# THE RIBOSOME & SKELETAL MUSCLE ANABOLISM

Submitted by Christopher Koscien, to the University of Exeter as a thesis for the degree of Master of Science by Research in Sport and Health Sciences

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I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

Signature: 

# ABSTRACT

Skeletal muscle hypertrophy is primarily determined by consistent increases in muscle protein synthesis in response to protein and resistance exercise. Any alterations in resistance training, such as load, and protein, such as dose, can impact the degree of muscle protein synthesis and therefore skeletal muscle hypertrophy.

Non-animal derived sources of protein have increased in popularity due to their ethical and environmental benefits. Previous evidence has shown that non-animal derived protein can stimulate hourly muscle protein synthesis to a lesser extent than animal-derived sources. However, this response can be rescued through increasing the amount of protein consumed. Indeed, two recent studies have shown that by consuming a high amount of daily protein with resistance exercise, the degree of daily muscle protein synthesis and skeletal muscle hypertrophy is unaffected by the type of protein consumed.

Muscle protein synthesis is modulated through sub cellular processes at the level of the ribosome, through either increasing ribosome efficiency (translational efficiency) or ribosomal number (translational capacity). Translational capacity has been thought to underpin the longer-term changes in muscle protein synthesis and skeletal muscle hypertrophy, however little is known about the time course and the transcriptional regulation of translational capacity during skeletal muscle hypertrophy. Therefore, the aim of this thesis is to characterise the abundance and regulation of indirect markers of translational capacity during a short (3 days) and long (10 weeks) of resistance training and

understand if this response is impacted by the type of protein (animal or non-animal derived) consumed.

The first study demonstrated that 3 days of resistance training and high protein consumption lead to a stimulation of all areas of ribosome biogenesis, including rDNA transcription and related signalling, ribosomal proteins and mature rRNA transcripts. However, this did not lead to significant increases in indirect markers of ribosome concentration and other macromolecules (DNA and protein). Additionally, this effect occurred irrespective of the type of protein (animal vs non-animal derived) consumed.

Building upon the first study, the second study measured the regulation and the concentration of the ribosome temporally during 10 weeks of consistent high protein consumption and resistance training. Again, it was found that all areas of ribosome biogenesis were stimulated during the 10 weeks of resistance training and high protein consumption. Indeed, this led to an increase in indirect markers of ribosome concentration at and around 2 weeks of resistance training and this response was maintained for the duration of the study. Similarly to study 1, the transcriptional regulation and concentration of the ribosome was unaffected by the type of protein consumed.

The present thesis reported the novel finding that the transcriptional regulation of all areas of ribosome biogenesis are increased following 3 days and during 10 weeks of high protein consumption and consistent resistance exercise training, irrespective of the type of protein (animal or non-animal) consumed.

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# DECLARATION

The *in vivo* human data collection was carried out by Dr Alistair Monteyne and myself. All of the molecular analysis within this thesis was carried out by myself.

Associate Professor Benjamin Wall, Dr Marlou Dirks, Professor Francis Stephens, and Dr Sarah Jackman conducted the skeletal muscle biopsy procedure described throughout, in that order of prominence. RNA extracted samples were run on a RT-PCR machine by Dr Benjamin Lee.

I hereby declare that the present thesis has been composed by myself, and that it is a record of work performed by myself, except where assistance has been acknowledged. No part of this thesis has been submitted in any other application for a higher degree and all sources of information have been appropriately referenced.

Christopher Koscien

15th September 2021

*The scientist does not study nature because it is useful.*

*He studies it because he takes pleasure in it,*

*and he takes pleasure in it because it is beautiful.*

Henri Poincaré, Science and Method, 1908

## CHAPTER 1

# GENERAL INTRODUCTION

Skeletal muscle tissue has a remarkable ability to adapt to various stimuli. The loss of skeletal muscle mass is associated with many disease states and can result in significant health care costs (Janssen *et al.*, 2004). As a result, adopting methods to reverse muscle mass loss would provide a benefit to the individual and wider society. But first, in order to fully appreciate what occurs in a muscle disease state, one must investigate the regulation of muscle growth (hypertrophy). Thereafter, one can fully make comparisons between states (healthy vs unhealthy) to understand areas of interest and differential regulation.

## MUSCLE PROTEIN TURNOVER

Overview

Skeletal muscle mass is dictated by the balance of muscle protein synthesis and protein breakdown, known as muscle protein turnover. As a result, skeletal muscle hypertrophy is initiated by a positive state of muscle protein turnover, i.e.: muscle protein synthesis is greater than muscle protein breakdown, and overtime muscle (specifically myofibrillar) fibres are accrued whereby it can be detected by instruments such as magnetic resonance imaging. On the other hand, muscle protein synthesis can be detected after only a few hours after a stimulus, utilizing stable isotope tracers and mass spectrometry. However, muscle protein breakdown is technically very challenging to measure and when is measured, can only be investigated over a short period of time. Consequently, the literature has favoured the measure of muscle protein synthesis but also from early work in the field found that muscle protein synthesis is the main driver of muscle protein turnover (Phillips *et al.*, 1997), in the case of skeletal muscle hypertrophy. Overall, the measurement of muscle protein

synthesis and skeletal mass allows a complete picture of the acute, to chronic alterations in muscle hypertrophy.

