r = -0.08, P = 0.66; **Figure 3.6F**). There was no relationship between change in fibre type-specific satellite cell content and increase in fibre CSA for Type I (r = 0.22, P = 0.23; **Figure 3.7A**), Type II (r = 0.24, P = 0.19; **Figure 3.7B**), or mixed fibres (r = 0.23, P = 0.22; **Figure 3.7C**).

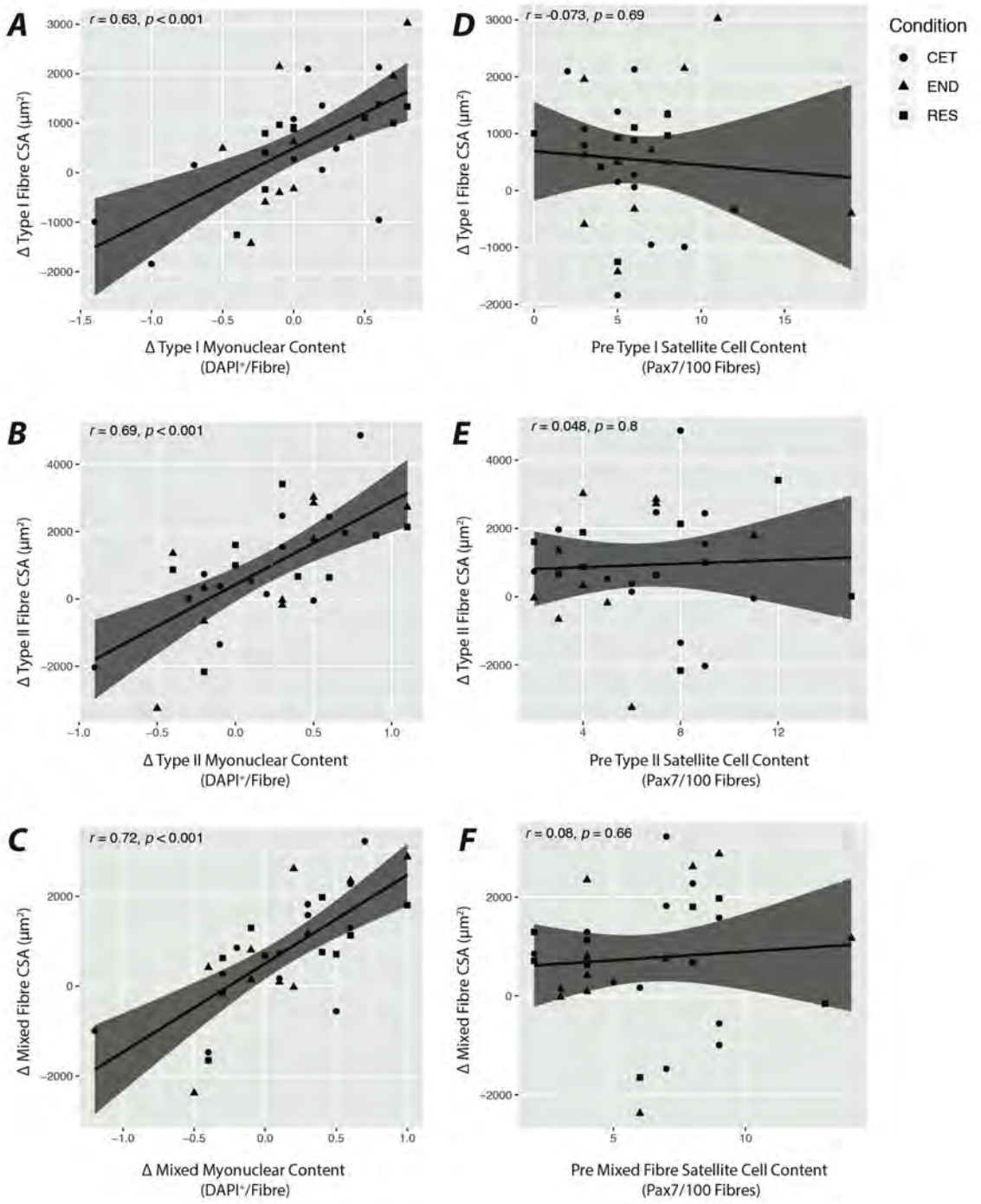

Figure 3.6 Pearson correlation coefficients (*r*) showing the relationship between fibre type-specific changes in cross-sectional area (CSA) and myonuclear content (A-C) and fibre type-specific changes in CSA and pre-intervention baseline satellite cell content (D-F). Statistical significance was set at $P < 0.05$. Abbreviations: CET, concurrent training; END, endurance training; RES, resistance training.

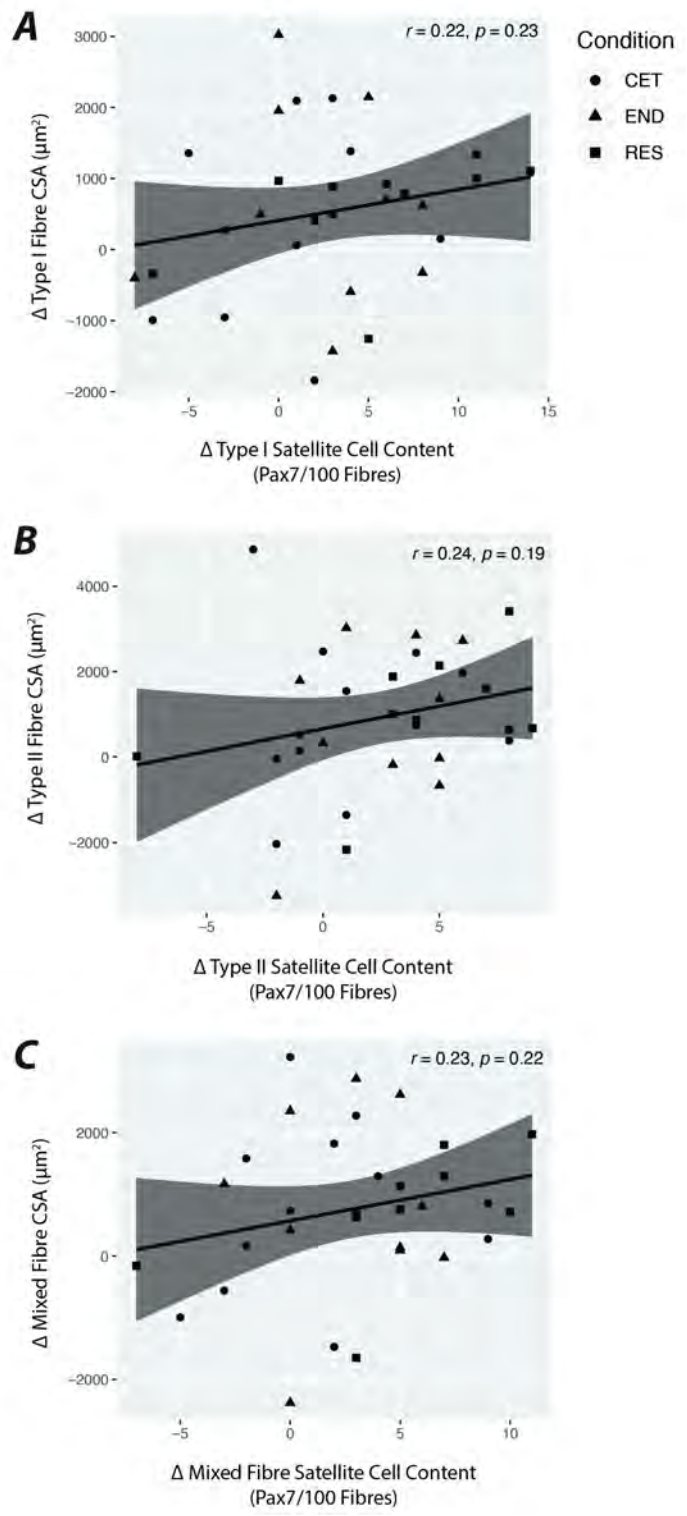

Figure 3.7 Pearson correlation coefficients (*r*) showing the relationship between fibre type-specific changes in cross-sectional area (CSA) and changes in satellite cell content (A-C). Statistical significance was set at $P < 0.05$. Abbreviations: CET, concurrent training; END, endurance training; RES, resistance training.

3.5 Discussion

The aim of the present investigation was to evaluate whether the magnitude of muscle hypertrophy achieved during concurrent training is affected by time-dependent changes in fibre type-specific satellite cell abundance. In response to 12 wk of endurance, resistance, and concurrent training, fibre CSA and myonuclear content are increased without a concomitant change in total satellite cell content. These results were contrary to the original research hypothesis given that it has been suggested that modulating concurrent training variables in association with protein availability may augment muscle hypertrophy compared to resistance training alone (Murach & Bagley, 2016). In the present study, Type II and mixed myofibre CSA hypertrophy increased over the duration of the training intervention for all training conditions. However, despite implementing recommendations to maximise hypertrophy with concurrent training (Murach & Bagley, 2016), no differences in the magnitude of myofibre hypertrophy achieved were observed between the different training interventions.

Previous reports of augmented hypertrophy (e.g. increased myofibre CSA) with concurrent training compared to resistance training alone have been without optimal nutritional support, and have been of shorter duration (Lundberg *et al.*, 2013; Kazior *et al.*, 2016). However, longer training interventions (i.e.,≥ 20 wk), have also failed to observe any differences in myofibre CSA hypertrophy compared to resistance training (Sale *et al.*, 1990; Häkkinen *et al.*, 2003). Therefore, it is possible that variance in the magnitude of myofibre hypertrophy observed with concurrent training between previous work (Sale *et al.*, 1990; Häkkinen *et al.*, 2003; Lundberg *et al.*, 2013; Kazior *et al.*, 2016) and the present study are due to differences in the length of the intervention or nutritional support provided. Accordingly, while the findings in the present study

support the recent recommendations for concurrent exercise training prescription to promote hypertrophy (Murach & Bagley, 2016), they do not appear to facilitate an augmented hypertrophic response compared to resistance or endurance training over 12 wk at the myofibre CSA level.

It is unclear why the greater volume of work completed by participants in the concurrent training group did not induce a greater increase in myofibre CSA, especially as it has been hypothesised that the potential for myofibre hypertrophy to occur with chronic concurrent training may be limited by satellite cells (Babcock *et al.*, 2012). Evidence for impaired satellite cell responses with concurrent exercise compared to resistance exercise alone has been observed when a bout of moderate intensity resistance exercise is followed immediately by moderate intensity continuous cycling in young, healthy males (Babcock *et al.*, 2012). However, performing moderate intensity resistance exercise followed immediately by high-intensity interval cycling results in an increase in Type I fibre satellite cell content comparable to resistance exercise alone in sedentary, overweight and obese, middle-aged individuals (Pugh *et al.*, 2018).

In the present study, both moderate intensity continuous and high intensity interval cycling was implemented and resulted in little difference in satellite cell content between CET and RES over the intervention. Contrary to the initial hypothesis, no increase in satellite cell content was observed in response to any training modality. While differences in exercise intensity, training status of participants, and baseline satellite cell content may partially explain disparities in satellite cell expansion previously observed with concurrent exercise (Babcock *et al.*, 2012; Pugh *et al.*, 2018), it is difficult to reconcile why no increase in satellite cell content was observed in response to either resistance or endurance training alone. Given the hypertrophy

observed in Type II and mixed fibre CSA in the absence of myonuclear addition, it is possible that the existing myonuclei were able to support the degree of hypertrophy achieved and that expansion of the satellite cell pool to promote myogenesis was not needed. While the current findings demonstrate that concurrent training does not result in an additive expansion in satellite cell numbers compared to isolated endurance or resistance training, without having observed an increase in satellite cell content in response to endurance or resistance training alone, the possibility that concurrent training impairs satellite cell expansion cannot be rejected, and deserves further investigation.

In addition to the lack of satellite cell pool expansion, activation of fibre type-specific satellite cells has also been shown to be limited by concurrent exercise compared to resistance exercise (Pugh *et al.*, 2018). As satellite cell activation can generate both progeny for self-renewal of the satellite cell pool and myogenic precursors to undergo terminal differentiation (Kuang *et al.*, 2007), a blunted response may underlie limited satellite cell expansion previously observed following concurrent exercise (Babcock *et al.*, 2012; Pugh *et al.*, 2018). While there appears to be little or no satellite cell activation 96 h after a bout of concurrent exercise (Babcock *et al.*, 2012; Pugh *et al.*, 2018), increases in satellite cell activation have been observed 9 h after a single bout of concurrent exercise in young, healthy males (Snijders *et al.*, 2012). Given the transient nature of satellite cell activation (Snijders *et al.*, 2015*a*), it is possible the absence of active satellite cells at 96 h after concurrent exercise previously reported (Babcock *et al.*, 2012; Pugh *et al.*, 2018) may be due to post-exercise biopsy timing. Though satellite cell activation was not directly assessed in the present study, a stable number of satellite cells and increased number of myonuclei was observed after eight weeks in Type I fibres in all training conditions. Therefore, satellite cell activation and

proliferation appear to have occurred in order to maintain a stable satellite cell pool. Furthermore, training-induced increases in myonuclear content in response to concurrent training were not different from isolated endurance or resistance training and occurred without fibre-type specific hypertrophy. Accordingly, concurrent training does not prevent satellite cell differentiation and myonuclear accretion. Collectively, these observations suggest that chronic concurrent training does not inhibit satellite cell activation or myogenesis.

Myonuclear content may regulate the capacity for myofibre hypertrophy (Petrella *et al.*, 2006, 2008). According to the myonuclear domain theory, a single myonucleus can only provide sufficient transcriptional capacity over a finite amount of cytoplasm (Cheek, 1985; Allen *et al.*, 1999). During periods of extensive myofibre hypertrophy, increases in myofibre CSA are accompanied by an increase in cell volume, which results in a strain on the myonuclear domain (i.e., μm^2 fibre area/myonucleus). In turn, myofibre hypertrophy can occur as a result of increasing the size of existing myonuclear domains or by increasing the absolute number of domains within the myofibre (Edgerton & Roy, 1991; Kadi & Thornell, 2000). While it has been hypothesised that myonuclear addition only occurs when myofibre hypertrophy exceeds a relative magnitude (~26%) (Kadi *et al.*, 2004) or the myonuclear domain exceeds an absolute 'ceiling' (~2,250 μm^2) (Petrella *et al.*, 2006, 2008), the concept of a universal myonuclear threshold has been challenged (Conceição *et al.*, 2018). In the present study, the average Type II and mixed fibre-type hypertrophy observed was ~15% and ~13%, respectively, which occurred in the absence of myonuclear addition or an expansion in the myonuclear domain. Indeed, this observation alone would be in agreement with the notion that a ~26% increase in CSA is needed to evoke myonuclear addition (Kadi *et al.*, 2004). However, when considered with the increased myonuclear

number in the absence of hypertrophy in Type I fibres observed in the present study, the current data do not support the rationale that hypertrophy must exceed a relative threshold to permit myonuclear addition. Likewise, the hypertrophy observed in Type II fibres occurred without a prior increase in myonuclear domain and demonstrates that changes in myonuclear domain size do not precede myofibre hypertrophy. These findings are supported by previous work demonstrating that myofibre hypertrophy in response to 12 wk of resistance training occurs without prior changes in myonuclear domain size in young, healthy men (Snijders *et al.*, 2016). While the average Type II myonuclear domain (~4,200 μm^2) in the present study greatly exceeds the suggested theoretical myonuclear domain 'ceiling' (Petrella *et al.*, 2006, 2008), this value is comparable to previous reports (Karlsen *et al.*, 2015). Collectively, these observations highlight that the myonuclear domain does not limit increases to myonuclear content, and does not appear to be a limiting factor in the degree of myofibre hypertrophy achieved with concurrent training.

While there is considerable debate around the notion that satellite cells are required to facilitate overload-induced myofibre hypertrophy (Petrella *et al.*, 2006, 2008; Verdijk *et al.*, 2009, 2014; Bellamy *et al.*, 2014; Dirks *et al.*, 2017; Reidy *et al.*, 2017*b*; McCarthy *et al.*, 2017; Karlsen *et al.*, 2015; Murach *et al.*, 2017), current evidence indicates that a positive correlation exists between satellite cell-mediated myonuclear accumulation and myofibre hypertrophy (Petrella *et al.*, 2006, 2008; Verdijk *et al.*, 2010, 2014; Bellamy *et al.*, 2014; Reidy *et al.*, 2017*b*). In accordance, higher baseline satellite cell content has been associated with a greater magnitude of myofibre hypertrophy achieved after a period of resistance training (Petrella *et al.*, 2008). In the current study, there was no relationship between baseline satellite cell content and increases in Type I, Type II, or mixed myofibre CSA. However, a positive

correlation was observed for increases in fibre type-specific myonuclear content and increases in myofibre CSA (**Figure 3.5**). These observations are consistent with previous works (Petrella *et al.*, 2006, 2008; Verdijk *et al.*, 2010, 2014; Bellamy *et al.*, 2014; Reidy *et al.*, 2017*b*) demonstrating that increases in myonuclear number are tightly coupled to increases in myofibre CSA. Collectively, these findings illustrate that myonuclear content, rather than satellite cells, is more likely related to limitations in the degree of myofibre hypertrophy achieved in following a concurrent training intervention.

There are several limitations in present study that need to be acknowledged. First, biopsies were collected 48 h after the last bout of endurance exercise in the CET condition. As the number of satellite cells has been shown to peak ~72 h after exercise (Snijders *et al.*, 2015*a*), consideration must be given to discrepancies in biopsy sampling time between CET and END conditions. Likewise, whether a prior bout of resistance exercise alters satellite cell and/or myonuclear content 72 h after endurance exercise in CET cannot be determined from the selected biopsy sampling time. Next, it is unclear whether training altered satellite cell activation. It has recently been shown that chronic resistance training enhances the activation of satellite cells in response to an acute bout of exercise (Nederveen *et al.*, 2017). Future investigations assessing markers of satellite cell activation (i.e., MyoD, Myf5, Myogenin) are required to understand if chronic concurrent training alters satellite cell activation in response to an acute exercise stimulus. Finally, dietary protein (Shamim *et al.*, 2018*c*) and other nutrients (Tachtsis *et al.*, 2018; Shamim *et al.*, 2018*a*) have been suggested to enhance satellite cell responses and promote exercise adaptations in skeletal muscle. However, without the inclusion of a placebo control, it is unclear if consuming a higher protein intake affected myogenesis throughout the course of the training intervention.

In conclusion, this is the first investigation to assess changes in satellite cell and myonuclear content following a period of chronic concurrent exercise training compared to isolated resistance and endurance training. The findings demonstrate that resistance, endurance, and concurrent training induce myofibre hypertrophy in the absence of expansion to the satellite cell and myonuclear pools. Implementing strategies to maximise hypertrophic potential with chronic concurrent training did not result in augmented myofibre hypertrophy, satellite cell pool expansion, or myonuclear accretion compared to endurance or resistance training alone. Likewise, myonuclear domain size remains stable throughout chronic endurance, resistance, and concurrent training, and, as such, does not appear to be a critical mediator in myonuclear accretion or limit the degree of hypertrophy achieved with concurrent training. The current data suggest that changes in myonuclear content are not prerequisite to changes in myofibre hypertrophy, but do appear to be associated with the magnitude of myofibre hypertrophy achieved in young, healthy males.

CHAPTER 4

SKELETAL MUSCLE TRANSCRIPTOMIC RESPONSES TO CHRONIC CONCURRENT EXERCISE TRAINING

4.1 Abstract

Combining resistance and endurance exercises in a training program (concurrent training) attenuates gains in muscle hypertrophy, strength, and power compared to when resistance training is undertaken alone. In order to understand which gene programs underpin adaptations to different training modes, skeletal muscle transcriptomic responses were explored following 12 wk endurance, resistance, and concurrent training in samples from eighteen (n = 6 per group) young, healthy male participants. Gene expression related to plasma membrane structures was enriched while gene expression related to regulation of mRNA processing and protein degradation was suppressed with concurrent training. Considerable overlap of gene expression related to extracellular matrix remodelling was observed between concurrent and endurance training. This is the first comparison of unique and overlapping gene sets enriched following chronic resistance, endurance, and concurrent training, and identifies known pathways with potential roles underpinning the limitations to adaptations made from concurrent exercise training.

4.2 Introduction

The molecular basis of skeletal muscle adaptations to exercise involves increased expression and/or activity of key proteins, mediated by an array of signalling events regulating pre- and post-transcriptional processes, protein translation, post-translational modifications, and intracellular localisation of proteins (Hawley *et al.*, 2014). Given the divergent stimuli associated with endurance- and resistance-based exercise, there are a variety of signalling kinases and downstream pathways/targets activated in response to each modality (Egan *et al.*, 2016). Adaptations to exercise training are hypothesised to result from the culmination of transient increases in mRNA transcripts encoding for various proteins after each successive exercise bout (Perry *et al.*, 2010). These repeated surges in mRNA abundance appear to be essential to drive the intracellular adaptive response to exercise training (Perry *et al.*, 2010). However, when endurance- and resistance-based training modalities are combined (concurrent training), there are impairments to resistance-based adaptations (Hickson, 1980; Craig *et al.*, 1991; Hennessy & Watson, 1994; Kraemer *et al.*, 1995; Dolezal & Potteiger, 1998; Bell *et al.*, 2000; Häkkinen *et al.*, 2003; Rønnestad *et al.*, 2012*a*; Mikkola *et al.*, 2012; Fyfe *et al.*, 2016*a*, 2018), a phenomenon known as the 'interference effect'.