Resistance training

One stimulus that can increase muscle protein synthesis is resistance exercise. After a single bout of resistance exercise protein synthesis of the of contractile, collagen and structural (sarcoplasmic) proteins can be elevated for up to 72 hours but usually peaks at 24 hours (Miller *et al.*, 2005). As resistance training progresses, the frequent stimulation of muscle protein synthesis after resistance exercise leads to an accrual of new muscle tissue (skeletal muscle hypertrophy). Typically, the rate of muscle growth is greatest at the early phase of training (usually between 0-3 weeks of training; Brook *et al.*, 2015). As the individual becomes more "trained" the duration of exercise-induced muscle protein synthesis becomes shorter but also becomes more specific in remodelling. Furthermore, in the trained state, resting (~72 hrs after resistance exercise) muscle protein synthesis is greater than in the untrained state (Kim, Staron and Phillips, 2005). This response is likely to accommodate the greater muscle turnover as a consequence of greater levels of muscle mass. Overall, the physiological response to resistance training is caused by frequent and consistent elevations in muscle protein synthesis (metabolic level). Furthermore, the metabolic level can be underpinned through the altered regulation on the molecular level. Yet, the specific molecular response that underpins muscle protein synthesis and as a result, skeletal muscle hypertrophy are not well characterised. Therefore, the upcoming sections will aim to determine what theoretical molecular mechanisms are driving the acute muscle protein synthetic response to resistance exercise as well as the elevated basal muscle protein synthesis levels in the trained state.

Protein

Resistance exercise provides the anabolic stimulus to stimulate muscle protein synthesis, but in order to grow new muscle tissue protein and/or amino acids must be consumed in sufficient quantity to provide the building blocks of muscle tissue. Protein can also serve as an anabolic stimulus to stimulate muscle protein synthesis. Therefore, protein and resistance exercise can work synergistically to prolong muscle protein synthesis. (Moore *et al.*, 2009). The type and dose of protein can have a significant impact on the muscle protein synthetic response. In regards to the type of protein consumed, previously, it has been shown that animal protein was superior to non-animal derived protein in stimulating muscle protein synthesis (Tang *et al.*, 2009). However, recent investigations have shown that when sufficient protein is consumed, the impact of the type of protein on muscle protein synthesis is negligible (Monteyne *et al.*, 2020; Monteyne *et al.*, 2020). It is important to look at the 'big picture' and investigate how weekly consumption of animal or non-animal derived protein impacts muscle growth. Currently, there is a scarcity of chronic studies investigating the impact of the type of protein consumed with resistance training on muscle growth and it has been hypothesised that when sufficient protein is consumed, the impact of the type of protein consumed is negligible. Only one study by Hevia-Larraín *et al.* (2021) has tested this hypothesis. Participants underwent a 12 week resistance training program and either consumed a vegan or omnivorous diet with a daily protein intake of ~1.7 g/kg of bodyweight/day. It was found that both diet conditions produced a similar increase in leg lean mass and lower body strength. Therefore, based on the limited evidence available, when sufficient protein is consumed the type of protein consumed has no impact on resistance exercise-induced muscle growth. However, more studies are required to strengthen these findings.

## THE RIBOSOME

Muscle protein synthesis occurs at the 80S ribosome in muscle cells. The 80S ribosome is a functional unit, which comprises of a small (40S) and large subunit (60S) with its sole purpose being to synthesise new muscle proteins through the translation messenger ribonucleic acid (mRNA). This process is highly complex in nature and requires a multitude of steps. Translation can be split into three key stages, initiation, elongation and termination. Prior to the initial formation of the mature 80S ribosome complex, a pre-initiation complex, composed of eukaryotic initiation factor (eIF) 3 and 2, transfer RNA (tRNA) and guanosine triphosphate (GTP) is recruited to the $m^7$GpppN cap structure of the mRNA after it binds with the eIF4F complex (eIF4F), composed of eIF4A, eIF4E and eIF4G. Upon binding to the cap structure, this complex scans the mRNA until it detects the initiation codon (AUG), thereby completing the initiation stage of protein synthesis. Thereafter, the 60S and 40S subunit are recruited to mRNA initiation code site, to begin the elongation stage. In this stage, the ribosome utilises amino acids to start building a protein and the choice of amino acids is dictated through the reading of the specific codons. The chain of amino acids is then released from the ribosome when the ‘stop’ codon is encountered (termination) and the chain of amino acids is subsequently folded to create a functional protein (Chaillou, Kirby and McCarthy, 2014).