The incompatibility of competing molecular signals following divergent exercise stimuli has been hypothesised to underlie the interference observed with concurrent training (Nader, 2006; Hawley, 2009; Hamilton & Philp, 2013; Baar, 2014*a*; Perez-Schindler *et al.*, 2015; Coffey & Hawley, 2017). Strategies focused on promoting anabolic intracellular signalling recommend that modifying training variables (i.e., recovery period between exercise modes) and nutrition (i.e., post-exercise protein) may prevent the interference effect (Nader, 2006; Hawley, 2009; Hamilton & Philp, 2013;

Baar, 2014*a*; Camera *et al.*, 2015; Perez-Schindler *et al.*, 2015; Murach & Bagley, 2016; Coffey & Hawley, 2017). The bases for these recommendations are centred on the discrete induction of two intracellular signalling mediators: mammalian target of rapamycin complex 1 (mTORC1) and 5' adenosine monophosphate-activated protein kinase (AMPK). Resistance-based exercise activates canonical mTORC1 signalling, stimulating protein synthesis and cell growth, while endurance-based exercise primarily activates AMPK signalling, promoting mitochondrial biogenesis and modifying substrate utilisation (Hawley *et al.*, 2014). However, a single bout of endurance exercise followed immediately by resistance exercise stimulates mTORC1 signalling to the same magnitude as resistance exercise alone, while simultaneously activating AMPK (Apró *et al.*, 2015), suggesting that exercise-induced AMPK does not prohibit activation of mTORC1. Furthermore, chronic concurrent training increases markers of translational capacity (i.e., ribosome content), independent of mTORC1 signalling or myofibre hypertrophy (Fyfe *et al.*, 2018). Given the complexity of molecular networks involved in contraction-induced training responses (Egan & Zierath, 2013; Coffey & Hawley, 2017), a binary relationship between exercise modality and molecular pathways is unlikely. Instead, it is probable that numerous interdependent pathways are responsible for eliciting any interference effect.

From a molecular perspective, it is obvious that training adaptation is a consequence of accumulation of specific proteins, with the gene expression initiating these changes in protein content crucial to any subsequent adaptation. Hence, contraction-induced perturbations at the level of the transcriptional machinery may provide a unique 'molecular signature' to help identify mechanisms underpinning divergent training adaptations (Camera *et al.*, 2010). To date, transcriptome-wide analysis of unique metabolic pathways in skeletal muscle has only been evaluated

following a single bout of concurrent exercise (Lundberg *et al.*, 2016). However, there is a clear disconnect between transcriptomic changes following acute exercise and subsequent adaptations to chronic training (Phillips *et al.*, 2013). Therefore the primary aim of this study was to explore skeletal muscle transcriptomic responses to a program of chronic concurrent training and discover whether changes in global gene expression reveal a molecular foundation for limited degree of hypertrophy observed with concurrent exercise training.

4.3 Methods

4.3.1 Experimental overview

The present work is a part of a larger study (Shamim *et al.*, 2018*b*), that used a parallel-groups design whereby participants were stratified according to lean body mass (LBM) and allocated to either a resistance only (RES), endurance only (END) or concurrent resistance and endurance exercise training (CET) group for 12 wk. Measures of maximal strength, aerobic capacity, and anaerobic power, as well as body composition were performed pre- and post-intervention. Resting biopsies (*vastus lateralis*) were taken pre-intervention, after 2 and 8 wk of training, and post-intervention. For the duration of the intervention, all participants consumed a high-protein diet (2 $g \bullet kg^{-1} \bullet d^{-1}$) to maximise gains in muscle mass. The study was approved by the Australian Catholic University Human Research Ethics Committee and was carried out in accordance with the latest revision of the Declaration of Helsinki. This trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12617001229369).

4.3.2 Participants

A subset of 18 participants (n = 6 per group) data were used for the current analysis. As the central purpose of the primary study was to assess changes in lean mass, six participants from each condition were selected to match the baseline leg lean mass to that of the full participant cohort (Shamim *et al.*, 2018*b*).

4.3.3 Training intervention

The training intervention has been described elsewhere (Shamim *et al.*, 2018*b*). In brief, participants in the RES and END groups performed training 3 d•wk^{-1}, while the CET group trained 6 d•wk^{-1}, alternating between resistance and endurance training each day. The resistance and endurance components of the training regime were matched between CET and RES, and CET and END, respectively.

4.3.4 Muscle biopsy

Resting skeletal muscle biopsies from the *vastus lateralis* were taken pre-intervention and 72 h after the last exercise session of weeks 2, 8, and 12. Biopsies in the CET condition were taken 72 h after resistance exercise to determine the effect of endurance exercise performed on alternate days on the muscle transcriptional profile. Samples were immediately frozen in liquid nitrogen and stored at -80^{o}C for subsequent analysis.

4.3.5 mRNA sequencing

Total RNA was isolated from ~40 mg of skeletal muscle using a commercially available kit (*mir*Vana™ PARIS™, Invitrogen) according to manufacturer's protocol.

RNA was eluted in PCR grade water. The RNA Integrity Number (RIN) was determined by automated electrophoresis (2100 Bioanalyzer™, Agilent Technologies). Average RIN value for all samples was 8.3 ± 0.6. Concentration of RNA was determined by fluorometric quantification using Qubit™ RNA HS Assay (Invitrogen). Single- and double-stranded DNA, as well as oligodeoxy-ribonucleotides containing a 5'-phosphate, were digested by Amplification Grade DNase I (Invitrogen) treatment according to manufacturer's protocol. Following DNase treatment, the concentration of RNA samples was adjusted to 50 ng•μL^{-1}.

RNA libraries were prepared for sequencing using the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (Life Technologies), following the manufacturer's recommendations. Briefly, 50 ng of total RNA was reverse-transcribed to generate first strand cDNA using SuperScript™ VILO™ (Invitrogen) reverse transcriptase and random primers. Following reverse transcription, target genes were amplified using Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel. After target amplification, primer sequences were partially digested and amplicons were phosphorylated. The amplicons were then ligated to unique Ion Xpress™ barcodes (Life Technologies) and the resulting constructs were purified using Agencourt™ AMPure™ XP Magnetic Beads (Beckman Coulter). Libraries were quantified by qPCR against a standard curve of *E. Coli* DH10B (Life Technologies) and normalized to 100 pM. Six libraries were pooled equally and 25 μL of the pooled mixture was used on one Ion 540™ Chip (Life Technologies) for template preparation using the Ion Chef™ Instrument (Life Technologies).

Ion semiconductor sequencing was performed on the Ion S5 XL system (Life Technologies). Read alignment was mapped back to target sequences of reference genome GRCh37.hg19 using the Tmap package for Torrent Suite™ software v5.8 (Life

Technologies) with default parameters. The resulting mapped read counts were used for subsequent bioinformatics analysis, and raw FASTQ files deposited in the Gene Expression Omnibus (#GSE137832).

4.3.6 Real-time PCR

Real-time PCR was carried out for selected mRNA targets using TaqMan™ probes and primers on the same RNA extract used for sequencing as previously reported (Camera *et al.*, 2017). Additionally, RNA was isolated from ~20 mg of skeletal muscle (using the aforementioned protocol) available for an additional 12 participants who completed the intervention (Shamim *et al.*, 2018*b*). Purity of RNA was determined by assessing the 260:280 ratio using a NanoDrop 2000 spectrophotometer (ThermoFisher). Quantification of RNA concentration, DNase treatment, and reverse transcription of 20 ng of RNA were performed as described above.

Quantification of mRNA, in duplicate, was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories). TaqMan™ Gene Expression Master Mix in combination with TaqMan™-FAM™ reporter dye labelled probes for secreted protein acidic and rich in cysteine (*SPARC*; Hs00234160_m1) and collagen, type IV, alpha 1 (*COL4A1*; Hs00266237_m1; Applied Biosystems) were used in a final reaction volume of 20 μL. Target gene expression relative to reference genes, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; Hs02786624_g1) and 18S ribosomal RNA (*18S*; Hs99999901_s1), was calculated using $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

4.3.7 Bioinformatics

Differentially expressed genes (DEG) between individual training conditions were obtained by analysing read counts using R/Bioconductor package *DESeq2* (v1.22.2) by applying regularised log transformation (Love *et al.*, 2014). DEG were identified as surpassing a false discovery rate (FDR) corrected *q*-value of ≤ 0.05 and subsequently considered for further analysis. Ingenuity Pathway Analysis (IPA; Qiagen) software was used for prediction of upstream regulators of DEG. Functional gene ontology (GO) terms from the Molecular Signature Database (v6.2) were determined using Gene Set Enrichment Analysis (GSEA) from ranked gene lists (Subramanian *et al.*, 2005). Analysis included GO sets with an FDR-corrected *q-value* ≤ 0.05. Intersecting GO terms between experimental conditions were visualized using the *UpSetR* package (Lex *et al.*, 2014).

4.3.8 Statistical analysis

Statistical analysis was performed using the *nlme* package on R (v3.5.2). Linear mixed-effect (LME) model, fit by restricted maximum likelihood estimate with random intercept for subject was used to test the effect of training condition on mRNA abundance measured by qPCR. Interactions for training condition × time were tested by the same LME. Where LME revealed significance, a Fisher's Least Significant Difference *post hoc* test for pair-wise comparisons was performed. Statistical significance was set at $p \leq 0.05$. Unless otherwise stated, data are presented as mean ± standard deviation of $n = 6$ per training intervention.

4.4 Results

4.4.1 Global gene expression responses to chronic exercise training

RNA sequencing was performed on pre- and post-intervention skeletal muscle biopsies. Principal component analysis revealed that the transcriptional profile across conditions shared considerable overlap (**Figure 4.1A**). Among all training conditions, CET induced the largest change in DEG, with 284 transcripts differentially expressed, versus 168 in END, and none for RES (**Figure 4.1B**). Of the DEG observed, 216 and 100 were unique to CET and END, respectively. Direction of change favoured upregulation, with CET and END displaying 197 and 103 increased transcripts, respectively, while 87 and 65 transcripts decreased, respectively (**Figure 4.1C**). Upstream regulator analysis of DEG identified major transcriptional regulators of myogenesis, such as Myc, hypoxia-inducible factor-1α, and myogenic determination protein 1, as well as growth factors, including transforming growth factor beta 1, fibroblast growth factors 1 and 2, and vascular endothelial growth factor (**Figure 4.1D**).

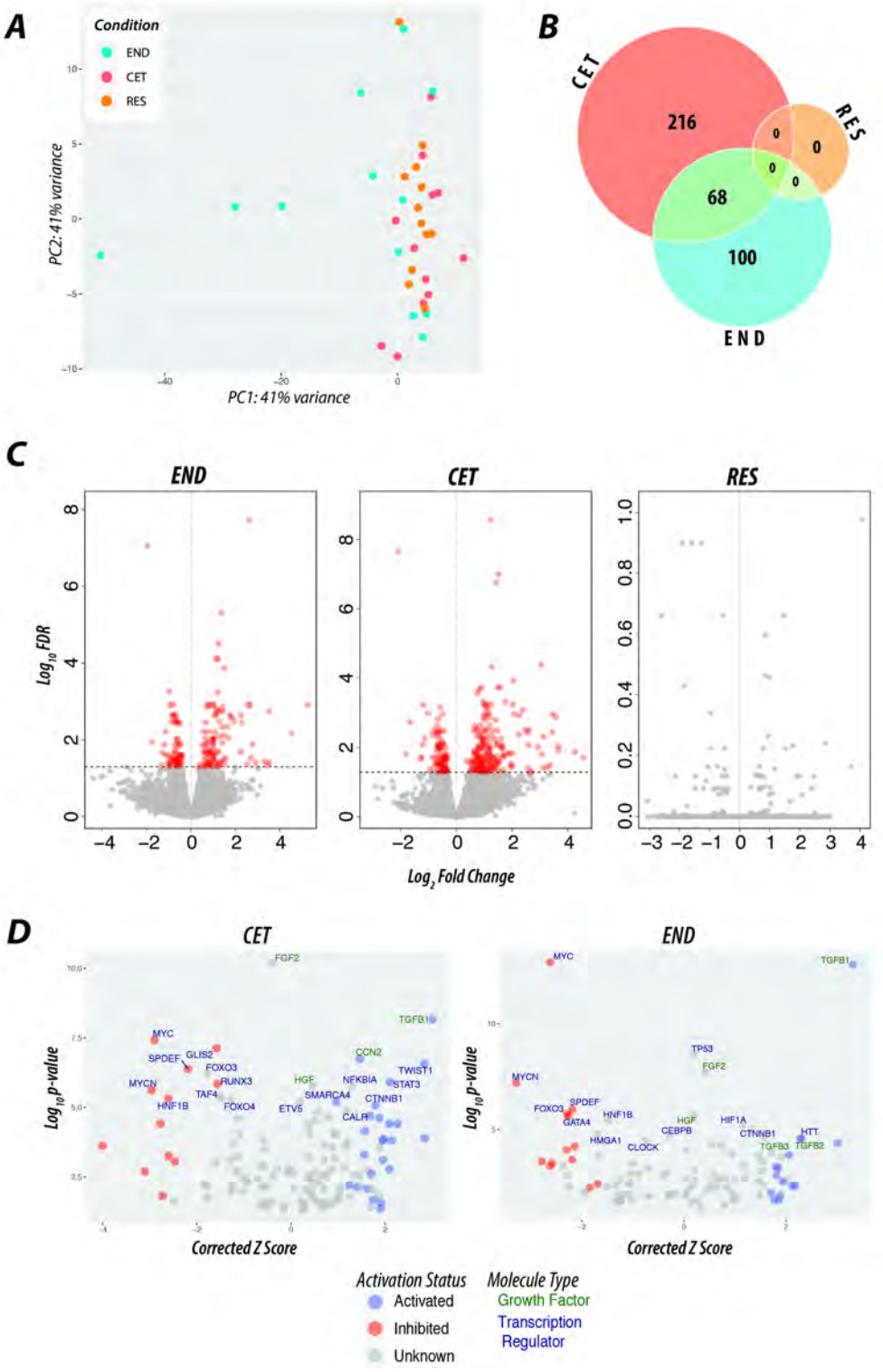

Figure 4.1 Differentially expressed gene and predicted upstream regulator analysis. Principal component analysis of individual samples designated by experimental condition (A). Venn diagram of unique and overlapping differentially expressed genes (B). Volcano plots of differentially expressed genes in individual experimental conditions (C). Ingenuity Pathway Analysis of predicted upstream regulators of differentially expressed genes composed of growth factors and transcription regulators (D).

To determine whether modulation of muscle gene transcripts following training contributed to the observed changes in metabolic phenotypes reported in **Chapter 2,** a gene set enrichment analysis (GSEA) was performed to assess potential gene sets contributing to distinct training phenotypes. Gene transcript changes were rank-ordered from most increased to decreased. Gene Ontology (GO) process annotations displayed the greatest enrichment in CET and END (**Figure 4.2**), with the most significant enrichment for terms associated with increased extracellular matrix (ECM) remodelling and angiogenesis and decreased ribosome biogenesis (**Figure 4.3A-B**). Gene sets unique to CET were most prominently upregulated for plasma membrane structures and downregulated relating to regulation of mRNA processing and protein degradation (**Figure 4.4A-B**).

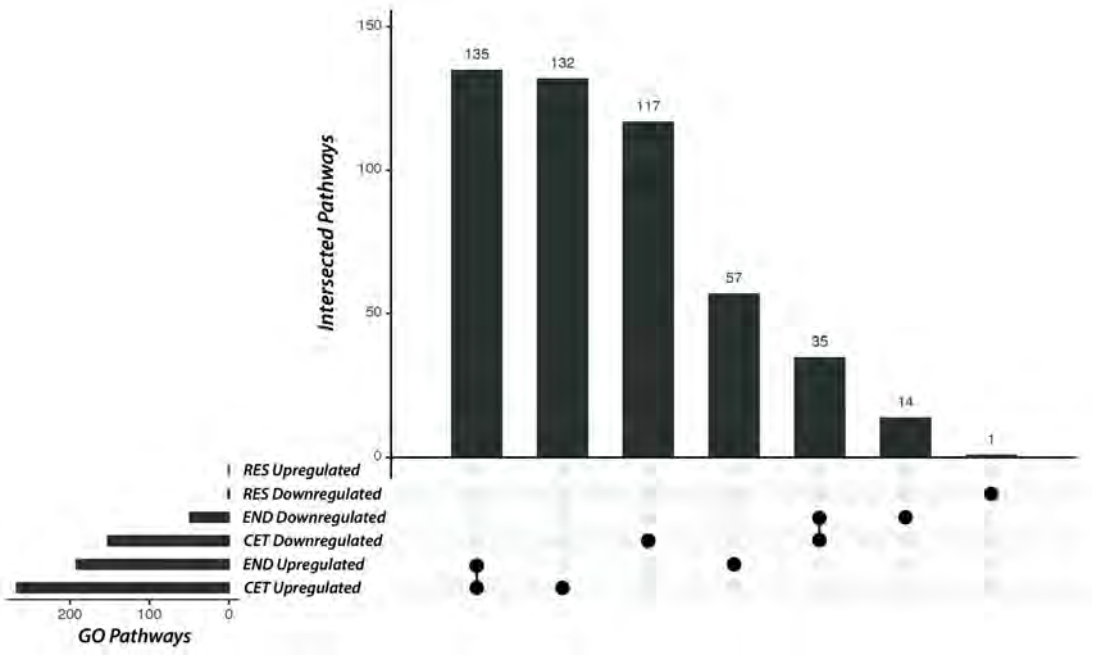

Figure 4.2 UpSet plot of the number of gene sets derived from gene set enrichment analysis (GSEA) that were common or exclusive between experimental conditions. Gene ontology terms for molecular function, biological process, and cell component were determined using GSEA software to search the Molecular Signature Database for enriched gene sets using the ranked gene list. Only gene sets with a false discovery rate (FDR) *q*-value of ≤ 0.05 were included. The FDR *q*-value was computed by using 1000 random permutations of the gene set.

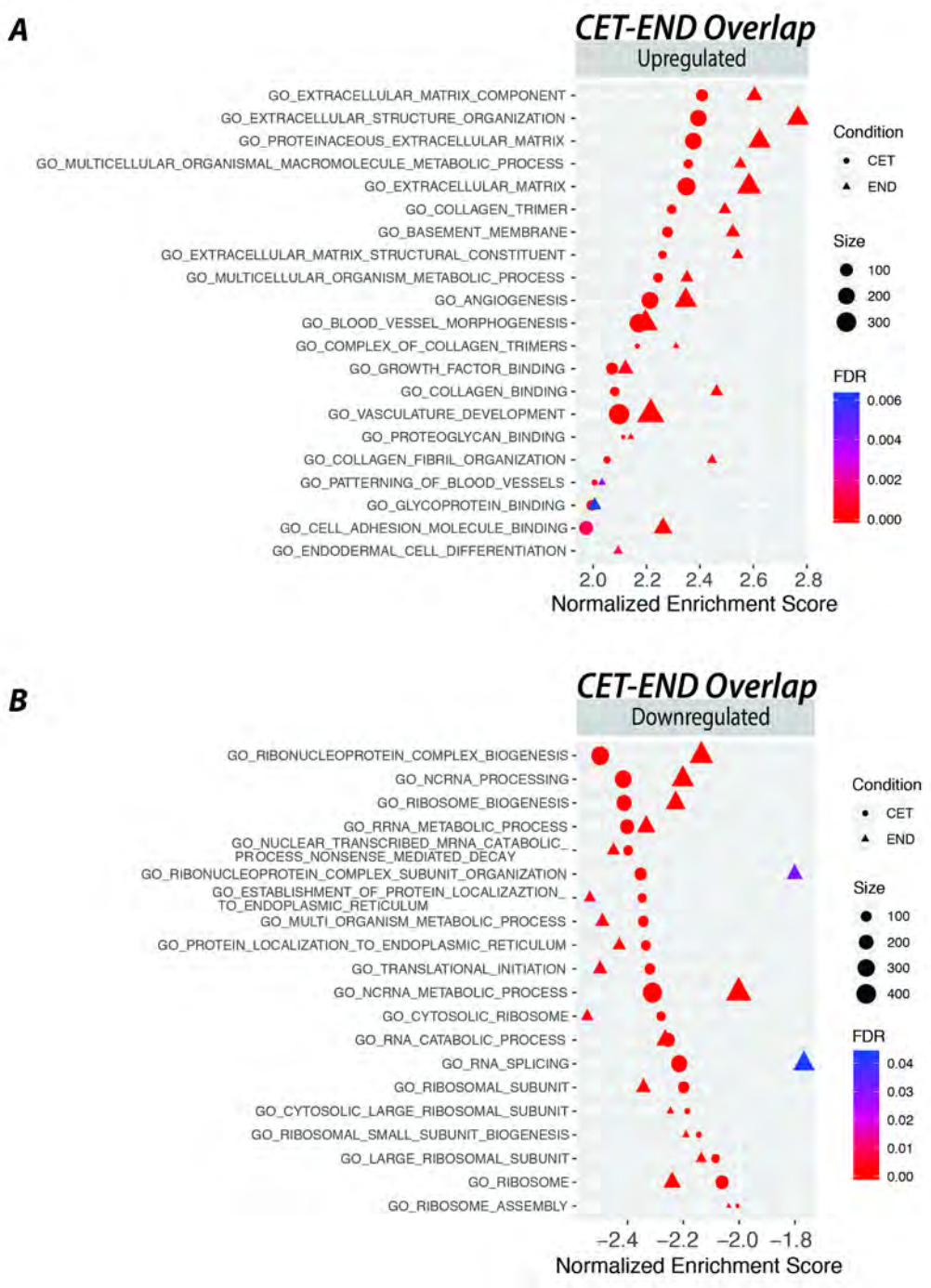

Figure 4.3 Top 20 enriched gene ontology terms up- (A) and down- (B) regulated overlapping between concurrent exercise training (CET) and endurance training (END). Gene ontology terms for molecular function, biological process, and cell component were determined using Gene Set Enrichment Analysis (GSEA) software to search the Molecular Signature Database for enriched gene sets using the ranked gene list. Size indicates the number of genes in the gene set. Only gene sets with a false discovery rate (FDR) *q*-value of ≤ 0.05 were included. The FDR *q*-value was computed by using 1000 random permutations of the gene set.

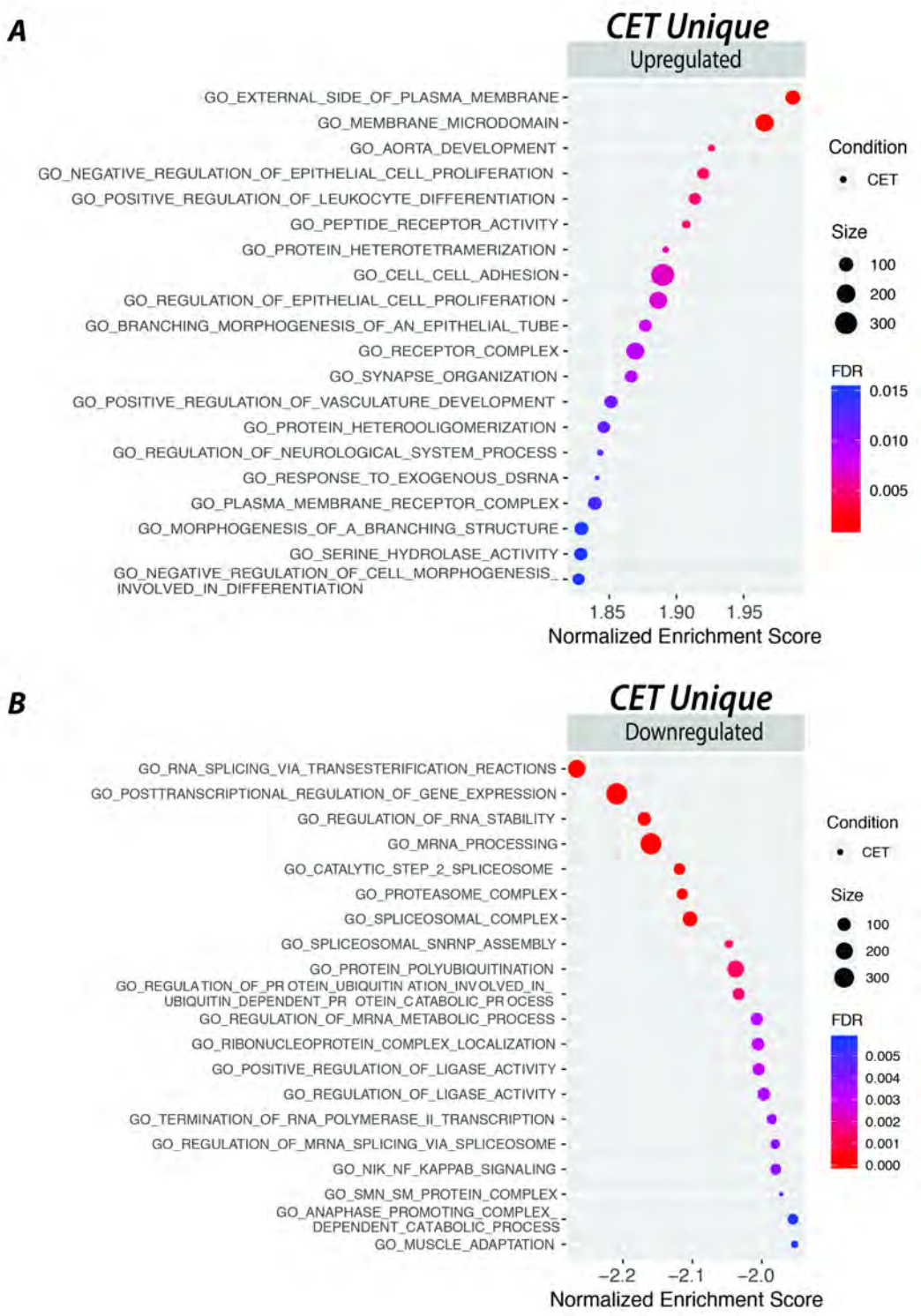

Figure 4.4 Top 20 enriched gene ontology terms up- (A) and down- (B) regulated unique to concurrent exercise training (CET). Gene ontology terms for molecular function, biological process, and cell component were determined using Gene Set Enrichment Analysis (GSEA) software to search the Molecular Signature Database for enriched gene sets using the ranked gene list. Size indicates the number of genes in the gene set. Only gene sets with a false discovery rate (FDR) *q*-value of ≤ 0.05 were included. The FDR *q*-value was computed by using 1000 random permutations of the gene set.

4.4.2 Targeted gene expression responses to chronic exercise training

Given the greatest degree of enrichment was observed in the GO extracellular structure organisation set (**Figure 4.3A**), targeted gene expression of members within this gene set, *SPARC* and *COL4A1*, was performed to confirm RNA sequencing results. These genes were differentially expressed in a consistent manner in both RNA sequencing and qPCR analyses (**Figure 4.5A-C**). Gene expression of *SPARC* and *COL4A1* measured in RNA extracts that were also used for RNA sequencing demonstrated an increased expression analogous to that reported by RNA sequencing, albeit non-significant (**Figure 4.5B**). However, when RNA extracts from the larger cohort of available participant samples were included (n = 30), *SPARC* and *COL4A1* were both found to be significantly differentially expressed from Pre to Post intervention. Specifically, *SPARC* expression was significantly increased in CET (p = 0.012) and END ($p < 0.001$), while *COL4A1* was substantially increased in CET (p = 0.067) and END ($p < 0.001$; **Figure 4.5C**).

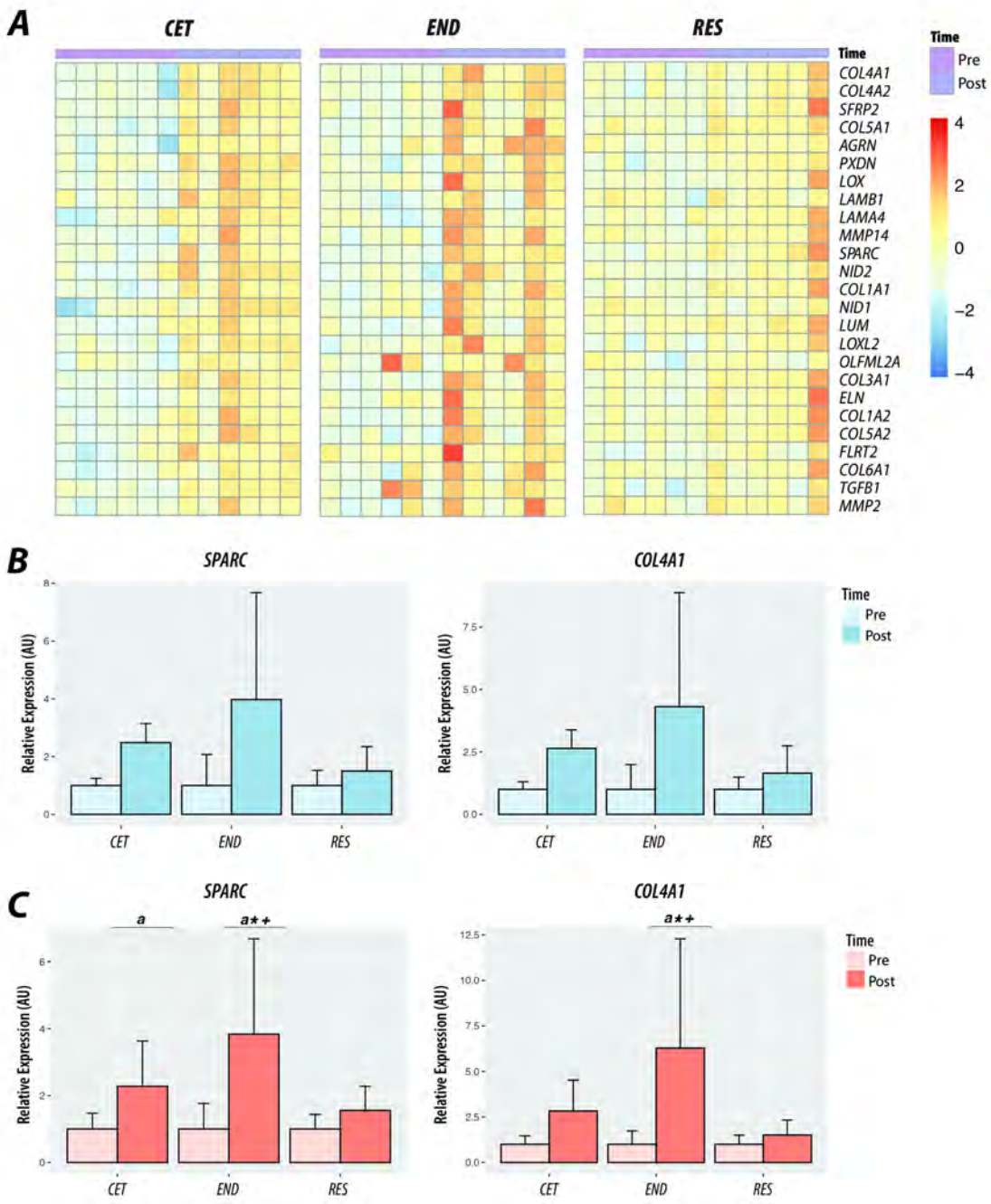

Figure 4.5 Targeted gene expression of members within top up-regulated gene set enrichment analysis annotation. Heat maps illustrating top 25 differentially expressed genes within the GO Extracellular Structure Organisation set for concurrent (CET), endurance (END), and resistance (RES) training (A). Gene expression of *SPARC* and *COL4A1* by qPCR from muscle of participants used for RNA sequencing (n = 6, 6, and 6 for CET, END, and RES, respectively) (B) and muscle from additional study participants (n = 11, 9, and 10 for CET, END, and RES, respectively) (C). Data are presented as mean ± standard deviation. *a* = $p < 0.05$ from Pre; * = $p < 0.05$ from CET; + = $p < 0.05$ from RES.

4.5 Discussion

The extent to which temporal changes in mRNA abundance patterns were associated with skeletal muscle adaptations to three different exercise training protocols was investigated using transcriptomics. The main finding was that concurrent training augments gene sets related to plasma membrane structures while suppressing those related to regulation of mRNA processing and protein degradation. Additionally, considerable overlap of gene sets enriched for terms related to ECM remodelling were observed in CET and END, which may underlie attenuations in maximal anaerobic power outputs. This study provides the first comparison of unique and overlapping gene sets enriched following chronic resistance, endurance, and concurrent training.

Both resistance (Damas *et al.*, 2018*b*) and endurance (Keller *et al.*, 2010) exercise have pronounced effects on transcriptome-wide gene expression, which may ultimately determine training adaptations (Timmons *et al.*, 2010). Previous work has demonstrated that a single bout of exhaustive aerobic exercise preceding resistance exercise augments the expression of genes with putative roles in oxidative metabolism, while concomitantly suppressing genes involved in regulating skeletal muscle tissue development (Lundberg *et al.*, 2016). Recently, Robinson and colleagues (Robinson *et al.*, 2017) demonstrated that 12 wk of resistance, endurance, or concurrent training in both young and older males and females upregulates a common subset of genes involved in angiogenesis 72 h after exercise. In the present study, a similar overlap of terms related to angiogenesis was observed amongst CET and END, but not RES. The reasons for the disparity in resistance exercise-induced gene expression between the two investigations is unclear, but may, in part, be due to differences in exercise volume, which has been shown to affect intracellular signalling (Burd *et al.*, 2010*a*). Similarly, differences in proximity (i.e., same-day versus alternate-day training) and order (i.e.,

endurance before resistance versus resistance before endurance) of exercise between concurrent training protocols makes direct comparison to previous work difficult.

In the present study, CET provoked a unique transcriptional profile with increased gene sets related to plasma membrane receptors. This finding is in contrast to previous observations of attenuated transcripts related to transmembrane receptors and mechanosensing measured after an acute bout of combined aerobic and resistance exercise (Lundberg *et al.*, 2016). Mechanotransduction involves the conversion of force applied across a cell membrane to biochemical signals that result in changes to gene expression, protein function, and cell architecture (Vogel & Sheetz, 2006). Acute concurrent exercise results in similar levels of phosphorylation of mechanosensitive transmembrane proteins, such as α7β1-integrin and focal adhesion kinase, compared to resistance exercise (Hansson *et al.*, 2019). However, downstream mechanically-sensitive transcriptional co-activators involved in cell growth and remodelling are active several days following mechanical overload (Goodman *et al.*, 2015). Previous microarray data suggest genes encoding for integrins are enriched up to 48 h following contraction-induced damage to protect myofibres from subsequent perturbations (Hyldahl *et al.*, 2015). Therefore, the enrichment of plasma membrane receptor genes following concurrent training observed in the current study suggests activation of remodelling processes to repair and stabilise the cell membrane for effective signal transduction.

Increased enrichment for gene sets related to substrate utilisation, as previously seen immediately following a bout of concurrent exercise (Lundberg *et al.*, 2016), was not observed in the present study. It is likely that acute transcriptomic responses to concurrent exercise are more reflective of transient metabolic perturbation, whereas myofibre remodelling predominates when energetic stress has subsided. In this regard,

these findings provide a new perspective and additional context for consideration when interpreting transcriptomic responses to concurrent exercise, and illustrate that transient pathways activated immediately after exercise alone may not be completely representative of chronic concurrent training adaptations.

In the current study, CET suppressed gene sets related to mRNA processing and protein degradation. Increasing mRNA stability allows cells to adjust mRNA content, without altering transcription, permitting rapid responses to internal and external stimuli. Numerous mRNA surveillance pathways coordinate stabilisation and posttranscriptional degradation (e.g., noncoding RNAs, processing bodies, stress granules, nonsense-mediated decay). MicroRNAs, small noncoding RNA species which target mRNA for degradation, are one regulatory mechanism known to be modulated by both nutrition (Camera *et al.*, 2016) and exercise (Fyfe *et al.*, 2016*b*). Concurrent exercise has been reported to modulate the expression of select microRNAs to promote a more 'anabolic' environment within skeletal muscle (Fyfe *et al.*, 2016*b*). Similarly, concurrent training decreases basal transcript levels of E3 ubiquitin ligases, MuRF-1 and Atrogin-1 (Fernandez-Gonzalo *et al.*, 2013). The reduction in transcripts related to ubiquitin processes observed in CET is therefore in agreement with previous findings (Fernandez-Gonzalo *et al.*, 2013).

It has been suggested that acute concurrent exercise increases the expression of genes involved in muscle breakdown, and may diminish hypertrophic responses (Apró *et al.*, 2015). The present transcriptomic analyses do not support such a hypothesis. Instead, it seems reasonable to suggest that concurrent training downregulates degradation pathways, allowing enhanced transcriptional and translational efficiency as a mechanism to expedite protein accretion. When considered with the observed transcript enrichment for plasma membrane receptors, these observations suggest the

high frequency and volume of contractile stimuli sustained when undertaking concurrent training suppresses degradation pathways during latent recovery to promote cellular remodelling. However, the mechanisms that determine RNA stability and gene expression are complex and not well understood. Future studies may look towards non-coding transcriptomes (Timmons *et al.*, 2018*a*) to fully appreciate RNA regulation networks in the context of training-induced skeletal muscle adaptations.

In the current study, upregulation of gene sets associated with ECM remodelling in CET and END may provide insight into the impaired development of maximal anaerobic power previously observed following concurrent training (Kraemer *et al.*, 1995; Häkkinen *et al.*, 2003; Chtara *et al.*, 2008; Wilson *et al.*, 2012; Fyfe *et al.*, 2016*a*; Shamim *et al.*, 2018*b*). During muscle contraction, force is transmitted to tendons longitudinally (Huxley & Niedergerke, 1954), along sarcomeres via the myotendinous junction, and laterally (Street, 1983) across costameres via the ECM (Pardo *et al.*, 1983; Danowski *et al.*, 1992). Strength training stimulates collagen synthesis in the ECM and tendon (Miller *et al.*, 2005), which increases tendon hypertrophy (Kongsgaard *et al.*, 2007; Rønnestad *et al.*, 2012*b*) and stiffness (Couppé *et al.*, 2008). Increased stiffness of these structures results in higher rates of force development and transfer (Hughes *et al.*, 2015) and is positively associated with power output and squat jump performance (Bojsen-Møller *et al.*, 2005). Previous work suggests that concurrent training compromises adaptations to connective tissue (Rønnestad *et al.*, 2012*b*) and therefore may limit improvements to maximal power output (Fyfe *et al.*, 2016*a*). Consistent with this hypothesis, in the current study attenuations to anaerobic power development in both the CET and END groups were observed (Shamim *et al.*, 2018*b*), despite attempts to circumvent the interference effect by implementing recommended strategies of

alternate day training, minimising exercise volume, and increasing dietary protein intake (Murach & Bagley, 2016).

Given the similar morphological changes of myofibres between the different training interventions (**Chapter 3**), it is possible that endurance-based contractile stimuli result in alterations to connective tissue that attenuate maximal power generating capacity, despite the inclusion of higher intensity (100-110% of maximal aerobic power) training stimuli. In support of this notion, cycle ergometer training has been shown to increase the expression of *SPARC* and *COL4A1* (Riedl *et al.*, 2010), indicating that ECM genes are responsive to endurance training. When considered with the enrichment for terms related to angiogenesis, it is likely that remodelling of the ECM is occurring to facilitate branching of the microvascular network in response to endurance exercise. While alterations to mechanical properties of the ECM, such as decreasing density and stiffness, promote angiogenesis (Bauer *et al.*, 2009), these adaptations may compromise improvements to force transfer and power output. In this regard, such observations prompt further investigation of structural changes to the ECM that contribute to force transfer within the paradigm of concurrent training.

It is unclear why there were no DEG in the RES group. While the precise turnover time of mRNA following exercise is equivocal (Yang *et al.*, 2005; Louis *et al.*, 2007; Rowlands *et al.*, 2011; Egan *et al.*, 2013; Neubauer *et al.*, 2014; Andersen & Gruschy-Knudsen, 2018; Karlsen *et al.*, 2019), changes in gene expression have been observed up to 4 days after a single bout of maximal effort resistance exercise in sedentary young men (Andersen & Gruschy-Knudsen, 2018). However, given participants in the present study were trained for 12 wk, it is possible that the resistance exercise protocol was not strenuous enough to elicit such a latent gene expression

response. Future work exploring the time-course of transcriptional responses in trained individuals is needed to appreciate how chronic training affects mRNA turnover.

Limitations within the present study are acknowledged. First, alternative splicing was not captured in the present analysis, and as such, previously characterised isoforms that are exercise mode-specific (Ruas *et al.*, 2012) may have been overlooked. Second, as the primary intention of the study was to provide a characterisation of the concurrent training transcriptome signature, protein-abundance of target genes was not assessed. Although the degree of correlation between changes in transcript levels and corresponding proteins remains contentious (Liu *et al*., 2016), transcriptomics analysis can be used as a tool to predict protein copy numbers per cell and may serve as a proxy for protein changes (Edfors *et al*., 2016). Nevertheless, future validation and cross-examination utilising multi-omics approaches (e.g., epigenomics, proteomics, etc.) may potentially reveal further significant information related to mechanisms underlying the interference effect.

4.6 Conclusion

This work provides the first characterisation of skeletal muscle transcriptomic responses to chronic concurrent training. The current findings reveal several unique and intersecting gene sets that may contribute to interferences in hypertrophy and power-based adaptations. Specifically, the findings herein highlight the modulation of gene transcripts relating to plasma membrane and extracellular matrix remodelling, as well as suppression of degradation processes, reflecting the high stress sustained during concurrent training. Collectively, these findings encourage further targeted investigation of pathways that have not been considered in the context of concurrent training adaptations.

CHAPTER 5

GENERAL DISCUSSION

5.1 Summary

The primary aim of the studies undertaken for this thesis was to investigate the efficacy of implementing recent concurrent training recommendations to prevent or attenuate the 'interference effect' compared to resistance training performed in isolation. A second aim was to explore the molecular responses to chronic concurrent exercise training in an attempt to try and identify potential molecular mechanisms contributing to blunted training adaptations. To address these aims, young, healthy, recreationally active males performed 12 wk of resistance, endurance, or alternate day concurrent training. Measures of exercise performance and muscle architecture were assessed to determine whether concurrent training produced an 'interference effect' compared to single-mode training (**Chapter 2**). Subsequently, muscle morphology was assessed to recognize, for the first time, whether fibre-type specific changes in myonuclear and satellite cell content following concurrent training influence the 'interference effect' (**Chapter 3**). Lastly, in an effort to expose previously overlooked molecular pathways that may contribute to the 'interference effect', transcriptome-wide molecular responses were interrogated (**Chapter 4**). Collectively, this thesis provides novel insight into the 'interference effect' from both a training-based and molecular prospective.

The principal findings from the study described in **Chapter 2** demonstrate that the current 'best practice' guidelines for designing concurrent exercise training programs are efficacious for protecting adaptations to strength and hypertrophy over 12 wk, but are not sufficient to avoid compromised adaptations to maximal anaerobic power. Furthermore, changes to *vastus lateralis* muscle architecture (i.e., CSA, pennation angle, fascicle length) do not differ between concurrent and resistance training, and cannot therefore explain impairments to maximal anaerobic power.

Notably, implementing the recommended strategies to optimise maximal hypertrophic responses to concurrent training did not augment myofibre hypertrophy compared to endurance or resistance training alone, despite a greater volume of work completed.

The findings from the experiment described **Chapter 3** demonstrate that increases to satellite cell content are not required to support myofibre hypertrophy achieved during concurrent training. Conversely, increases in myonuclear content, while not prerequisite to myofibre hypertrophy, appear to be associated with the magnitude of myofibre hypertrophy achieved in young, healthy males. Collectively, these findings illustrate that limitations in the degree of myofibre hypertrophy achieved following a concurrent training intervention may be due to myonuclear content rather than satellite cell content as previously hypothesised.

The results from the study described in **Chapter 4** highlight an increased enrichment of gene sets related to plasma membrane structures, regulation of mRNA processing, and protein degradation following concurrent exercise training. However, it is currently unclear whether these gene programs cause limitations in myofibre hypertrophy, and therefore further research is required. Additionally, a considerable overlap of increased enrichment for gene sets related to extracellular matrix structure was observed between concurrent and endurance training and may be related to impairments in adaptations to maximal anaerobic power. Collectively, these findings suggest that muscle hypertrophy and maximal anaerobic power may be compromised by concurrent training, despite implementing aggressive strategies to prevent an 'interference effect'. These findings emphasise the need to revise protocols to promote hypertrophy responses and maximal anaerobic power adaptations within a concurrent training paradigm. Given the translational nature of this investigation, the results have broad implications for athletes and coaches striving to maximise physical performance,

non-athletes simply looking to reap the greatest benefits from their exercise efforts, and clinicians who may prescribe concurrent training for rehabilitation and physical therapy.

5.2 Study Limitations

In the study described in **Chapter 2**, the effectiveness of implementing a high protein diet in combination with concurrent training in an effort to prevent the 'interference effect' was examined. A limitation of this study was the lack of an appropriate nutritional control group. As a result, it is not possible to determine the contribution of dietary protein alone in promoting training adaptations. A recent investigation into alternate day concurrent training demonstrated that higher protein consumption (~2.2 $g \bullet kg^{-1} \bullet d^{-1}$) during a concurrent training program improved lean mass and strength in men to a greater degree than an energetically equivalent, lower protein intake (~1.1 $g \bullet kg^{-1} \bullet d^{-1}$) over 24 wk (Ormsbee *et al.*, 2018). However, no isolated resistance training condition was included in that study, making it unclear whether an 'interference effect' was present. Notwithstanding, it is clear that protein is a critical nutrient for supporting both resistance (Morton *et al.*, 2018) and endurance (Breen *et al.*, 2011) training adaptations. To date, no study has investigated parallel concurrent, resistance, and endurance training with both protein and placebo supplementation comparisons for each training condition. Such an enquiry would provide more refined evidence to the efficacy of protein within a concurrent training paradigm and prove useful for optimising nutrition to support training adaptations.

In the study described in **Chapter 3**, fibre type-specific skeletal muscle morphology, satellite cell content, and myonuclear content were assessed to determine whether such factors can explain the similar gains in lean mass observed across training

conditions, despite performing a greater training volume during concurrent training. A limitation associated with investigating changes to satellite cell content by immunohistochemistry is the restricted number of antibodies that can be simultaneously visualised under fluorescence microscopy. As satellite cell populations are heterogeneous in their expression of different molecular markers (i.e., Pax7 vs. NCAM), using a single molecular marker for their identification may underestimate total satellite cell content (Lindström & Thornell, 2009; Lindström *et al.*, 2010). In addition, multiple labelling methods allow for the detection of subpopulations of satellite cells progressing through terminal differentiation (i.e., $Pax7^{-}/NCAM^{+}$) (Lindström & Thornell, 2009). As Pax7 alone was used for satellite cell enumeration in the present work, it is possible that subpopulations of satellite cells progressing through various stages of myogenesis were not detected, and, as a result, total satellite cell content was underestimated. Furthermore, while the immunohistochemistry has been routinely used to measure changes in myofibre cross-sectional area (Murach *et al*., 2019), the reliability of this measure was not determined in the present work. However, care was taken to quantify >150 fibres per fibre-type in order to minimise variability and provide an accurate representation of physiological changes in myofibre hypertrophy (Nederveen *et al*., 2020). Lastly, a linear mixed-effect model was used to allow estimations by restricted maximum likelihood and account for random inter-subject variability in molecular analyses to produce unbiased estimates for variance components in the model. Despite implementing a distinct statistical model, the results in **Chapter 3** remain consistent with those observed using two-way ANOVA with repeated measures in **Chapter 2**.

In the study described in **Chapter 4**, skeletal muscle transcriptomic responses to chronic concurrent training to determine whether changes in global gene expression

reveal a molecular foundation for the 'interference effect'. A major challenge associated with this approach is the selection of sampling time points and the 'snapshot' nature of information contained within any post-exercise biopsy. As no resting pre-exercise biopsy was collected during week 12 of training in the current investigation, it cannot be determined whether changes observed at the level of the transcriptome are due to transient fluctuations after exercise or accumulated over the course of training. Consequently, it is unclear whether gene sets enriched at 72 h were also prevalent throughout earlier time points during post-exercise recovery. However, based on previous findings demonstrating that transcriptomic responses 3 h after concurrent exercise are highly enriched for pathways associated with metabolic stress (Lundberg *et al.*, 2016), it is likely that pathways associated with re-establishing cellular energy homeostasis predominate the immediate post-exercise recovery period to allow for successive structural remodelling pathways to prevail. Notably, contraction-induced myofibrillar damage can persist for up to 48 h after exercise, with much of this damage localised around the Z-disc (Gibala *et al.*, 1995). Given that force transmission occurs via the Z-disc (Hughes *et al.*, 2015), and that rate of torque development is impaired for up to 96 h (Gibala *et al.*, 1995), it is possible that considerable structural remodelling is occurring beyond time points measured in the present thesis and contributes to impairments in maximal anaerobic power output following concurrent training. However, damage and remodelling processes after concurrent training have gained little attention. Future studies examining myofibrillar and ECM remodelling by electron microscopy and picroserius red staining, respectively, are necessary fully appreciate the post-exercise time course of myofibre recovery.

Lastly, it has been suggested that impaired adaptive responses with concurrent training compared to single-mode training are exacerbated with increased training

history (Coffey & Hawley, 2017). As athletes become more highly trained, the biochemical and mechanical signals produce adaptive signals become more distinct and skeletal muscle morphs into a phenotype that coincides with the specificity of training. In turn, the divergent phenotype becomes more sensitive to antagonistic signalling (Coffey *et al*., 2009*b*), which may result in exacerbated impairments to training adaptations with concurrent training. Thus, it is possible that the interference to maximal anaerobic power and the limited degree of muscle hypertrophy in response to concurrent training observed in this thesis are influenced by the training status of the participants and may potentially be exaggerated in highly trained athletes. Therefore, the findings of any interference effect observed must be interpreted with caution and placed in context with the training status of the individual or athlete.

5.3 Future Directions

From a theoretical perspective, it is recommended that endurance and resistance exercise within a concurrent training program should be separated by 6-24 h to optimise training responses (Murach & Bagley, 2016). However, a lack of time is commonly cited as a barrier to meeting physical activity recommendations for a large portion of adults (Trost *et al.*, 2002; Bauman *et al.*, 2012). Thus, the practicality of completing multiple training sessions in a single day or alternate-day training may not be realistic in recreational populations. In this regard, combining both endurance and resistance exercises into a single session may serve as a time-efficient means to improve the feasibility of reaching and adhering to physical activity guidelines. Notably, time-matched concurrent training has been shown to be more efficacious in increasing lower limb strength than endurance or resistance training alone in untrained older adults (>65 y), despite performing only half the work volume of each training mode (Timmons *et*

al., 2018*b*). However, the efficacy of time-matched concurrent training in younger populations remains to be evaluated. A major challenge in prescribing time-matched concurrent exercise sessions is the balance of work volume completed between training modes. As concurrent training typically results in a larger total training volume completed than single-mode training, it is unclear whether differences in adaptations between concurrent and single-mode training are due to the volume of work completed or the combined exercise stimulus. In the present work, the additional volume of work completed during concurrent training did not augment any training adaptations. Whether differences in adaptations occur if total work volume is matched across all training modes has yet to be determined. Thus, future investigations implementing work-matched training are needed to appreciate how total training volume influences concurrent training adaptations and the 'interference effect'.

Emerging evidence suggests that several micronutrients and supplements (e.g., creatine, Omega-3 polyunsaturated fatty acids, collagen) may facilitate recovery from strenuous exercise (Heaton *et al.*, 2017). In particular, collagen and vitamin C supplementation have been shown to augment collagen synthesis (Shaw *et al.*, 2017), which is imperative for connective tissue remodelling and training adaptations (Kjær *et al.*, 2006). In this regard, stiffness of the tendinous structures is positively correlated with rate of torque development and performance (power, force, and velocity) during high-force isometric and dynamic contractions (Bojsen-Møller *et al.*, 2005). While resistance training increases tendon CSA and connective tissue stiffness (Kongsgaard *et al.*, 2007; Couppé *et al.*, 2008; Rønnestad *et al.*, 2012*b*), it appears that concurrent weightlifting and cycle training impairs such remodelling (Rønnestad *et al.*, 2012*b*). When considered with the observations that concurrent training interferes with rate of torque development (Häkkinen *et al.*, 2003; Rønnestad *et al.*, 2012*a*), it is possible that

alterations to connective tissue stiffness and/or tendon structure may be the basis for impaired improvements to maximal anaerobic power output with concurrent training. Albeit speculative, collagen and vitamin C supplementation during a concurrent training program may promote an increase in tendinous structure CSA and stiffness, resulting in more effective force transmission and superior rate of torque development. Such evidence may be of considerable interest to athletes where a high degree of strength and power are necessary for performance, including events such as rowing, martial arts, and sprinting, and deserves further investigation.

Increases in myonuclear content to support myofibre hypertrophy are dependent on the differentiation of satellite cells to myonuclei through myogenesis (Snijders *et al.*, 2015*a*). While satellite cell content does not limit hypertrophy, obstructions during the progression of satellite cells through terminal differentiation of myogenesis may pose a potential restraint on the hypertrophic process. As satellite cells exist in heterogeneous populations (Lindström & Thornell, 2009), developing a comprehensive understanding of such heterogeneity will begin to reveal how satellite cells navigate through the myogenic lineage and how they are maintained under homeostasis and in response to an exercise stimulus. A recent investigation using a combination of single cell RNA-Seq and flow cytometry to distinguish disparate satellite cell populations from human skeletal muscle identified 12 transcriptionally distinct clusters of cells within the satellite cell pool of adult muscle under resting conditions (Barruet *et al.*, 2020). While it remains unclear whether the distinct transcriptional profile of these subpopulations is retained throughout the lifespan of the satellite cell or represents a transient state along myogenesis, future work characterising the expression of discrete satellite cells populations in response to exercise is needed to appreciate if exercise modulates specific subpopulations of satellite cells.

Few studies exist on how life-long physical activity affects myofibre size and satellite cell content. Recently, McKendry and colleagues (2019) demonstrated that endurance-trained master athletes with ~37 y of training experience display no difference in satellite cell content compared to untrained young or age-matched individuals. Additionally, myofibre CSA was not different between the masters athletes and young untrained individuals, suggesting that life-long endurance training may preserve myofibre size. However, young endurance-trained athletes were not included in the analysis, making it difficult to reconcile if no change occurs or if there is a loss in myofibre CSA or satellite cell content compared to young endurance-trained athletes. Likewise, studies on how life-long resistance training affects myofibre CSA and satellite cell content are needed. Skeletal muscle from well-trained powerlifters displays greater satellite cell content compared to sedentary individuals, and is positively correlated to mean myofibre CSA (Lindström & Thornell, 2009). Yet, it remains unknown if resistance training-induced increases in myofibre CSA and satellite cell content from young adulthood are preserved and can delay sarcopenia in resistance-trained masters athletes. In this regard, whether declines in satellite cell content cause myofibre atrophy, or is simply a consequence, remains a topic of debate (Snijders *et al.*, 2014*b*; Arentson-Lantz *et al.*, 2016). However, given observations that age-related myofibre atrophy is accompanied by declines in satellite cell content (Verdijk *et al.*, 2007, 2014), satellite cells present a potential therapeutic target in the management of sarcopenia.

The notion that skeletal muscle may possess an intrinsic ‘muscle memory’ of earlier life encounters with hypertrophy has recently gained considerable attention (Bruusgaard *et al.*, 2010; Egner *et al.*, 2013; Lee *et al.*, 2018; Seaborne *et al.*, 2018; Turner *et al.*, 2019; Dungan *et al.*, 2019). The extent to which satellite cells contribute

to such a phenomenon is unclear from murine models (Bruusgaard *et al.*, 2010; Egner *et al.*, 2013; Dungan *et al.*, 2019), but remains to be explored in human skeletal muscle. Recently, the concept of an 'epi'-memory in skeletal muscle has been proposed, whereby epigenetic modifications to gene expression, such as DNA methylation, may contribute to the muscle memory (Sharples *et al.*, 2016). Genome-wide DNA methylation after an initial period of resistance training, followed by cessation of resistance training, and a subsequent later period of resistance training (i.e., loading, de-loading, re-loading) offers initial evidence for an 'epi'-memory in previously untrained young men (Seaborne *et al.*, 2018). Remarkably, hypomethylation from the initial loading period was maintained during de-loading, despite muscle mass returning to baseline levels. Upon re-loading, the frequency of hypomethylation was enhanced in association with the largest increases in lean mass, suggesting that 'muscle memory' occurs at the epigenetic level within human skeletal muscle. The implications of such muscle memories in young adulthood on the maintenance of muscle mass in later life, or potential to counteract sarcopenia, still remain to be explored. Additionally, how concurrent training affects epigenetic modifications and the 'muscle memory' has yet to be identified. Likewise, whether epigenetic modifications contribute to the 'interference effect' is currently unknown.

As breakthroughs in technology continue, it will become feasible to apply multi-omics approaches to map the biological complexity of exercise training adaptations. Indeed, a number of investigations have already begun to employ combined –omic approaches (Lindholm *et al.*, 2014; Robinson *et al.*, 2017; Laker *et al.*, 2017; Turner *et al.*, 2019). However, a major challenge as we progress rapidly through the –omics renaissance will be the integration of datasets across multi-omic platforms to reconstruct global biochemical networks *in silico*. While initial efforts to establish

'trans-omic' analyses and propositions for 'trans-ome-wide association studies' covering genetic and environmental factors are underway (Yugi *et al.*, 2016), technological and analytical advances must strive to improve the breadth and reliability of pathway information available. In this regard, efforts to amalgamate publically available data and curate repositories of uniformly processed –omics datasets are starting to emerge (Ziemann *et al.*, 2019), and are a critical development to ensure consistency and reproducibility across experiments.

5.4 Concluding Remarks

In this thesis, recommendations to minimise the 'interference effect' during a concurrent training regimen were tested. For the first time it has been demonstrated that concurrent training impairs the development of maximal anaerobic power output compared to resistance training, despite recommended strategies to optimise training variables and protein availability. From a molecular perspective, the data provide the first assessment of time course-dependent changes to satellite cell and myonuclear content in response to concurrent exercise training. Notably, the findings illustrate that satellite cell content does not limit the degree of myofibre hypertrophy achieved, and that myonuclear content may more likely be related to limitations in the degree of hypertrophic gains following a concurrent training intervention. Furthermore, studies in this thesis explored the use of transcriptomics to provide an unbiased analysis of potential genes and gene sets that may regulate the ability of skeletal muscle to adapt to divergent training modalities. Namely, data from the exploratory –omics-based investigation delivers the first comparison of unique and overlapping gene sets enriched following chronic resistance, endurance, and concurrent training and highlights remodelling of the ECM as a contributing factor potentially linked to impaired maximal

anaerobic power output. To date, no studies have investigated ECM remodelling in response to concurrent exercise training. Therefore, investigating structural remodelling to non-contractile (i.e., connective tissue) in addition to contractile (i.e., myofibrillar) apparatuses contributing to post-exercise recovery may expose previously uncharacterised adaptations.

In conclusion, this thesis demonstrates that concurrent exercise training is a potent stimulus to improve skeletal muscle hypertrophy, strength, and oxidative capacity, but impairs maximal anaerobic power development. Still, it remains unclear why chronic concurrent exercise training does not result in augmented hypertrophic responses. In theory, it seems reasonable that hypertrophic responses to concurrent training should be the sum of those observed by endurance and resistance training alone, yet this appears not to be the case in practice. But is this evidence of an 'interference effect'? In many respects, this will depend on how the 'interference effect' is defined. Nevertheless, it is clear that some degree of an 'interference effect' does exist following concurrent training, and deserves further attention. Collectively, the findings within this thesis present a new view of the 'interference effect' and offer prospective mechanisms for future investigation ranging from training to transcriptome.

CHAPTER 6

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APPENDIX A

CHAPTER 2: SUPPLEMENTAL METHODS

This chapter has been adapted from the following published article:

Baubak Shamim, Brooke L. Devlin, Ryan G. Timmins, Paul Tofari, Connor Lee Dow, Vernon G. Coffey, John A. Hawley, and Donny M. Camera. Adaptations to Concurrent Training in Combination with High Protein Availability: A Comparative Trial in Healthy, Recreationally Active Men. *Sports Medicine*. 2018 Dec;48(12):2869-2883.

A.1 Performance Testing

The following performance tests were conducted prior to, and upon completion of the 12-wk intervention to determine maximal power output:

A.1.1 Anaerobic power test

Participants completed a Wingate test ~20 min after VO_{2peak} testing. The Wingate test was performed on an externally loaded stationary cycle ergometer (Monark 894E, Monark, Sweden), with the resistance of the flywheel equivalent to 0.075 kg•kg^{-1} body weight. Participants were familiarized with the test and instructed to remain seated in the saddle for the duration of the test. Briefly, participants began pedalling as fast as possible against the inertial resistance of the ergometer. Once pedalling reached 150 revolutions•min^{-1}, the external load was automatically applied to the flywheel by the ergometer-computer interface (ATS, Monark, Sweden). Participants were verbally encouraged to continue pedalling as hard, and fast as possible throughout the whole 30-s test. Where stringent confines of the test were not met due to participants either rising from saddle (n = 1) or incorrect seat position (n = 1), data were excluded from analysis.

A.1.2 Countermovement and squat jumps

Participants completed the countermovement jump (CMJ) and squat jump (SJ) on the force plate prior to IMTP and 1RM testing. Following a standardized warm-up consisting of 5 min of low-intensity cycling (~50 W) and dynamic stretching of the lower limbs, three attempts of each jump were performed, as previously described (Tofari *et al.*, 2017). For the CMJ, participants started from a standing position and were instructed to maintain their hands on hips throughout the jump. Upon verbal

command, participants descended quickly to a self-selected depth then accelerated as rapidly as possible from the bottom position to achieve maximal jump height and velocity. Following the CMJ, SJ's were performed on the force plate to determine concentric-only jump performance. Participants were instructed to maintain their hands on their hips, squat down to a self-selected depth and hold the position for an audible 3-s count prior to a maximal jump. All efforts were separated by at least 60 s of passive recovery. Variables including jump height (cm), absolute and relative peak power (W and $W \bullet kg^{-1}$) and peak velocity ($m \bullet s^{-1}$) were recorded from the trial with the greatest jump height.

A.2 Architectural assessment of *Vastus Lateralis*

Segmental muscle thickness, pennation angle, fascicle length and volume changes of the VL were assessed from ultrasound images taken along the longitudinal axis of the muscle belly utilizing a two dimensional, B-mode ultrasound (frequency, 12 Mhz; depth, 8 cm; field of view, 14 x 47 mm; GE Healthcare Vivid-i, USA) at baseline, after wk 2, 4, 8, and post-intervention. Images were taken at 75 (proximal), 50 (mid), and 33% (distal) of the distance between the central palpable point of the greater trochanter and the lateral condyle of the femur. Once the scanning sites were determined, the distances from various anatomical landmarks were recorded to ensure reproducibility for future testing sessions. These landmarks included the ischial tuberosity, fibula head and the greater trochanter. On subsequent visits the scanning sites were determined and marked on the skin and then confirmed by replicated landmark distance measures. All architectural assessments were performed with participants in a supine position with the hip and knee in a neutral position following at least 5 min of inactivity and prior to any testing or exercise sessions. To obtain

ultrasound images, the linear array ultrasound probe was aligned parallel to the muscle fascicles and perpendicular to the skin with a layer of conductive gel. Care was taken to ensure minimal pressure was placed on the skin by the probe as this may influence measurement accuracy (Klimstra *et al.*, 2007). Finally, the probe orientation was manipulated slightly by the assessor if the superficial and deep aponeuroses were not parallel.

Once the images were collected, analysis was undertaken off-line (MicroDicom version 0.7.8, Bulgaria). For each image and site, fascicle length estimation was performed as described elsewhere (Blazevich *et al.*, 2006; Kellis *et al.*, 2009). At each site, muscle thickness was defined as the distance between the superficial and deep aponeuroses of the VL. A fascicle of interest was outlined and marked on the image, and the angle at which it inserted onto the deep aponeurosis was determined as the pennation angle. The superficial and deep aponeurosis angles were determined as the angle between the line marked as the aponeurosis and an intersecting horizontal reference line across the captured image (Blazevich *et al.*, 2006; Kellis *et al.*, 2009).

The same assessor collected and analysed all scans and was blinded to participant identifiers (name and group) during the collection and analysis of the images. Day-to-day reliability of the assessor was determined prior to data collection in a small pilot study (n = 9). Measures of reliability included intraclass correlation coefficients (ICC), typical error (TE) and TE as a coefficient of variation (%TE). Minimum detectable change at a 95% confidence interval (MDC_{95}) was calculated as TE x 1.96 x √2. Based on previous quantitative reliability literature, it was determined that an ICC ≥ 0.90 was regarded as high, between 0.80 and 0.89 as moderate and ≤0.79 as poor. A %TE ≤10% was considered to represent an acceptable level of reliability. Assessments for the pilot study were taken one day apart at the same time of day.

Across all three sites for muscle thickness ICCs ranged from 0.97 to 0.99, TE from 0.09 to 0.22, %TE from 1.0 to 3.9% and MDC_{95} from 0.25 to 0.61cm. For pennation angle ICCs ranged from 0.90 to 0.98, TE from 0.16 to 0.33, %TE from 2.1 to 4.0% and MDC_{95} from 0.44° to 0.91°. For fascicle length ICCs ranged from 0.90 to 0.98, TE from 0.18 to 0.30, %TE from 3.9 to 4.9% and MDC_{95} from 0.49 cm to 0.83 cm.

Muscle thickness measures from ultrasound have been used to estimate muscle volume at a single time point and following training interventions (Miyatani *et al.*, 2002, 2004; Franchi *et al.*, 2017). In the current study, thigh length and VL thickness measures at the mid-point of the thigh were utilised with the following validated equation (Miyatani *et al.*, 2002) to estimate muscle volume:

$$MV\ (cm^3) = (MT \times 311.732) + (TL \times 53.346) - 2058.529$$

Where MV = muscle volume, MT = muscle thickness in centimetres, and TL = thigh length in centimetres.

A.3 Diet

A free-living, high-protein (2 $g \bullet kg^{-1} \bullet d^{-1}$) eating plan was implemented over the 12-wk intervention. Energy intake was based on the Cunningham Equation (using fat free mass from DXA) and Physical Activity Level (PAL) of 1.6 (RES and END) or 1.8 (CET), and modified accordingly depending on individual weight changes over the 12-wk intervention. Macronutrient composition was monitored throughout the intervention with total energy intake (TEI) and protein intake a focus. Carbohydrate and fat intake were recommended to be within the Acceptable Macronutrient Distribution Range for these macronutrients (45-65% and 20-35% TEI for carbohydrate and fat, respectively).

Prior to and throughout the intervention, participants were provided the following guidelines to reach protein and energy targets: 1) distribute protein intake evenly throughout the day across 4-6 meals (Areta *et al.*, 2013; Loenneke *et al.*, 2016) and 2) consume ~20-30 g of protein prior to bed to maximize potential for muscle protein synthesis (Moore *et al.*, 2009*a*; Snijders *et al.*, 2015*b*; Trommelen *et al.*, 2018). Participants were provided with ~34 g of whey protein (Pure Warrior 100% WPI, Swisse™, Australia) following every training session to maximally stimulate post-exercise rates of muscle protein synthesis (Macnaughton *et al.*, 2016). In addition, all participants were provided with a whey protein supplement (Whey Protein Concentrate, Bulk Nutrients, Australia) to consume as needed throughout the 12-wk intervention. Both protein supplements are commercially available and undergo batch testing for banned substances by independent organizations in compliance with the World Anti-Doping Authority. To further assist in reaching the 2 $g \cdot kg^{-1} \cdot d^{-1}$ protein amount, participants were also provided with weekly allotments of yoghurt from Chobani (Chobani LLC, Australia) and Jalna (Jalna Dairy Foods Pty Ltd, Australia), as well as almonds (Almond Board of Australia).

Participants attended consultations with an Accredited Practicing Dietitian on a fortnightly basis for a total of 8 consultations (Baseline prior to intervention, wk 1, 3, 5, 7, 9, and 11, as well as wk 13 to conclude the study). Consultations lasted ~20-30 min and provided participants with education, support, and advice to ensure nutrient targets were met, protein intake was evenly distributed throughout the day, and to monitor, and assess, dietary adherence. Advice was tailored and individualized to each participant depending on food preference, as well as eating habits and behaviours. On alternate weeks, the Accredited Practicing Dietitian contacted participants via text message and phone call to ensure dietary compliance and food record maintenance.

Daily food records were kept by participants through mobile phone applications Easy Diet Diary (Xyris Software Pty Ltd, Australia) for participants with iPhones® (Apple Inc., USA; n = 20) and MyFitnessPal (MyFitnessPal Inc., USA) for participants with Android-based (Google Inc., USA) devices (n = 12). All dietary intake data was analysed using FoodWorks 8© (Xyris Software Pty Ltd, Australia) to ensure the same food database was used for all analysis. Diet records were analysed for energy ($kJ \cdot kg^{-1}$), protein, carbohydrate, and fat ($g \cdot kg^{-1}$ for all macronutrients) to provide a daily average for the entire 12-wk intervention. Habitual dietary intake at baseline was assessed prior to commencing the study and analysed for energy and macronutrient intakes.

A.4 Resistance training program

Table A.1 Overview of 12-week resistance training program. Abbreviations: 1RM, 1-repetition maximum; RPE, rating of perceived exertion; BB, barbell; DB, dumbbell; RDL, Romanian deadlift; SL, straight-legged; CG, close grip; WG, wide grip; DXA, dual-energy X-ray absorptiometry.

	Session								
	1			*2*			*3*		
Week	**Movement**	**Sets x Reps**	**Intensity**	**Movement**	**Sets x Reps**	**Intensity**	**Movement**	**Sets x Reps**	**Intensity**
1	Leg Press	5 x 10-15	@ 65% 1RM	Bench Press	5 x 10-12	@ 60% 1RM	Leg Press	5 x 10-15	@ 70% 1RM
	RDL	4 x 10	Up to RPE ~6-8	Seated DB Overhead Press	4 x 10-12	Up to RPE ~6-8	RDL	4 x 10	Up to RPE ~6-8
	Knee Extensions	3 x 12	@ 65% 1RM	BB Rows	4 x 12	Up to RPE ~6-8	Knee Extensions	3 x 12	@ 67.5% 1RM
	SL Sit Ups	3 x 15	Unweighted	DB Triceps Extension	3 x 15	Up to RPE ~6-8	DB Biceps Curl	3 x 15	Up to RPE ~6-8
2	Bench Press	5 x 10-12	@ 62.5% 1RM	Leg Press	5 x 10	@ 72.5% 1RM	Leg Press	5 x 10	@ 75% 1RM
	Incline DB Bench Press	4 x 8	Up to RPE ~6-8	BB Step Back Lunge	4 x 15	Up to RPE ~6-8	BB Step Back Lunge	4 x 16	Up to RPE ~6-8
	Chest Supported DB Row	4 x 12	Up to RPE ~6-8	Knee Extensions	3 x 10	@ 70% 1RM	Knee Extensions	3 x 10	@ 75% 1RM
	DB Triceps Extension	3 x 15	Up to RPE ~6-8	Single Arm DB Row	3 x 15 ea	Up to RPE ~6-8	Single Arm DB Row	3 x 15 ea	Up to RPE ~6-8
3	*No training - Ultrasound*			Leg Press	5 x 10	@ 80% 1RM	Leg Press	4 x 10, 1 x Failure	@ 80% 1RM
				BB Step Back Lunge	4 x 16	Up to RPE ~7-9	BB Hip Thruster	3 x 10	Up to RPE ~7-9
				RDL	3 x 10	Up to RPE ~7-9	Lat Pull Down	4 x 10	Up to RPE ~7-9
				DB Biceps Curl	3 x 15	Up to RPE ~7-9	DB Biceps Curl	3 x 12	Up to RPE ~7-9
4	Bench Press	4 x 8	@ 70% 1RM	Leg Press	5 x 10	@ 82.5% 1RM	Leg Press	4 x 8, 1 x Failure	@ 85% 1RM
	WG Paused Bench Press	4 x 8	@ 60% 1RM	BB Hip Thruster	4 x 10	Up to RPE ~7-9	Rope Pull Through	4 x 8	Up to RPE ~7-9
	CG Bench Press	3 x 8	@ 50% 1RM working up to 8RM	RDL	3 x 10	Up to RPE ~7-9	Knee Extensions	4 x 8	@ 72.5% 1RM
	DB Shrugs	4 x 12	Up to RPE ~7-9	DB Biceps Curl	3 x 15	Up to RPE ~7-9	BB Rows	4 x 10	Up to RPE ~7-9
5	*1/2 Session - DXA/Ultrasound*			Leg Press	5 x 10	@ 85% 1RM working up to 10RM	Paused Leg Press	4 x 8, 1 x Failure	@ 87.5% 1RM
				BB Step Back Lunge	4 x 16	Up to RPE ~8-10	Rope Pull Through	4 x 8	Up to RPE ~8-10
	Bench Press	4 x 8	@ 75% 1RM	SL Deadlift	3 x 10	Up to RPE ~5	Knee Extensions	4 x 8	@ 75% 1RM
	Incline DB Bench Press	4 x 8	Up to RPE ~8-10	DB Biceps Curl	3 x 15	Up to RPE ~8-10	BB Rows	4 x 10	Up to RPE ~8-10
6	Bench Press	4 x 6	@ 80% 1RM	Leg Press	4 x 6	@ 90% 1RM	*1RM Testing*		
	Incline DB Bench Press	4 x 8	Up to RPE ~8-10	Bench Press	4 x 6	@ 85% 1RM			
	Seated DB Overhead Press	3 x 8	Up to RPE ~8-10	Chest Supported DB Row	4 x 8	Up to RPE ~8-10			
	DB Triceps Extension	3 x 15	Up to RPE ~8-10						

Table A.1 (Continued)

7	Speed Leg Press	7 x 3	@ 70% 1RM	Bench Press	7 x 3	@ 70% 1RM	Paused Leg Press	5 x 3	@ 77.5% 1RM
	Knee Extensions	4 x 5	@ 70% 1RM	WG Paused Bench Press	3 x 8	@ 60% 1RM working up to 8RM	BB Step Back Lunge	4 x 12	Up to RPE ~8-10
	RDL	4 x 6	Up to RPE ~8-10	CG Speed Bench Press	4 x 6	@ 50% 1RM working up to 6RM	SL Deadlift	4 x 6	Up to RPE ~6-8
8	Leg Press	5 x 5	@ 85% 1RM	Paused Bench Press	5 x 5	@ 80% 1RM	Leg Press	5 x 5	@ 87.5% 1RM
	Knee Extensions	4 x 6	@ 80% 1RM	CG Bench Press	4 x 6	@ 60% 1RM working up to 8RM	BB Step Back Lunge	4 x 12	Up to RPE ~8-10
	RDL	4 x 6	Up to RPE ~8-10	Chest Supported DB Row	3 x 8	Up to RPE ~8-10	Knee Extensions	3 x 8	@ 87.5% 1RM
	DB Biceps Curl	2 x 15	Up to RPE ~8-10	DB Triceps Extension	3 x 12	Up to RPE ~8-10	Lat Pull Down	3 x 12	Up to RPE ~8-10
9	*No training - DXA/Ultrasound*			Paused Bench Press	5 x 4	@ 82.5% 1RM	Paused Leg Press	5 x 4	@ 87.5% 1RM
				CG Bench Press	4 x 6	@ 65% 1RM	Knee Extensions	4 x 6	@ 82.5% 1RM
				BB Rows	3 x 8	Up to RPE ~8-10	SL Deadlift	4 x 8	Up to RPE ~8-10
				DB Triceps Extension	3 x 12	Up to RPE ~8-10	DB Overhead Press	2 x 8	Up to RPE ~8-10
10	Leg Press	4 x 4	@ 90% 1RM	Paused Bench Press	6 x 4	@ 85% 1RM	Paused Leg Press	4 x 3	@ 90% 1RM
	BB Hip Thrusters	4 x 6	Up to RPE ~8-10	CG Bench Press	4 x 6	@ 70% 1RM	Knee Extensions	4 x 5	@ 85% 1RM
	RDL	4 x 6	Up to RPE ~8-10	BB Rows	4 x 8	Up to RPE ~8-10	SL Deadlift	4 x 8	Up to RPE ~8-10
	DB Biceps Curl	3 x 10	Up to RPE ~8-10				Lat Pull Down	3 x 8	Up to RPE ~8-10
11	Leg Press	3 x 3	@ 92.5% 1RM	Paused Bench Press	3 x 2	@ 90% 1RM	Leg Press	3 x 2	@ 95% 1RM
	Knee Extensions	3 x 5	@ 87.5% 1RM	CG Bench Press	1 x 10, 1 x 8, 1 x 6, 1 x	@ ~55%, ~62.5%, ~70%, ~75% 1RM	SL Deadlift	3 x 6	Up to RPE ~8-10
	RDL	3 x 8	Up to RPE ~8-10				DB Shrugs	4 x 10	Up to RPE ~8-10
	Single Arm DB Row	3 x 15 ea	Up to RPE ~8-10	Chest Supported DB Row		Up to RPE ~8-10	DB Biceps Curl	2 x 15	Up to RPE ~8-10
12	Leg Press	2 x 2	@ 97.5%	Paused Bench Press	2 x 2	@ 90% 1RM	Leg Press	5 x 5	@ 90% 1RM
	BB Hip Thrusters	3 x 5	Up to RPE ~8-10	CG Bench Press	1 x 8, 1 x 6, 1 x 4, 1 x 2	@ ~62.5%, ~70%, ~75%, ~77.5% 1RM	Knee Extensions	3 x 8	@ 85% 1RM
	Knee Extensions	3 x 4	@ 90% 1RM				BB Step Back Lunge	3 x 12	Up to RPE ~8-10
	Single Arm DB Row	2 x 15 ea	Up to RPE ~8-10	BB Rows	5 x 6	Up to RPE ~8-10			

A.5 Endurance training program

Table A.2A Overview of 12-week endurance training program. Abbreviations: MAP, maximum aerobic power; HIIT, high-intensity interval training, DXA, dual-energy X-ray absorptiometry.

	Session		
Week	*1*	*2*	*3*
1	Hill Simulation	8 x 2.5	Hill Simulation
2	8 x 2.5	Hill Simulation	8 x 2.5
3	*No training - Biopsy/Ultrasound*	Hill Simulation	10 x 2.5
4	6 x 5	Hill Simulation +5%	6 x 5
5	*1/2 Session - DXA/Ultrasound*: 4 x 5	Hill Simulation +10%	7 x 5
6	Hill Simulation +15%	Steady State	VO_{2peak} Test
7	Hill Simulation	8 x 5	Hill Simulation
8	HIIT A	Hill Simulation	HIIT A
9	*No training - Biopsy/Ultrasound*	Hill Simulation	HIIT A
10	Hill Simulation +5%	Steady State	Hill Simulation +5%
11	HIIT B	Hill Simulation +10%	HIIT B
12	HIIT B	Hill Simulation +10%	HIIT B

Table A.2B Breakdown of specific endurance training sessions.

Session Outlines

Session Descriptor	Work Interval	Rest Interval	Work Power % MAP	Rest Power % MAP	Repeats
Hill Simulation	8 min	-	40.0%	-	-
	5 min	-	52.5%	-	-
	3 min	-	62.5%	-	-
	3 min	-	70.0%	-	-
	5 min	-	25.0%	-	-
	10 s	-	40.0%	-	-
	10 s	-	50.0%	-	-
	10 s	-	60.0%	-	-
	10 s	-	70.0%	-	-
	10 s	-	80.0%	-	-
	10 s	-	90.0%	-	-
	30 s	-	100.0%	-	-
	10.5 min	-	25-40%	-	-
8 x 2.5	2.5 min	1 min	70%	40%	8
10 x 2.5	2.5 min	1 min	70%	40%	10
4 x 5	5 min	1 min	70%	40%	4
6 x 5	5 min	1 min	70%	40%	6
7 x 5	5 min	1 min	70%	40%	7
8 x 5	5 min	1 min	70%	40%	8
HIIT Session A	10 s	50 s	100%	40%	6
	60 s	60 s	100%	40%	1
	20 s	40 s	100%	40%	3
	-	4 min	-	40%	-
	Repeat all parts 2x after 4 min rest				
HIIT Session B	5 min	1 min	70%	40%	3
	10 s	50 s	100%	40%	6
	60 s	60 s	100%	40%	1
	20 s	40 s	100%	40%	3
	-	4 min	-	40%	-
	Repeat all 100% efforts 2x after 4 min rest				
Steady State	45 min	-	50%	-	-

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APPENDIX B

CHAPTER 2: SUPPLEMENTAL RESULTS

This chapter has been adapted from the following published article:

Baubak Shamim, Brooke L. Devlin, Ryan G. Timmins, Paul Tofari, Connor Lee Dow, Vernon G. Coffey, John A. Hawley, and Donny M. Camera. Adaptations to Concurrent Training in Combination with High Protein Availability: A Comparative Trial in Healthy, Recreationally Active Men. *Sports Medicine*. 2018 Dec;48(12):2869-2883.

B.1 Muscle architecture

Table B.1 Changes to *vastus lateralis* muscle architecture throughout the 12 week training intervention as measured by two-dimensional B-mode ultrasound.

		Measure									
		Thickness (cm)			Pennation (°)			Fascicle (cm)			Estimated Volume (cm^3)
Group		*Proximal*	*Middle*	*Distal*	*Proximal*	*Middle*	*Distal*	*Proximal*	*Middle*	*Distal*	
PRE	**CET**	2.8 ± 0.4	2.7 ± 0.4	2.0 ± 0.4	18.5 ± 2.4	18.3 ± 1.6	17.4 ± 2.5	9.3 ± 0.8	9.0 ± 0.9†	7.1 ± 0.9	956 ± 164
	RES	2.7 ± 0.3	2.6 ± 0.2	2.0 ± 0.3	18.2 ± 2.1	18.0 ± 1.3	16.2 ± 0.7	8.9 ± 0.6	8.9 ± 0.8	7.4 ± 0.9	990 ± 122
	END	2.6 ± 0.3	2.4 ± 0.2	1.9 ± 0.2	17.3 ± 1.3	17.5 ± 2.1	16.3 ± 1.7	9.2 ± 1.0	8.3 ± 0.9	7.2 ± 0.6	940 ± 124
WK2	**CET**	3.0 ± 0.4^{a†}	3.0 ± 0.4^{a†}	2.4 ± 0.4^{a†}	20.1 ± 2.1^{a}	19.8 ± 2.3^{a†}	18.6 ± 2.4^{a}	9.1 ± 0.6	9.1 ± 0.6	8.0 ± 0.6^{a}	1040 ± 163^{a}
	RES	2.9 ± 0.3^{a}	2.8 ± 0.3^{a}	2.2 ± 0.3^{a}	19.2 ± 2.1	18.8 ± 2.3	18.1 ± 2.4^{a}	9.1 ± 0.4	9.0 ± 0.6	7.6 ± 0.7	1040 ± 133^{a}
	END	2.7 ± 0.2	2.5 ± 0.3	2.1 ± 0.1^{a}	18.4 ± 1.5	17.6 ± 1.4	16.9 ± 1.1	8.8 ± 0.6	8.6 ± 0.6	7.4 ± 0.6	966 ± 133
WK4	**CET**	3.1 ± 0.4^{a†}	3.0 ± 0.6^{a†}	2.4 ± 0.4^{a}	20.3 ± 2.3^{a}	19.9 ± 2.2^{a}	19.1 ± 3.2^{a}	9.2 ± 0.9†	9.2 ± 0.7	7.6 ± 0.7^{a}	1060 ± 171^{a}
	RES	2.9 ± 0.3^{a†}	2.8 ± 0.3^{a}	2.2 ± 0.3^{a}	19.5 ± 2.1^{a}	19.4 ± 2.1^{a}	17.7 ± 2.6^{a}	9.1 ± 0.5$^{(\dagger)}$	8.9 ± 0.6	7.8 ± 0.9	1060 ± 129^{a}
	END	2.6 ± 0.2	2.6 ± 0.2^{a}	2.1 ± 0.2^{a}	18.6 ± 1.6	18.0 ± 1.1	17.3 ± 1.9	8.5 ± 0.6^{a}	8.6 ± 0.7	7.3 ± 0.7	992 ± 115^{a}
WK8	**CET**	3.2 ± 0.4^{ab†}	3.1 ± 0.4^{ab†}	2.5 ± 0.5^{a†}	21.6 ± 2.1^{abc}	21.0 ± 2.6^{a}	20.1 ± 3.5^{ab}	9.2 ± 0.7†	9.2 ± 0.6†	7.7 ± 1.0^{a}	1090 ± 166^{abc}
	RES	3.0 ± 0.3^{abc†}	2.9 ± 0.3^{ab†}	2.3 ± 0.4^{a}	20.9 ± 1.8^{abc}	20.5 ± 1.8^{ab}	18.4 ± 2.4^{a}	8.9 ± 0.7†	8.7 ± 0.6	7.6 ± 1.2	1080 ± 132^{ab}
	END	2.7 ± 0.2^{a}	2.6 ± 0.3^{ab}	2.1 ± 0.2^{a}	20.0 ±2.1^{abc}	19.1 ± 2.1^{abc}	18.0 ± 1.5^{a}	8.3 ± 0.8^{a}	8.4 ± 0.7	7.2 ± 0.9	1010 ± 135^{ab}
POST	**CET**	3.2 ± 0.4^{abc†}	3.2 ± 0.4^{abc†}	2.4 ± 0.4^{a†}	21.5 ± 2.3^{abc}	20.5 ± 1.9^{a}	19.5 ± 3.2^{a}	9.3 ± 0.7†	9.5 ± 0.5†	7.8 ± 0.8^{a}	1100 ± 162^{abc}
	RES	3.1 ± 0.3^{abc†}	3.0 ± 0.3^{abc†}	2.4 ± 0.3$^{abcd(\dagger)}$	20.9 ± 1.7^{abc}	20.1 ± 1.9^{ab}	19.3 ± 1.6^{ac}	9.0 ± 0.5	9.1 ± 0.5†	7.7 ± 1.1	1100 ± 134^{abc}
	END	2.7 ± 0.2^{ac}	2.6 ± 0.2^{ab}	2.1 ± 0.2^{a}	19.8 ± 2.3^{abc}	19.8 ± 2.0^{abc}	18.3 ± 1.6^{a}	8.5 ± 0.6^{a}	8.1 ± 0.6	7.1 ± 0.7	1010 ± 115^{ab}

Values are presented as means ± SD. a = $P < 0.05$ from PRE. b = $P < 0.05$ from WK2. c = $P < 0.05$ from WK4. d = $P < 0.05$ from WK8. ǂ = $P < 0.05$ from END at time point. (ǂ) = $P < 0.055$ from END at time point. Abbreviations: CET, concurrent exercise training; RES, resistance training; END, endurance training.

B.2 Isometric mid-thigh pull

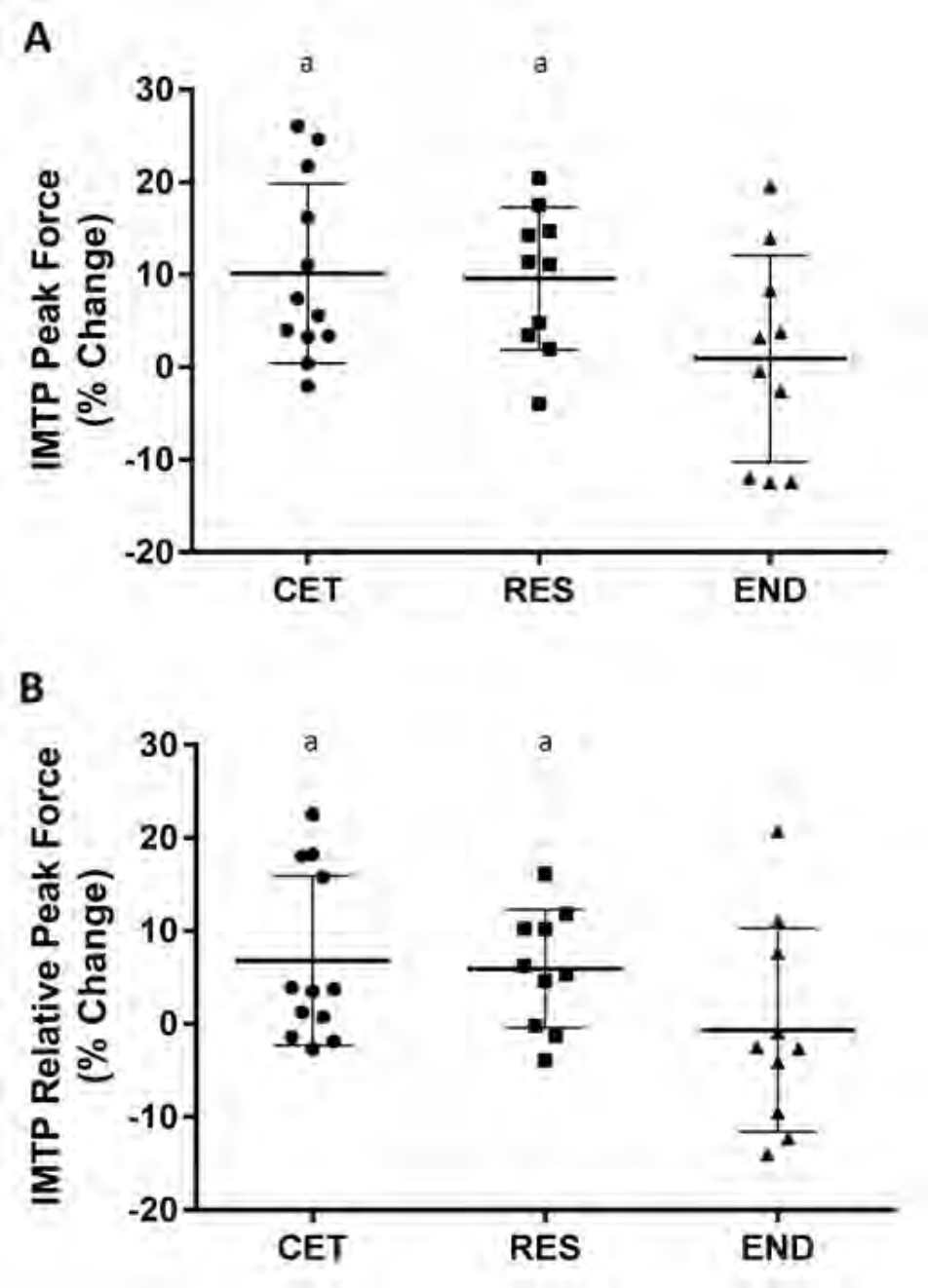

Figure B.1 Change to A) absolute and B) relative isometric mid-thigh pull (IMTP) peak power. Values are presented as percent change from PRE to POST and presented as individual data with group mean ± SD. a = $P < 0.05$ from PRE. Abbreviations: CET, concurrent exercise training; RES, resistance training; END, endurance training.

B.3 Training variables

Table B.2 Session averages for training variables throughout the 12-week training intervention.

Variable	Training Group		
	CET	**RES**	**END**
Resistance Variables			
Time To Complete Set ($s \bullet set^{-1}$)	42 ± 9	40 ± 3	-
Rest Interval (s)	185 ± 2	185 ± 3	-
Rating of Perceived Exertion	7 ± 0	7 ± 1	-
Endurance Variables			
Training Hours ($h \bullet wk^{-1}$)	1.44 ± 0.02	-	1.43 ± 0.04
Heart Rate (bpm)	159 ± 7	-	155 ± 8
Rating of Perceived Exertion	6 ± 1‡	-	5 ± 1
Time Between Session (h)	23.6 ± 0.85$^{\wedge\ddagger}$	47.7 ± 3.13	48.0 ± 1.76

$\ddagger$ = $P < 0.05$ from END. ^ = $P < 0.05$ from RES. Abbreviations: CET, concurrent exercise training; RES, resistance training; END, endurance training.

B.4 Training volume

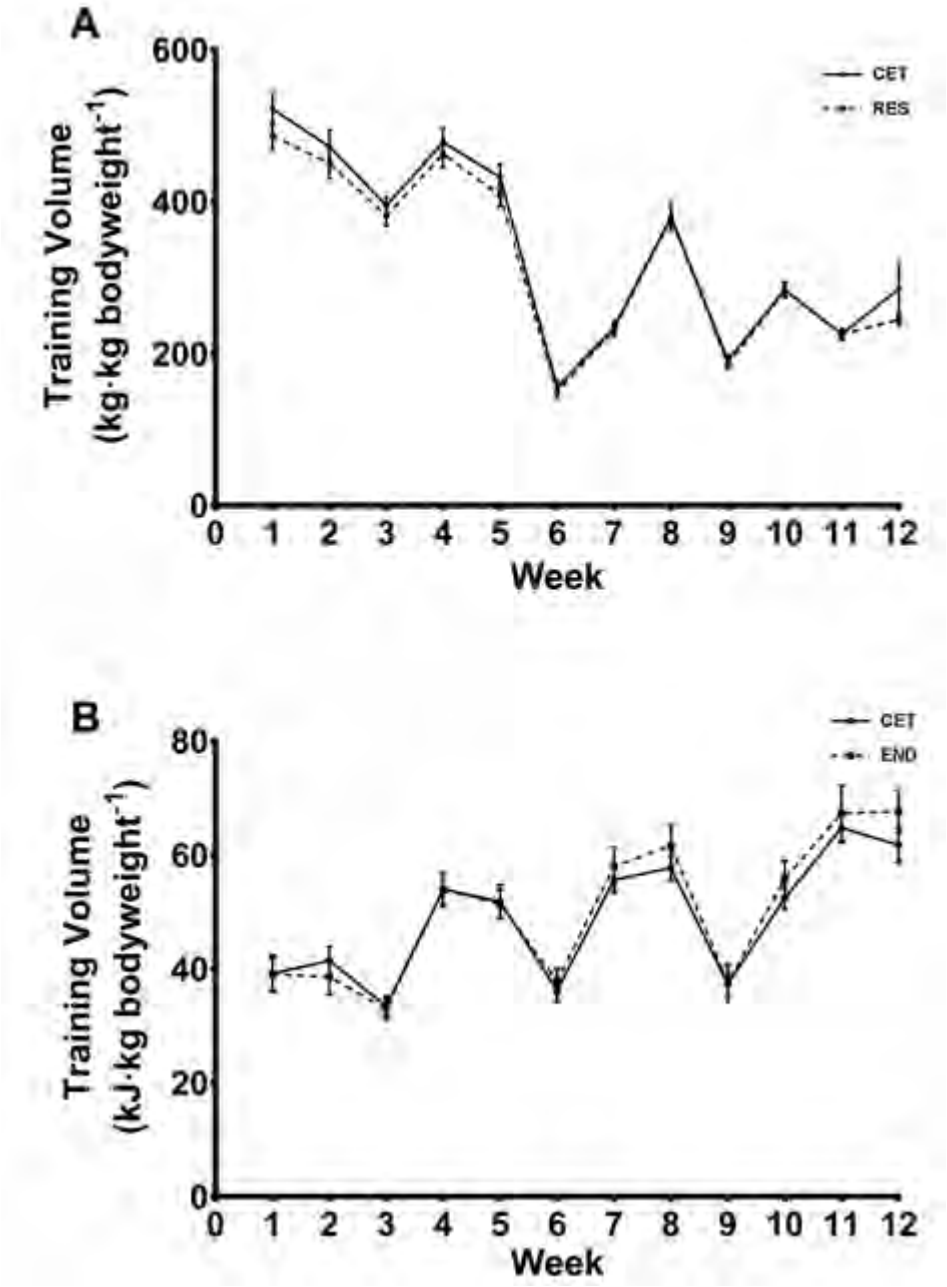

Figure B.2 Weekly average training volume for A) resistance-based and B) endurance-based training programs. Abbreviations: CET, concurrent exercise training; RES, resistance training; END, endurance training.

APPENDIX C

WHERE DO SATELLITE CELLS ORBIT? AN ENDOMYSIUM SPACE ODYSSEY

This chapter has been adapted from the following published article:

Baubak Shamim, Miguel S. Conceição, Marcus J. Callahan, and Donny M. Camera. Where do satellite cells orbit? An endomysium space odyssey. *Journal of Phsyiology*. 2018 May;596(10):1791-1792.

The regenerative capacity of skeletal muscle following injury is dependent on myogenic specific precursor cells, referred to as satellite cells. Under homeostatic conditions in adult muscle, satellite cells lie quiescent between the sarcolemma and basal lamina of myofibers. Upon perturbations to their surroundings, such as exercise-induced mechanical stress, satellite cells are summoned to an active state. Subsequently, active satellite cells can proliferate and differentiate by fusing with existing myofibers and donating their nuclei, or fusing with one another to form new fibres in a process known as myogenesis. Conversely, satellite cells may exit the myogenic lineage before differentiation and return to quiescence to replenish the satellite cell reserve for restitution of future damage.

Satellite cells were first identified using electron microscopy. However, the development of high quality antibodies for specific detection of satellite cell markers has made the use of light microscopy possible for evaluating satellite cells. Major technical advances in immunohistochemistry and fluorescence microscopy have allowed for simultaneous measurement of multiple antibodies on a single specimen. As a result, studies utilizing multiple labelling methods provide greater resolution into fibre type specific satellite cell enumeration and activation status.

Satellite cells are most commonly detectable by the expression of transcription factor Paired-box protein 7 (Pax7), which, in skeletal muscle, is exclusively expressed in satellite cells. However, the progression of satellite cells through myogenesis is choreographed by a transcriptional network of myogenic regulatory factors (MRF), particularly myogenic differentiation 1 (MyoD), myogenic factor 5, myogenin, and MRF4. Thus, through combined detection of Pax7 with MRFs, the number of satellite cells progressing through particular stages of myogenesis can be quantified. While the

role of individual MRFs is well established, the precise mechanisms regulating their manifestation remain largely unidentified.

It has been hypothesised that myogenesis is influenced by the delivery of growth factors and inflammatory cytokines (e.g., insulin-like growth factor-1, hepatocyte growth factor, interleukin 6, etc.) to satellite cells via capillaries. Initial work from Christov and colleagues (2007) demonstrated that myogenesis is spatiotemporally associated with angiogenesis, with active satellite cells located in closer proximity to capillaries compared to quiescent satellite cells. Such symbiosis appears to be, in part, mediated by the "cross-talk" between endothelial cells and satellite cells to deliver growth factors during myogenic differentiation. However, the extent of satellite cell responses controlled by the microvascular network during recovery from acute injury (i.e., exercise) in human muscle had not been explored.

A recent article published in *The Journal of Physiology* by Nederveen and colleagues (2018) assessed the expansion and activation status of the satellite cell niche following exercise-induced muscle fibre damage. Twenty-nine previously untrained healthy young men undertook a bout of unilateral lower-limb resistance exercise. For each participant, one leg was randomly selected to perform 30 sets of 10 maximal isokinetic eccentric knee extensions on a dynamometer at 180 $deg \bullet s^{-1}$ with 1 minute of rest between sets; a protocol designed to induce a significant level of myocellular damage. Percutaneous needle biopsies of the *vastus lateralis* and venous blood samples were taken pre-exercise and at select points over a 96 h post-exercise recovery period. Satellite cell content and activation status were measured concomitantly with capillarisation via immunohistochemistry. Specifically, quantification of capillary contacts (number of capillaries adjacent to a fibre), capillary-to-fibre ratio of individual fibres, number of fibres sharing contact with each capillary, and capillary density were

performed to determine the impact of muscle fibre capillarisation on satellite cell responses following exercise. Additionally, the capillary-to-fibre perimeter exchange (CFPE; derived as the quotient of the individual capillary-to-fibre ratio and the fibre perimeter) of mixed muscle was determined as an index of fibre perfusion.

Participants were retrospectively divided into tertiles based on their mixed muscle CFPE index into a High (n = 10; ~7.6 capillaries•1000μm^{-1}), Mid (n = 9; ~6.4 capillaries•1000μm^{-1}) or Low (n = 10; ~5.2 capillaries•1000μm^{-1}) CFPE group. At rest, cross-sectional area, perimeter, myonuclear domain, and myonuclear content of type I and II fibres were not different between High and Low groups. Additionally, neither total (Pax7^{+} cells•100 myofibers^{-1}) nor activated (Pax7^{+}/MyoD^{+} cells•100 myofibers^{-1}) satellite cell content were different between the High and Low cohorts. Notably, differences in fibre type proportions were observed, whereby High displayed ~22% more type I fibres than Low, indicating a potentially more oxidative muscle. In addition, the High group presented with a greater capillary-to-fibre ratio, larger number of capillary contacts, and shorter satellite cell distance to nearest capillary in both type I and II fibres compared to Low.

As expected, eccentric contractions resulted in significant muscle damage. In both the High and Low groups, creatine kinase activity was increased at 24 h post-exercise, while force production was significantly reduced over 72 h post-exercise. However, force production returned to baseline by 96 h post-exercise in the High group, whereas such recovery was absent in the Low group. Distinct satellite cell responses were also observed whereby a greater expansion of total satellite cell content was seen in High as compared to Low at 6 h (~48% vs. ~1%, respectively) and 24 h (~73% vs. ~10%, respectively) of recovery. Likewise, greater activation of satellite cells was observed in High compared to Low at 6 h (~750% vs. ~450%, respectively) and 72 h

(~750% vs. ~300%, respectively). Together, these findings indicate that individuals with a higher CFPE index appear to recover more quickly following an acute bout of damaging exercise.

When data from all participants was collated, a positive correlation was identified between the activation of satellite cells at 6 h and 72 h post-exercise and CPFE index. Similarly, there was a positive correlation between the expansion of total satellite cell content 24 h post-exercise and CFPE index. Collectively, these correlations suggest that the greatest satellite cell response to exercise was experienced by individuals with the highest capacity for muscle fibre perfusion. Thus, the authors postulate that there appears to be a link between juxtavascular position of satellite cells and microvasculature that influences satellite cell responses following muscle damage.

These novel insights provided by Nederveen and colleagues (2018) extend on the group's previous work demonstrating that satellite cells are located significantly further away from capillaries in muscle of older (~67 y) compared to younger (~24 y) individuals (Nederveen *et al.*, 2016). Given that older muscle typically exhibits impaired satellite cell responses to exercise as well as decrements to capillary structure and function (Verdijk *et al.*, 2016), interventions that proliferate the microvasculature network in elderly individuals may improve, to a degree, satellite cell responses following injury and preservation of skeletal muscle mass. In turn, a greater capillary supply may precondition skeletal muscle to support myofiber hypertrophy and combat anabolic resistance. In this regard, Verdijk and colleagues (2016) demonstrated that 12-wk of resistance training can effectively augment muscle fibre capillarisation and whole muscle cross-sectional area in older men. This work also reported increases in capillary contacts and CFPE index were associated with an expansion of satellite cell content after 12-wk of training. Accordingly, these findings exhibit resistance training as an

effective strategy to improve muscle size and satellite cell function as well as fibere capillarisation in older muscle.

Similar improvements in individual capillary-to-fibre ratio and satellite cell activation have been observed following 16-wk of resistance training in young men (Nederveen *et al.*, 2017). Considering the concomitant augmentation in satellite cell content and muscle perfusion after training, it was speculated that enhanced delivery of circulating growth factors results in more rapid initiation of myogenesis. Indeed, it has been shown that activated satellite cells are positioned in closer proximity to capillaries compared to their quiescent antecedents (Christov *et al.*, 2007; Nederveen *et al.*, 2016). However, to date, no studies have investigated spatial proximity of activated satellite cells with capillaries in older adults. Similarly, whether increasing muscle fibre capillarisation through exercise training results in augmented satellite cell responses during post-exercise recovery in older adults remains unidentified.

Indeed, many questions on the interaction of satellite cells and capillaries with regards to exercise modality (i.e., resistance, endurance, or concurrent exercise), nutrient availability (i.e., dietary protein), and clinical populations (i.e., sarcopenic adults) remain to be answered. The propinquity between satellite cells and capillaries provokes the notion that implementing strategies to improve vasodilation may augment delivery of valuable circulating factors to sites of injury and potentially expedite myogenesis. Thus, the capacity for dietary inorganic nitrates (e.g., beetroot juice), which are known vasodilators, to enhance satellite cell activation warrants exploration. Furthermore, investigations utilising high-throughput pathway platforms are needed to comprehensively profile and disinter circulating biochemical factors that may prospectively influence satellite cell responses following exercise.

Nonetheless, Nederveen and colleagues (2018) are to be commended for their novel insights into the critical relationship between muscle capillarisation and satellite cells during skeletal muscle repair following exercise. Characterising the satellite cell's "orbit" around capillaries provides one small step in understanding mechanisms regulating skeletal muscle repair, and one giant leap for exploration of the endomysium space.

C.1 References

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Competing Interests

None Declared

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APPENDIX D

PROTEIN AVAILABILITY AND SATELLITE CELL DYNAMICS IN SKELETAL MUSCLE

This chapter has been adapted from the following published article:

Baubak Shamim, John A. Hawley, and Donny M. Camera. Protein Availability and Satellite Cell Dynamics in Skeletal Muscle. *Sports Medicine*. 2018 Jun;48(6):1329-1343

D.1 Abstract

Human skeletal muscle satellite cells are activated in response to both resistance and endurance exercise. It was initially proposed that satellite cell proliferation and differentiation were only required to support resistance exercise-induced hypertrophy. However, satellite cells may also play a role in muscle fibre remodelling after endurance–based exercise and extracellular matrix regulation. Given the importance of dietary protein, particularly branched chain amino acids, in supporting myofibrillar and mitochondrial adaptations to both resistance and endurance-based training, a greater understanding of how protein intake impacts satellite cell activity would provide further insight into the mechanisms governing skeletal muscle remodelling with exercise. While many studies have investigated the capacity for protein ingestion to increase post-exercise rates of muscle protein synthesis, few investigations have examined the role for protein ingestion to modulate satellite cell activity. Here the molecular mechanisms controlling the activation of satellite cells in response to mechanical stress and protein intake in both *in vitro* and *in vivo* models are reviewed. A mechanistic framework that describes how protein ingestion may enhance satellite activity and promote exercise adaptations in human skeletal muscle is provided.

D.2 Key Points

- The regenerative capacity of skeletal muscle is dependent on an undifferentiated niche of myogenic specific precursor cells, referred to as satellite cells. The role of satellite cells in skeletal muscle remodelling following exercise has long been known. However, whether dietary protein ingestion can modulate satellite cell responses is less well understood.
- *In vitro* literature indicates that amino acids improve satellite cell dynamics; however, results *in vivo* remain ambiguous. Findings from human trials suggests that dietary protein may have the most pronounced effect on satellite cell activity after unaccustomed exercise when most myocellular damage and structural repair occurs, but may have diminishing returns with prolonged periods of training.
- The potential for protein supplementation to accelerate satellite cell responses after acute muscle damage may be of clinical and economic significance by expediting skeletal muscle remodelling processes and recovery from injury.

D.3 Introduction

The regenerative capacity of skeletal muscle is dependent on an undifferentiated niche of myogenic specific precursor cells, referred to as satellite cells. In adult skeletal muscle, satellite cells exist in a quiescent state and are located between the sarcolemma and basal lamina (Mauro, 1961). Classically, they are activated in response to muscle damage, such as mechanical stress caused by exercise (Crameri *et al.*, 2004; Dreyer *et al.*, 2006; Babcock *et al.*, 2012; Joanisse *et al.*, 2013; Snijders *et al.*, 2014*a*; Farup *et al.*, 2014*a*). Once activated, satellite cells proliferate and differentiate in order to contribute to the repair of existing muscle fibres through the formation of new myonuclei, a process known as myogenesis (Blaauw & Reggiani, 2014). In turn, the addition of new myonuclei increases the transcriptional capacity of the fibre to support further hypertrophy. However, evidence for the requirement for satellite cells in supporting overload hypertrophy is equivocal. McCarthy and colleagues (McCarthy *et al.*, 2011) demonstrated that in a novel mouse strain developed to deplete >90% of satellite cells, short-term (2 wk) mechanical overload-induced hypertrophy was not blunted compared to wild type mice, suggesting satellite cells are not required for load-induced hypertrophy. In contrast, results from other investigations show that satellite cell depletion effectively attenuates muscle fibre hypertrophy over both short-term (2 wk) (Egner *et al.*, 2016) and long-term (8 wk) (Fry *et al.*, 2014*a*) overload. While the notion that satellite cells are required to facilitate muscle growth responses is a topic of considerable debate (Petrella *et al.*, 2006, 2008; Verdijk *et al.*, 2009, 2014; Bellamy *et al.*, 2014; Dirks *et al.*, 2017; Reidy *et al.*, 2017*b*; McCarthy *et al.*, 2017; Karlsen *et al.*, 2015; Murach *et al.*, 2017), current evidence indicates that the presence and activation of satellite cells are obligatory for supporting training-induced adaptations.

The activation of satellite cells is influenced by the delivery of growth factors to muscle such as insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), and the myokine interleukin 6 (IL-6) (McKay *et al.*, 2008, 2009; Nederveen *et al.*, 2016, 2017). Changes to the concentrations of circulating cytokines or growth factors can induce satellite cell activation (Conboy *et al.*, 2005; Merritt *et al.*, 2013; Corrick *et al.*, 2015; Rodgers *et al.*, 2017). However, information on the effect of nutrient delivery, specifically amino acids from dietary protein consumption, on satellite cell activation is lacking. This is surprising considering the numerous studies demonstrating the stimulatory effects of protein ingestion on muscle hypertrophy with exercise (Cermak *et al.*, 2012; Morton *et al.*, 2018) and the purported roles of satellite cells to promote muscle hypertrophy. Given *in vitro* findings showing that leucine availability can promote myocyte proliferation and differentiation (Averous *et al.*, 2012; Chen *et al.*, 2013; Dai *et al.*, 2015; Duan *et al.*, 2017), protein ingestion in conjunction with exercise may provide an additional stimulus to promote satellite cell activation *in vivo*. This review focuses on the role of protein availability to regulate satellite cell dynamics in both cell and animal models and in the adaptive response to both resistance- and endurance-based exercise in human skeletal muscle. Studies are discussed that have determined the effects of protein ingestion on satellite cell activation following exercise and provide putative mechanistic insight into the regulation of exercise adaptation responses through increased satellite cell activity with protein availability.

D.4 The role of satellite cells in exercise adaptations

Adaptations to exercise training are specific to the mode, intensity, frequency, and loading pattern of activity being undertaken (Hawley *et al.*, 2014; Coffey &

Hawley, 2017). For example, endurance-based exercise classically results in increased skeletal muscle oxidative capacity and improved whole-body maximal oxygen uptake (VO_{2max}) (Holloszy & Coyle, 1984; Hawley, 2002). This is predominantly due to an increase in mitochondrial proteins (e.g., energy-producing oxidative enzymes) to facilitate metabolic adaptations, leading to a more fatigue–resistant muscle (Wilkinson *et al.*, 2008). Conversely, resistance-based exercise (i.e., weightlifting) is characterized by its ability to induce skeletal muscle hypertrophy and maximal force-generating capacity (McDonagh & Davies, 1984), particularly via the synthesis of contractile myofibrillar proteins (e.g., myosin heavy chain proteins). Though the specificity of training produces phenotypically divergent adaptations (Hawley *et al.*, 2014; Coffey & Hawley, 2017), both endurance and resistance exercises stimulate the turnover of skeletal muscle tissue.

Myonuclei are post-mitotic, and therefore the addition of new myonuclei to support fibre adaptations is ultimately dependant on satellite cell differentiation. Accretion of myonuclei with exercise training is assumed to accommodate the increased demands for transcriptional activity and synthesis of new proteins to support hypertrophy. It has been suggested that a single myonucleus only has control over a limited volume of cytoplasm, known as the myonuclear domain (Cheek, 1985). During robust hypertrophy, expansion of the myofibre volume places strain on the myonuclear domain. Accordingly, additional myonuclei are hypothesised to permit muscle fibre hypertrophy beyond a definite extent (~2250 μm^2), a postulate referred to as the 'ceiling theory' (Petrella *et al.*, 2006, 2008). Similarly, it has been speculated that only when the relative magnitude of fibre hypertrophy exceeds a certain threshold (≥ ~25% of cross-sectional area) are additional myonuclei required to sustain growth (Kadi *et al.*, 2004). However, myonuclear accretion has been observed during periods of

hypertrophy (~18% of cross-sectional area) where this threshold is not met (Snijders *et al.*, 2016). Furthermore, myonuclear content and fibre size are linearly related, whereas myonuclear domain and fibre size share a logarithmic relationship, with smaller fibres possessing disproportionately smaller myonuclear domains (Karlsen *et al.*, 2015). Though the reason for this relationship is unclear, it indicates that myonuclear domains may be different between smaller and larger fibres, and raises question to the scope of satellite cell behaviour dictated by previously established thresholds.

In human skeletal muscle, the activation of satellite cells following resistance exercise is well accepted (Kadi *et al.*, 2004; Olsen *et al.*, 2006; Verdijk *et al.*, 2007; Petrella *et al.*, 2008; Verdijk *et al.*, 2009; Mackey *et al.*, 2011; Snijders *et al.*, 2012; Babcock *et al.*, 2012; Snijders *et al.*, 2014*b*, 2014*a*; Fry *et al.*, 2014*b*; Bellamy *et al.*, 2014; Farup *et al.*, 2014*a*; Snijders *et al.*, 2016; Nederveen *et al.*, 2017; Reidy *et al.*, 2017*b*). Following a single bout of resistance exercise, increases in satellite cell proliferation are typically detectable after 24 h, with these responses peaking 72 h post–exercise (Snijders *et al.*, 2015). However, the precise timing of initial satellite cell proliferation is equivocal, and early (≤ 24 h) increases in satellite cell number may likely be due to an increased cell size prior to division, as suggested by *ex vivo* data (Rodgers *et al.*, 2014; Charville *et al.*, 2015), which may increase the likelihood of detection though immunohistochemistry. Nevertheless, a positive correlation exists between satellite cell-mediated myonuclear accumulation and muscle fibre hypertrophy (Petrella *et al.*, 2006, 2008; Verdijk *et al.*, 2010, 2014; Bellamy *et al.*, 2014; Reidy *et al.*, 2017*b*), which has led some (Petrella *et al.*, 2008; Bellamy *et al.*, 2014) to hypothesise that an individual's 'responsiveness' to resistance exercise may be based on satellite cell activation. Indeed, Petrella and colleagues (Petrella *et al.*, 2008) reported that individuals with the highest basal quantity of satellite cells achieved the

greatest magnitude of myonuclear addition and hypertrophy after 16 wk of resistance training. Bellamy and associates (Bellamy *et al.*, 2014) also demonstrated that an acute expansion of the satellite cell pool, rather than basal number, after a single bout of resistance exercise was associated with the magnitude of hypertrophy achieved over 16 wk of resistance training. However, it should be noted that Petrella and colleagues (Petrella *et al.*, 2008) used the membrane-bound satellite cell marker neural cell adhesion molecule (NCAM) while Bellamy and colleagues (Bellamy *et al.*, 2014) used the paired-box transcription factor Pax7, which is confined to the satellite cell nucleus. As a result of their cellular locations, staining of successive 7 μm cryosections shows that the same satellite cell is detectable through only two sections for Pax7, whereas NCAM is detectable through four or five sections (Mackey *et al.*, 2009). Thus, discrepancies regarding baseline satellite cell enumeration between studies may be attributable to inherent differences in staining profiles of satellite cell markers and thickness of cryosections. Irrespective of the marker used, these data collectively suggest that satellite cell activation and muscle fibre size may be closely related over chronic periods of resistance training.

While the majority of investigations that have determined the role of satellite cells in adaptations to exercise have focused on muscle hypertrophy, less is known regarding the role of satellite cells during less 'anabolic' stimuli, such as endurance exercise and high-intensity interval training (Charifi *et al.*, 2003; Verney *et al.*, 2008; Snijders *et al.*, 2011; Babcock *et al.*, 2012; Joanisse *et al.*, 2013, 2015; Fry *et al.*, 2014*b*; McKenzie *et al.*, 2016). However, recent evidence suggests a contribution of satellite cells to muscle fibre remodelling in the absence of hypertrophy (Joanisse *et al.*, 2013, 2015). Following 6 wk of sprint cycle interval training (10 x 60 s at ~90% of maximal heart rate, three times per week) in untrained women, the number of satellite cells

associated with hybrid fibre types (type I/II myosin heavy chain isoforms) increased as a mechanism hypothesised to assist in fibre type remodelling (Joanisse *et al.*, 2013). Similarly, both continuous moderate-intensity and high-intensity sprint interval cycle training have been shown to increase the number of activated and differentiating satellite cells post exercise without an expansion of the satellite cell pool or myonuclear content (Joanisse *et al.*, 2015). Though less predominant, unaccustomed aerobic training can result in muscle hypertrophy which is accompanied by increases in both satellite cell and myonuclear content in type I fibres (Fry *et al.*, 2014*b*; McKenzie *et al.*, 2016). While discrepancies regarding the increase in myonuclear content may have been driven by the robust hypertrophy seen in the latter investigation, these studies collectively demonstrate the recruitment of satellite cells in response to endurance-based exercise stimuli. Though the heightened activity and constant turnover of satellite cells in the absence of hypertrophy remains ambiguous, it may be required for myonuclear turnover during enucleation processes (McLoon *et al.*, 2004) or regulation of the extracellular matrix required during myofibre remodelling (Fry *et al.*, 2017).

The molecular basis for this response with both endurance and sprint interval exercise may centre on the activation of the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) (**Figure D.1**). As a key regulator in endurance exercise adaptations through its co-activation of several DNA binding transcription factors including the nuclear respiratory factors (NRF-1 and NRF-2) (Hood, 2009) and peroxisome proliferator activated receptors (PPARs) (Gilde & Van Bilsen, 2003), PGC-1α may play a role in regulating satellite cell activation by increasing both the mitochondrial content and activity of satellite cells (Rodgers *et al.*, 2014). Additionally, PGC-1α may also be involved in remodelling the extracellular matrix composition, thereby improving the propensity for satellite cells to proliferate

(Dinulovic *et al.*, 2016). However, several isoforms of PGC-1α are known to exist and are differentially activated based on the mode of exercise performed (Ruas *et al.*, 2012). For example, PGC-1α4 becomes activated only after resistance or combined resistance and endurance exercise (termed concurrent exercise) and promotes muscle fibre hypertrophy (Ruas *et al.*, 2012). Whether the effects of PGC-1α on satellite cell regulation are isoform specific is currently unknown. Similarly, the transcription factor prospero-related homeobox-1 (Prox1) has been proposed as a critical regulator of satellite cell differentiation in slow-twitch type I fibres, while also being responsible for fast- to slow-fibre type gene programing through modulation of the nuclear factor of activated T-cells (NFAT) signalling pathway (Kivelä *et al.*, 2016). Whether endurance exercise modifies Prox1 activity has yet to be determined. Indeed, the precise role(s) of satellite cells during adaptation to endurance training requires further investigation. Notably, an acute bout of concurrent exercise impairs satellite cell proliferation (Babcock *et al.*, 2012). While this attenuated response may be linked to the 'interference' in muscle hypertrophy typically observed when resistance and endurance exercise are performed concurrently over several months, the precise mechanisms directing this response and whether this phenomenon manifests after a chronic concurrent training program (i.e., 12-16 wk) is unknown.

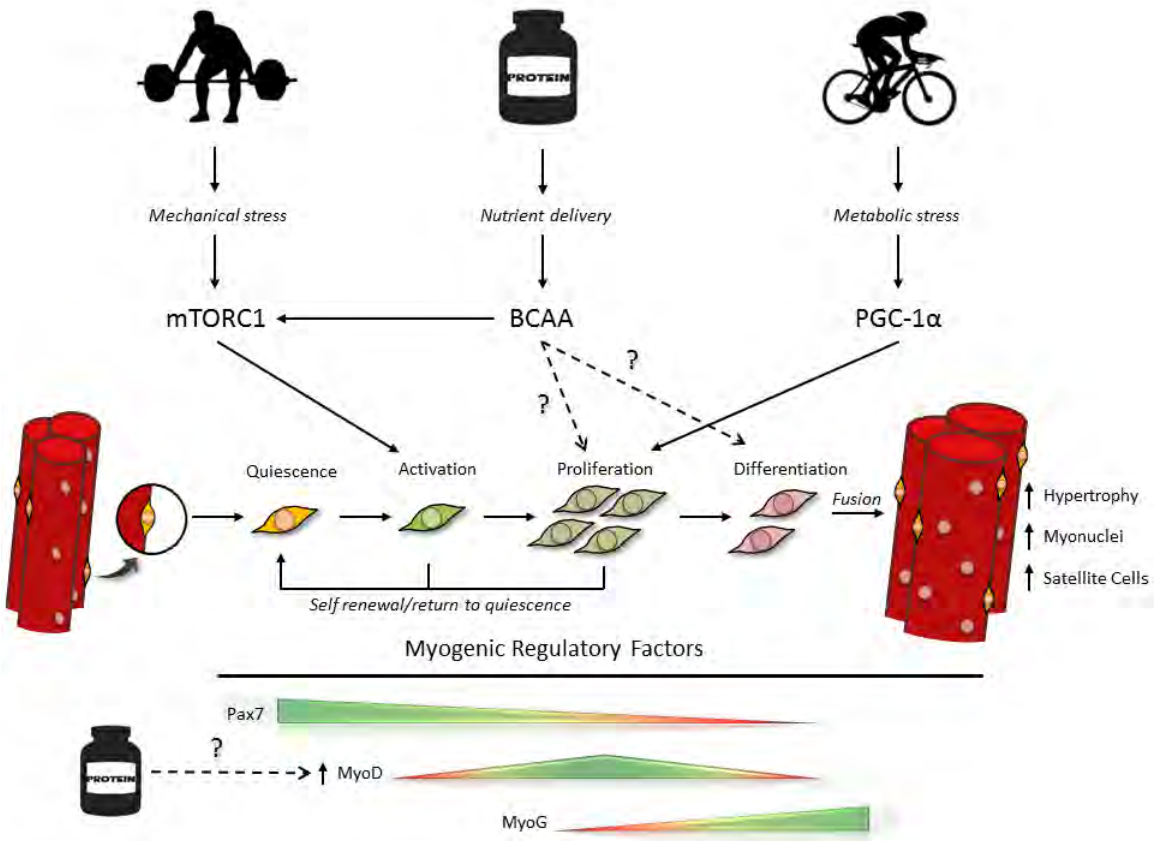

Figure D.1 Graphical representation of the potential mechanistic underpinning for satellite cell stimulation by resistance exercise, endurance exercise, and protein ingestion, as well as the expression pattern of associated transcription factors based on evidence presented from *in vitro* and murine models. Following a bout of resistance exercise, mechanical stress results in the activation of the mechanistic target of rapamycin complex 1 (mTORC1), which, in turn, assists in the transition of satellite cells from a quiescent state into an active state. Upon activation, satellite cells can either continue along the path of myogenic commitment to proliferate into myoblasts, or return to quiescence and self–renew to maintain the satellite cell pool. Metabolic stress caused by endurance exercise stimulates the activity of the transcriptional coactivator peroxisome proliferator–activated receptor–γ coactivator 1α (PGC–1α), which can promote the proliferation of satellite cells. Protein/branch chained amino acid (BCAA) supplementation may enhance both proliferation and differentiation of satellite cells. Though the mechanisms are not fully understood, potential pathways of satellite cell modulation through protein/BCAA supplementation have been included as dashed arrows. Myogenic regulatory factor expressions are present in higher levels (green) through specific stages and become suppressed (red) as the myogenic process advances as depicted by the shift from green to red in representative expression bars. Solid black arrows indicate increases/activation of downstream target proteins/ processes.

D.5 The impact of protein ingestion on satellite cell responses to exercise

Dietary protein is a critical substrate for providing amino acids to facilitate skeletal muscle repair and regeneration during recovery from exercise. Accordingly, sufficient protein needs to be consumed to facilitate the synthesis of new proteins during the immediate (2-3 h) post–exercise recovery period, which provides the basis for both resistance and endurance training–induced adaptations in skeletal muscle (Moore *et al.*, 2009*b*; Breen *et al.*, 2011; Camera *et al.*, 2015). Moreover, the addition of new satellite cell-derived nuclei through exercise-induced myonuclear turnover is essential to the continued contribution of genetic information for protein synthesis (Burd & De Lisio, 2017). Several interrelated factors including the dose (Moore *et al.*, 2009*a*), type (Tang *et al.*, 2009), timing (Res *et al.*, 2012) and distribution (Morton *et al.*, 2015; Areta *et al.*, 2013) of protein ingestion directly impact the anabolic effects of post-exercise protein ingestion. An in-depth discussion on these factors is beyond the scope of this review and readers are referred to several comprehensive reviews on this topic (Phillips & van Loon, 2011; Moore *et al.*, 2014; Phillips, 2016).

D.5.1 In vitro and animal models of satellite cell activity in response to amino acids

Work from as early as the 1970's reported the branched-chain amino acid (BCAA) leucine accelerates muscle regeneration in crushed animal skeletal muscle (Rogulska & Kurasz, 1975). *In vitro*-based models demonstrate C2C12 myoblast proliferation and differentiation are enhanced with BCAAs (Duan *et al.*, 2017) or leucine supplementation alone (Chen *et al.*, 2013). Leucine treatment has also been shown to promote myotube formation and increase MyoD and myogenin (MyoG) expression in primary preterm rat satellite cells (Dai *et al.*, 2015), while leucine withdrawal from culture media blunts C2C12 myoblast and primary satellite cell

differentiation (Averous *et al.*, 2012). Kornasio and colleagues (Kornasio *et al.*, 2009) investigated the effects of adding various concentrations of the leucine metabolite β-hydroxy-β-methylbutyrate (HMB) on serum–starved myoblasts and observed enhanced proliferation, differentiation, and accelerated fusion, indicating a capacity for HMB to drive quiescent adult myoblasts into the cell cycle. Similarly, HMB supplementation in neonatal pigs results in increased satellite cell proliferation and protein synthesis (Kao *et al.*, 2016) during a period of rapid growth that is accompanied by myonuclear addition (Davis & Fiorotto, 2009) and may serve as an effective strategy to increase muscle mass in clinical settings such as low-birth weight or preterm births.

Leucine induces hypertrophy on tissue engineered skeletal muscle as evidenced by increases in myotube width in supplemented constructs compared to a rapamycin control (Martin *et al.*, 2017). In regards to animal models, Alway and co-workers (Alway *et al.*, 2013) reported enhanced muscle stem cell proliferation exclusively in type II skeletal muscle of aged rats during recovery from disuse with HMB supplementation. Leucine ingestion has also been shown to improve muscle force production and increase the number of proliferating satellite cells of regenerating young and old skeletal muscles after cryolesion independent of modulating rates of muscle protein synthesis (Pereira *et al.*, 2015). Collectively, these findings provide strong evidence for a beneficial effect of leucine supplementation on muscle regenerative processes.

Recently, Rodgers and colleagues (Rodgers *et al.*, 2014) demonstrated that the leucine sensitive mechanistic target of rapamycin complex 1 (mTORC1) controls the transition of satellite cells between a quiescent and an initial ‘alert’ phase of the cell cycle in mice. This finding is noteworthy as subsequent investigations have demonstrated that mTORC1 signalling is rapidly activated during skeletal muscle

regeneration (Jash *et al.*, 2014) and is not only required for the adaptive transition of cell cycle phases, but necessary for satellite cell proliferation, differentiation, and overall skeletal muscle regeneration (Han *et al.*, 2008; Zhang *et al.*, 2015). Given the ability of leucine to both activate mTORC1 directly (Sancak *et al.*, 2008) and promote proliferation and differentiation *in vitro* through an mTORC1-MyoD cascade (Dai *et al.*, 2015), protein ingestion in conjunction with an appropriate exercise stimulus may provide an additional signal to promote satellite cell activation *in vivo* (**Figure D.1**).

D.5.2 Satellite cell activity in response to protein availability in human skeletal muscle

To date, few studies have investigated the interaction between protein supplementation and satellite cell activity in human skeletal muscle. In the following section, acute (defined here as a single exercise session), short-term (< 2 wk exercise training), and chronic (> 2 wk training intervention) exercise protocols that have determined the effects of protein ingestion/supplementation on markers of satellite cells activity in human skeletal muscle are discussed. Studies that have investigated how acute and short–term protein restriction can impact satellite cells activity are also reviewed.

D.5.3 Acute and short–term exercise

Following a single bout of resistance exercise in elderly men, Hulmi and colleagues (Hulmi *et al.*, 2008) reported that the ingestion of 15 g of whey protein immediately before and after exercise increased the gene expression of myogenic regulatory factors and cell cycle regulators in the 48 h post-exercise (**Table D.1**). Likewise, in elderly men, ingesting 10 g of essential amino acids after a single bout of resistance exercise increased the number of proliferating satellite cells during 24 h of

post-exercise recovery compared to a non-caloric placebo beverage (Reidy *et al.*, 2017*a*). Specifically, an increase in the number of $MyoD^+$ cells was observed only in the essential amino acid supplemented condition at 24 h post-exercise. Likewise, only essential amino acid supplementation resulted in an increase in $Pax7^+/Ki67^+$ cells post-exercise, which was significantly greater than the placebo condition. Though an increase in type I satellite cell content was observed with essential amino acid supplementation at 24 h, there was no difference in satellite cell content of type II fibres between groups. Similarly, when all myofiber types were pooled, no significant difference in satellite cell content was apparent between groups. There may be several explanations for these findings. First, the timing of analysis may have been too early to detect new satellite cells which typically occurs later (i.e., 48-72 h) in human skeletal muscle (Snijders *et al.*, 2015). Second, two separate essential amino acid supplements were used and were not matched for amino acid composition, particularly leucine (1.85 g, n = 4 versus 3.5 g, n = 7). Third, immunohistochemistry was only performed on nine participants in the essential amino acid group and five control participants and thus may have underpowered the analysis. Nevertheless, it appears that essential amino acids can accelerate proliferation compared to a placebo. In line with these findings, consumption of 28 g of protein during the post-exercise recovery period increased satellite cell content compared to a placebo control for up to 48 h in healthy young men (Farup *et al.*, 2014*a*)(**Table D.2**). Notably, exercise alone was unable to stimulate a satellite cell response in the placebo group. This is surprising given previous investigations have shown robust satellite cell proliferation within 48 h of completing exercise in the absence of protein supplementation (McKay *et al.*, 2009, 2010). However, it may be that a delayed response occurred in the placebo group as others have shown satellite cells to accumulate as late as 4-8 days after exercise (Crameri *et al.*, 2004, 2007). When

considering the homogeneity in number of satellite cells between the protein and placebo group at 168 h, it is possible that the satellite cell response was not completely captured across the selected sampling time points, highlighting the difficulty with biopsy sampling collection for timing of satellite cell proliferation.

Publication	*N* (Sex)	Age	Exercise Mode	Daily Protein Intake ($g \cdot kg^{-1} \cdot d^{-1}$)	Protein Bolus (g)	Satellite Cell Gene Marker	0.5 h	1 h	2 h	4 h	6 h	12 h	24 h	48 h	72 h
Hulmi et al. 2008	18 (M)	62 ± 4	RES	1.2	30	MyoD		↔					↔	↔	
						MyoG		↔					↔	↔	
						cdk2		↔						↑ ~250%	
						MSTN		↔					↔	↔	
Roberts et al. 2010	10 (M)	22 ± 4	RES	Not specified	25	MyoD			↔		↔				
						p21			↔		↔				
Snijders et al. 2014	12 (M)	21 ± 2	RES	0.1	N/A	MyoD						↔	↔	↔	↔
						MyoG						↔	↔	↔	
						MSTN						↔	↔	↔	
Rowlands et al. 2016	12 (M)	30 ± 7	END	Not specified	70	MyoD1	↑ ~120%								
						MyoG				↑ ~120%					
Reidy et al. 2017	19 (M)	72 ± 2	RES	Not specified	10 (EAA)	Pax7							↔		
						MyoD		↑ ~64%							

Table D.1 Change in myogenic gene expression in human skeletal muscle in response to acute and short-term high and low protein intakes with exercise. Arrows pointed upwards (↑) indicate a significant increase relative to non-protein control at each time point. Arrows pointed left–right (↔) indicate no difference between protein supplement and controls at each time point. Exercise modalities are abbreviated as RES (resistance training) or END (endurance training). Essential amino acid supplementation is abbreviated as EAA.

Publication	*N* (Sex)	Age	Exercise Mode	Daily Protein Intake (g•kg^{-1}•d^{-1})	Protein Bolus (g)	Satellite Cell IHC Marker	12 h	24 h	48 h	72 h	168 h
Farup et al. 2014	24 (M)	23 ± 1	RES	1.2	28	Pax7+		↔	↑ ~67%		↔
Snijders et al. 2014	12 (M)	21 ± 2	RES	0.1	N/A	Pax7+	↔	↔	↔	↔	
McKenzie et al. 2016	6 (M) / 2 (F)	25 ± 7	END	2.6	67	Pax7+			↔		
Reidy et al. 2017	19 (M)	72 ± 2	RES	Not specified	10 (EAA)	Pax7+		↑ ~100% (type I)			
						Pax7+/Ki67+		↑ ~300%			
						MyoD+		↔			

Table D.2 Change in satellite cell content measured through immunohistochemistry (IHC) in human skeletal muscle in response to acute and short-term high and low protein intakes with exercise. Arrows pointed upwards (↑) indicate a significant increase relative to non-protein control at each time point. Arrows pointed left–right (↔) indicate no significant difference between protein supplement and control at each time point. Values are presented for mixed muscle fibres, unless specified. Exercise modalities are abbreviated as RES (resistance training) or END (endurance training). Essential amino acid supplementation is abbreviated as EAA.

Consuming a 70 g bolus of milk protein (providing 15 g of leucine) following prolonged endurance exercise upregulates MyoD and MyoG gene signalling networks in the first 4 h of post-exercise recovery compared to a lower protein intake (23 g, providing 5 g of leucine) or an isoenergetic carbohydrate placebo (Rowlands *et al.*, 2016). Though not a direct measure of satellite cell content, the augmented gene expression of these myogenic regulatory factors may be indicative of a greater propensity for satellite cell proliferation and differentiation. Collectively, the results from these studies suggest that protein ingestion may accentuate myogenic regulatory factor gene expression and promote satellite cell activation and proliferation following single bouts of resistance and endurance–based exercises.

Not all findings have been analogous amongst all studies. For example, in a crossover study in which healthy young men completed three separate bouts of lower–body resistance exercise before ingesting 25 g of whey protein isolate or placebo (maltodextrin or artificial sweetener) beverages, no differences in MyoD mRNA expression were observed between conditions (Roberts *et al.*, 2010). While not directly measured, the isolated gene expression of MyoD suggests an absence of satellite cell proliferation. Similarly, results from a crossover trial in a cohort of well-trained cyclists (VO_{2max} ~63 mL•kg^{-1}•min^{-1}) performing 10 days of intensified cycle training (120% of average daily training volume) followed by a period of reduced volume training (~60% of average daily training volume) in combination with intra-session (38 g) and post-session (29 g) whey protein or carbohydrate placebo supplementation have shown limited effects of protein availability on satellite cell function (McKenzie *et al.*, 2016). Specifically, following intensified training with protein supplementation, an increase in the number of satellite cells associated with type I fibres in the absence of myonuclear addition was observed, whereas the carbohydrate placebo condition

elicited a rapid increase in both type I satellite cells and myonuclear density (McKenzie *et al.*, 2016). Additionally, both satellite cell and myonuclear number increased following reduced volume training in the carbohydrate placebo condition. Whether a similar response is also apparent in the protein condition was not determined due to insufficient tissue yield from the small sample size (n = 8). Nonetheless, protein supplementation resulted in type I and II myofibre hypertrophy (D'Lugos *et al.*, 2016) following intensified training. Collectively, the increase in satellite cell content and myofibre hypertrophy suggests that protein supplementation may be beneficial for skeletal muscle during periods of heavy endurance training.

While the factor(s) responsible for discrepant outcomes between acute exercise trials is unclear, they may partially be explained by inherent differences in study design and methodology (i.e., type and volume of exercises performed, training status of participants, sex of participants, biopsy timing, analytical measurements, etc.). In particular, several investigations conducted satellite cell analyses as secondary measures and selected biopsy time points around separate primary outcomes (such as cell signalling and gene expression, as well as muscle protein synthesis analyses). As a result, measurement time points across the aforementioned studies ranged from 0-144 h post-exercise (**Table D.2**). Likewise, the use of different satellite cell markers (i.e., Pax7 vs. NCAM, gene vs. protein, etc.) between studies can also introduce considerable variability due to potential issues with differences in antibody sensitivity and detection between markers. Furthermore, satellite cell populations are heterogeneous in their expression of different molecular markers and using a single molecular marker for their identification may underestimate total satellite cell content (Lindström & Thornell, 2009; Lindström *et al.*, 2010). Therefore, multiple labelling methods should be implemented to improve detection of subpopulations of satellite cells progressing

through terminal differentiation (i.e., $Pax7^{-}/NCAM^{+}$)(Lindström & Thornell, 2009). Although multiple labelling will most likely provide a more comprehensive identification of the total satellite cell pool, use of multiple markers in immunohistochemistry can be cumbersome, especially when combining multiple nuclear markers with surface proteins for activation status and myosin heavy chain isoforms for fibre type specific analysis. Such inconsistencies in methodological approaches highlight the need to design studies with satellite cell dynamics as primary outcomes and establish consistent analytical techniques between investigations in order to accurately evaluate satellite cell responses to exercise.

D.5.4 Chronic training

Olsen and colleagues (Olsen *et al.*, 2006) were the first to demonstrate that chronic protein supplementation in combination with strength-based resistance training amplifies the expansion of satellite cell and myonuclei numbers in human skeletal muscle compared to a placebo control (**Table D.3**). In that study, healthy young male participants performed lower body periodised strength training (external loads corresponding to 6-12 repetition maximum) three times per week and consumed 20 g of cow milk protein in close proximity to each training session (10 g pre- and 10 g post-exercise) and once daily on non-training days. Whilst robust increases in muscle fibre cross sectional area were observed both with and without protein supplementation, the increase in number of satellite cells per fibre was significantly greater with protein supplementation. However, data on habitual dietary intake for participants was not provided, making it unclear whether the larger expansion in satellite cell content was a result of a greater daily protein intake or due to protein availability in close proximity to exercise. Furthermore, whether protein feeding influenced satellite cells in a fibre-

type-specific manner was not determined. Nonetheless, findings from this study provided the first evidence that consuming a bolus amount of additional protein around resistance exercise bouts could augment long-term training–induced satellite cell expansion and yield concomitant increases in myonuclear accretion and fibre hypertrophy.

Publication	*N* (Sex)	Age	Intervention Length	Daily Protein Intake ($g \cdot kg^{-1} \cdot d^{-1}$)	Protein Bolus (g)	Biopsy Time (h)	Satellite Cell Marker	Satellite Cell Density	Myonuclear Density
Olsen et al. 2006	32 (M)	24 ± 2	16 wk	Not specified	20	N/A	NCAM	↑ ~39%	↔
Molsted et al. 2015	16 (M) / 13 (F)	55 ± 14	16 wk	1.3	9.4	48-72	Pax7	↔	↔
Mobley et al. 2017	75 (M)	21 ± 1	12 wk	1.95	26	72	Pax7	↑ ~67%	↔
Reidy et al. 2017	54 (M)	25 ± 1	12 wk	1.6	22	72	Pax7	↔	↔
Dirks et al. 2017	12 (M) / 22 (F)	77 ± 1	24 wk	1.3	30	72	Pax7	↔	↔
Reidy et al 2017	9 (M) / 14 (F)	23 ± 1 and 66 ± 1	8 wk	Not specified	17	72-120	Pax7	↔	↔
Farup et al. 2014	22 (M)	24 ± 1	12 wk	Not specified	19.5	72-144	Pax7	↑ ~25% (type I)	↔

Table D.3 Change in satellite cell and myonuclear content measured through immunohistochemistry in human skeletal muscle in response to chronic resistance exercise with protein supplementation. Arrows pointed upwards (↑) indicate a significant increase relative to non-protein control at each time point. Arrows pointed left–right (↔) indicate no significant difference between protein supplement and control at each time point. Values are presented for mixed muscle fibres, unless specified.

To further explore how increased protein availability may influence satellite cell numbers in response to chronic resistance training, Farup and colleagues (Farup *et al.*, 2014*b*) investigated the effect of contraction mode (i.e., concentric versus eccentric) on fibre type specific satellite cell response in the presence of a protein supplement. Using a within-subject design, healthy young male participants undertook 12 wk of unilateral resistance training of the knee extensors, three times per week, with one leg performing eccentric (lengthening) contractions only and the contralateral leg performing concentric (shortening) contractions only. For the duration of the training program, participants were randomised into either a protein supplement (~20 g of whey protein) or a control group (isocaloric carbohydrate placebo). On all training days, participants ingested half of their supplement before and the remaining half after training (providing ~10 g protein pre- and ~10 g protein post-exercise). Though both protein and placebo supplementation resulted in equivalent increases to type I fibre cross-sectional area and number of satellite cells per unit of fibre cross-sectional area, protein supplementation elicited a significantly greater satellite cell expansion compared to the placebo group. Though not directly measured, the greater increase in satellite cell content with concentric contractions was hypothesised to be caused by the larger metabolic demands and greater transcription of IGF-1 with concentric versus eccentric contractions. Additionally, concentric contractions combined with protein supplementation lead to increases in type II fibre cross sectional area with parallel myonuclear accretion. Notably, a similar degree of myonuclear addition was also observed in type II fibres in the absence of hypertrophy with eccentric training in the placebo group. While it is unclear why nutrient intake resulted in contraction mode specific changes to myonuclear content, the similar increase in type II fibre myonuclei suggests any potential ergogenic effects of protein to drive hypertrophy may not have been

responsible for myonuclear addition. However, information regarding changes to myonuclear domain were not presented and therefore cannot be ruled out as a possible explanation for expansion of myonuclear number. Nevertheless, the results provide further evidence for the consideration of protein supplementation to augment satellite cell content with chronic training.

The findings of Farup and colleagues (Farup *et al.*, 2014*b*) raise the possibility that increasing supplemental protein availability around concentric-based exercise could amplify long-term training-induced increases in satellite cell and myonuclei numbers and promote fibre hypertrophy. It has previously been reported that an increase in myogenic gene expression and satellite cells associated with type I fibres manifests after acute bouts of cycling exercises (McKenzie *et al.*, 2016; Rowlands *et al.*, 2016). Given the reliance upon type I fibres for aerobic-based contractile activity, consumption of additional protein after endurance exercise may be a useful strategy to promote type I fibre hypertrophy and myonuclear turnover to support tissue repair through increased satellite cell proliferation. To date, no investigation has assessed the effects of chronic endurance training with protein supplementation on satellite cell function, and is an area that deserves further attention.

The type of protein ingested has also recently received attention in regards to satellite cell response to chronic resistance training in young healthy men (Mobley *et al.*, 2017). In a study involving 12 wk of periodised whole-body resistance training, participants were randomly allocated to either a leucine supplementation (~3 g), one of two leucine-matched protein supplements (whey protein: ~26 g, or soy protein: ~39 g), or a carbohydrate placebo supplement (~44 g) condition to be consumed twice daily that resulted in habitual daily protein intakes of ~1.35 g•kg^{-1}•d^{-1}, ~1.95 g•kg^{-1}•d^{-1}, and ~1.3 g•kg^{-1}•d^{-1}, respectively, throughout the intervention. Regardless of dietary

condition, all participants increased total lean body mass and muscle strength, as well as fibre cross sectional area and myonuclear number in both type I and II fibres at the end of the 12 wk. However, only participants consuming whey or soy protein supplements significantly increased satellite cell count (~94%) in mixed muscle fibre types. These results suggest that consumption of intact protein influences the satellite cell response to chronic training.

Not all studies, however, have reported added benefits of protein supplementation on satellite cell activity during periods of chronic resistance training in young men (Spillane & Willoughby, 2016; Reidy *et al.*, 2017*b*, 2017*c*) or elderly men and women (Dirks *et al.*, 2017). Work from Reidy and colleagues (Reidy *et al.*, 2017*b*) found 12 wk of resistance exercise training (including both concentric and eccentric contractions) in the presence of protein supplementation (22 g of either whey or a soy-dairy protein blend ingested immediately post–exercise on training days, and once between meals on non-training days) resulted in similar increases in mean fibre satellite cell content, proportion (percentage of satellite cells per myonuclei), and domain (satellite cells per mm^2) compared to an isocaloric maltodextrin placebo condition. Notably, habitual protein intake for participants across conditions in this study was ~1.3 $g \bullet kg^{-1} \bullet d^{-1}$, and was increased to ~1.6 $g \bullet kg^{-1} \bullet d^{-1}$ in the protein supplemented group. The authors concluded that habitual protein intake without supplementation was sufficient to promote skeletal muscle remodelling and satellite cell activity following chronic resistance training. Therefore, it appears that when adequate protein is available, additional protein supplementation is otherwise of negligible benefit. However, the authors did observe a trend for greater satellite cell content increases in myosin heavy chain type I fibres with protein supplementation compared to the placebo control. Thus, it is also plausible that these studies did not

observe any added (or synergistic) benefits of protein supplementation due to the high individual variability in responses to protein ingestion and potential low effect size for protein supplementation to enhance muscle anabolism and associated satellite cell responses (Reidy & Rasmussen, 2016).

Recent work from the Dirks and associates (Dirks *et al.*, 2017) examined whether protein supplementation over a 24-wk whole-body resistance-training program in frail elderly men and women modulates satellite cell content. Participants were randomly allocated to either a protein (30 g of milk protein) or placebo (non-protein containing dairy beverage) supplement group and trained twice weekly over the course of the intervention. Baseline habitual protein intake for participants was 1.0 $g \cdot kg^{-1} \cdot d^{-1}$ and was increased to 1.3 $g \cdot kg^{-1} \cdot d^{-1}$ in the protein–supplemented group. While there was a trend for muscle fibre hypertrophy in the placebo group after training (no change in type I and ~20% in type II; $P = 0.051$), only significant hypertrophy was observed in the protein-supplemented group (~23% in type I and ~33% in type II, $P < 0.01$). Despite the marked increase in fibre cross sectional area, no changes in satellite cell or myonuclear content were observed in either group. The authors attribute the lack of changes in satellite cell and myonuclear content to smaller baseline myonuclear domains, which may have allowed fibre hypertrophy to occur without the need for additional myonuclei. These findings are in contrast to previous reports in elderly individuals (Verdijk *et al.*, 2009), whereby resistance training-induced hypertrophy is accompanied by an increase in satellite cell content.

Incorporating protein supplementation to chronic exercise rehabilitation programmes following short-term bed rest has also been equivocal (Reidy *et al.*, 2017*c*). Following five days of bed rest, both young and older adults completed 8 weeks of eccentric knee extensor training three times per week. During the rehabilitation

programme, half of the young participants and all of the older participants were provided with 17 g of a BCAA-enriched (4.6 g leucine, 2.4 g isoleucine, 2.3 g valine) whey protein. Though only the older participants increased myofiber cross-sectional area from the cessation of bed rest to completion of training, all participants demonstrated significant increases in satellite cell density and number per fibre, regardless of protein supplementation. Despite no further benefit of protein supplementation to satellite cell content in the young cohort, these results are inconclusive in regards to older individuals as there was no non-supplemented older participant group to determine whether the satellite cell response in these older subjects was solely due to exercise training, protein supplementation, or a combination of the two.

Protein supplementation in combination with 16 wk of resistance exercise in clinical populations undergoing dialysis has also shown no effect on satellite cell content, myonuclear number, or myonuclear domain compared to a placebo condition (Molsted *et al.*, 2015). In this study, habitual protein intake for participants was 1.3 $g \bullet kg^{-1} \bullet d^{-1}$ in both groups, and was unchanged with the additional 9.4 g of whey protein ingested by the protein supplement group. Considering the equivalent daily protein intake between conditions, it is possible that the protein supplement consumed may not have been an effective dose to elicit a meaningful change to satellite cell activity given the relatively low leucine content. However, a dose response study has yet to be performed to determine if a protein threshold exists to stimulate satellite cell activity. Additionally, dialysis patients have reduced type I fibre satellite cell content, but not fibre area or myonuclear content, compared to healthy untrained men (Mackey *et al.*, 2014). Consequently, the satellite cell pool of dialysis patients may be under excessive stress in order to maintain fibre size and myonuclear numbers. Accordingly, the results

related to satellite cell content/activity from investigations involving disease-states must be interpreted with caution when making comparisons to healthy populations.

Protein overfeeding (~2.5 g•kg^{-1}•d^{-1}) has little effect on markers of satellite cell activity (Spillane & Willoughby, 2016). Following 8 wk of whole-body resistance training in healthy young men supplemented daily with either a mixed protein-carbohydrate-fat beverage (94 g, 196 g, and 22 g of protein, carbohydrate, and fat, respectively) or carbohydrate beverage (312 g) before and after each resistance exercise session, no changes to c-Met content, a proxy for satellite cell quantification, were observed in either condition. However, c-Met is expressed in several epithelial cell types and is not exclusive to satellite cells (Lindström *et al.*, 2010). Thus, without having also directly measured satellite cell specific markers (i.e., Pax7), it is unclear whether the training stimulus or protein supplementation affected satellite cell content. Nevertheless, the findings from this study suggest that high protein intakes provide no benefit to satellite cell responses during chronic training.

D.5.5 Protein restriction

Several studies have investigated the effects of protein restriction on satellite cell activity in human skeletal muscle. Four days of severe protein restriction (0.1 g•kg^{-1}•d^{-1}) in healthy young men had little impact on satellite cell content during post-exercise recovery following a single bout of resistance exercise compared to a protein intake of a 1.2 g•kg^{-1}•d^{-1} (Snijders *et al.*, 2014*a*) (**Table D.2**). While there were no differences in satellite cell content or myogenic regulator factor gene expression between the low and higher protein diets over the 72 h post-exercise recovery period, a pronounced reduction in the number of satellite cells expressing myostatin protein was observed in the low protein group at 72 h. Myostatin is a member of the transformation

growth factor-β (TGF-β) superfamily and is known to be a negative regulator of satellite cell activity (Taylor *et al.*, 2001; McKay *et al.*, 2012) as well as muscle protein synthesis (Welle *et al.*, 2006). The authors speculated that in the absence of dietary protein, the co-localization of myostatin with satellite cells remains repressed for a prolonged period as a compensatory mechanism to allow muscle remodelling to occur when an adequate concentration of amino acids becomes available. To conclude that protein has no effect on satellite cell activity based on studies in which protein intake has been severely restricted may be an over-simplification. Previous reports have indicated that short-term (7-18 days) protein restriction results in decreased transcription of genes associated with satellite cell proliferation and increased transcription profile of genes related to ubiquitin–dependant protein catabolism and apoptosis (Thalacker-Mercer *et al.*, 2007, 2010). Thus, it would appear that several protective mechanisms exists to allow for skeletal muscle remodelling during an acute period of protein deprivation, although these mechanisms appear to be down-regulated over time.

D.6 Conclusions and future directions

The role of satellite cells in skeletal muscle remodelling has long been known. However, the role for protein ingestion to modulate satellite cell activity is less well understood. Based on current *in vitro* literature, there are clear indications that amino acids improve satellite cell activity; however, results from *in vivo* work remain ambiguous. Data from human trials suggests that dietary protein has the most pronounced effect on satellite cell activity under acute exercise conditions, but may have diminishing returns with prolonged periods of training (i.e., months or years). One potential explanation for this is that acutely increasing dietary protein intake

simply accelerates the myogenic response to exercise, likely through increasing MyoD gene expression, which will eventually be reached with adequate protein intake (**Figure D.1**). Further, the effects of protein on satellite cell response after initiating unaccustomed exercise training, when most myocellular damage occurs, is most pronounced during acute structural repair to combat unfamiliar stress and may not be predictive of long term responses (Damas *et al.*, 2016). In this regard, these potential acute effects with protein supplementation may be of clinical and economic significance by enhancing skeletal muscle remodelling processes that reduce injury occurrence, muscle damage and soreness (Pasiakos *et al.*, 2014). Macrophage activity is also closely tied to satellite cell activity and may be modulated with amino acid availability (Rowlands *et al.*, 2016; Drummond *et al.*, 2017). Thus, the regulation of immunity pathways with protein ingestion following unaccustomed exercise stimuli may in part be responsible for accelerated satellite cell activity.

Little is currently known about the potential mechanistic bases that may govern enhanced satellite cell dynamics with protein ingestion following either resistance or endurance exercise. Therefore, an emphasis on designing studies in which satellite cell responses (i.e., time course of response) are primary outcome measures is essential to critically evaluate such mechanisms. Future studies in which diets are tightly monitored by daily food records in conjunction with supervised exercise training are also required to advance the current understanding of how nutrition (specifically protein) can stimulate satellite cell contribution to support exercise adaptations. Similarly, how variable protein intake affects satellite cell activity in response to divergent modes of exercise (e.g., resistance, endurance, or combined resistance and endurance) is a topic that warrants further exploration. In this regard, it has been shown that protein ingestion following a bout of concurrent resistance and endurance exercise increases rates of

muscle protein synthesis and attenuates markers of muscle catabolism compared to a placebo control (Camera *et al.*, 2015). Whether increased protein availability during a chronic concurrent training program can rescue the inhibition of satellite cell activity previously observed after a single bout is unknown. Thus, future investigations combining concurrent exercise and protein consumption with regards to satellite cell activity are needed to improve the translation from research to practice when prescribing exercise and dietary interventions to promote skeletal muscle health and quality with this training modality. Likewise, how the distribution of daily protein intake affects satellite cell activity after exercise is currently unknown. Low protein diets affect satellite cell activity and there are important implications of this for clinical populations. Accordingly, studies are needed to determine how changes to feeding patterns may impact the time course of satellite cell activity and skeletal muscle remodelling. Additionally, whether specific amino acids have potential regulatory roles in the return of satellite cells to quiescence is unknown and deserves consideration to improve our understanding of how satellite cells maintain regenerative capacity. Finally, a better understanding of the association, if any, between amino acid transporter expression/activation and satellite cell activity is warranted to determine whether the capacity for these transporters may be a limiting factor for the inward transport of amino acids to subsequently regulate satellite cell dynamics.

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APPENDIX E

RESEARCH PORTFOLIO

In accordance with Australian Catholic University Higher Degree Research policies for the degree of Doctor of Philosophy with publication, the following Research Portfolio has been included to summarise and clearly identify the nature and extent of the intellectual input contributed to research outputs by the candidate and any co-authors.

E.1 Statement of Contribution

Baubak Shamim, Miguel S. Conceição, Marcus J. Callahan, and Donny M. Camera. Where do satellite cells orbit? An endomysium space odyssey. *Journal of Physiology*. 2018 May 15;596(10):1791-1792.

I acknowledge that my contribution to the above paper is 97 per cent.

[redacted] Date: 6/1/20

I acknowledge that my contribution to the above paper is 1 per cent.

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I acknowledge that my contribution to the above paper is 1 per cent.

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Baubak Shamim, John A. Hawley, and Donny M. Camera. Protein Availability and Satellite Cell Dynamics in Skeletal Muscle. Sports Medicine. *Sports Medicine*. 2018 Jun;48(6):1329-1343.

I acknowledge that my contribution to the above paper is 70 per cent.

[redacted] Date: 6/1/20

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I [redacted] acknowledge that my contribution to the above [redacted] cent.

D.M. Camera: Date: 6/1/20

Baubak Shamim, Brooke L. Devlin, Ryan G. Timmins, Paul Tofari, Connor Lee Dow, Vernon G. Coffey, John A. Hawley, and Donny M. Camera. Adaptations to Concurrent Training in Combination with High Protein Availability: A Comparative Trial in Healthy, Recreationally Active Men. *Sports Medicine*. 2018 Dec;48(12):2869-2883.

I acknowledge that my contribution to the above paper is 55 per cent.

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