There are two primary avenues in which muscle protein synthesis can be altered at the level of the ribosome. The first being an increased efficiency of the ribosome (translational efficiency) and the second being an increased ribosome concentration (translational capacity; Figueiredo and McCarthy, 2019). Therefore, these two routes

are important in the regulation muscle protein synthesis and skeletal muscle hypertrophy. However, there exact contribution to the abovementioned processes are not well defined. As a result, the next subsequent sections will aim will explore translational efficiency and capacity in greater detail and establish the current theories surrounding their contribution to the regulation of muscle protein synthesis and skeletal muscle hypertrophy.

## TRANSLATIONAL EFFICIENCY

Translational efficiency can be defined as increases in protein synthesis per unit of mRNA (Figueiredo and McCarthy, 2019). It is currently believed that translational efficiency is driving the resistance exercise-induced increases in muscle protein synthesis (Figueiredo, 2019). This process is primarily regulated through the Mammalian Target Of Rapamycin (mTOR) pathway. mTOR is a serine/threonine kinase involved in cell growth and division in response to amino acids, growth factors and mechanical stimuli (Saxton and Sabatini, 2017; Kirby, 2019). mTOR forms part of two complexes, mTORC1 and mTORC2. mTORC1 is made up of mTOR, regulatory protein associated with mTOR (raptor) and mammalian lethal with Sec13 protein 8 (mLST8; Saxton and Sabatini, 2017). A key paper by Drummond *et al.* (2009) displayed causal evidence that muscle protein synthesis is activated through an mTOR dependent mechanism in humans. In the study, an mTOR inhibitor, rapamycin, was administered to participants prior to resistance training and the confirmation of mTOR inhibition was shown through western blotting of the mTOR protein. As a result of the rapamycin administration, muscle protein synthesis was blunted, whilst the control group, who did not take any rapamycin, showed a significant elevation in muscle protein synthesis in response to resistance training. Overall, this

unprecedented paper highlighted that mTOR's role in muscle protein synthesis regulation is causal.

mTOR increases translational efficiency through several downstream targets (figure 1-1). As mentioned previously, the enhancement of muscle protein synthesis in response to acute resistance exercise is achieved through an increase in translational efficiency, specifically an increased efficiency of the initiation, elongation and termination stages of translation. A well-established downstream target of mTOR is the ribosomal protein S6 kinases (S6K) 1 and 2. However, S6K1 seems to contribute more significantly to mTOR regulated protein synthesis (Ma and Blenis, 2009), therefore this will be discussed in further detail. In humans, the inhibition of mTOR through rapamycin administration resulted in a delayed activation of S6K1, therefore providing further evidence that S6K1 acts in a mTOR dependent manner (Drummond *et al*., 2009). S6K1 is thought to mediate translation initiation through the activation of eIF3, which forms part of the pre-initiation complex. mTORC1 phosphorylation of S6K1 on the Thr389 site results in its dissociation from eIF3 (Ma and Blenis, 2009). Thereafter, mTORC1-eIF3 signalling releases 4E Binding Protein 1 (4E-BP1) from eIF4E (Gingras *et al.*, 2001). Overall, this process results in the final completion of pre-initiation complex, ready for the translation initiation stage. Ribosomal Protein S6 (rps6) is another protein involved in regulating translation efficiency. Rps6 is one of the many ribosomal proteins that form part of the 40S ribosomal subunit, however it was one of the first substrates to undergo inducible phosphorylation (Ruvinsky and Meyuhas, 2006). Rps6 is one of the many targets of S6K1 and this signal pathway appears to be rapamycin sensitive (Drummond *et al.*, 2009). There are several effects of rps6, such as regulating cell size, glucose homeostasis and protein synthesis. In

regards to protein synthesis, rps6 interacts with tRNA, initiation factors and mRNA, which suggests that its phosphorylation regulates translation initiation (Ruvinsky and Meyuhas, 2006).

From the Drummond *et al.* (2009) paper, muscle protein synthesis was not entirely blunted due to rapamycin administration, this would therefore indicate that another pathway independent of mTOR can activate muscle protein synthesis. This pathway is likely the extracellular signal-regulated protein kinase 1 and 2 (ERK 1/2) pathway. ERK 1/2 acts upon translational efficiency through three different avenues. First, ERK 1/2 has been shown to activate rps6 via ribosomal protein S6 kinase A1 (RSK1; Roux *et al.*, 2007). Second, ERK 1/2 has been shown to directly activate S6K1 (Iijima *et al.*, 2002). Third, ERK 1/2 phosphorylates eIF4E via MAP kinase-interacting serine/threonine-protein kinase 1 (MNK1) (Bianchini *et al.*, 2008). Overall, the ERK 1/2 plays a small but important role in regulating muscle protein synthesis.

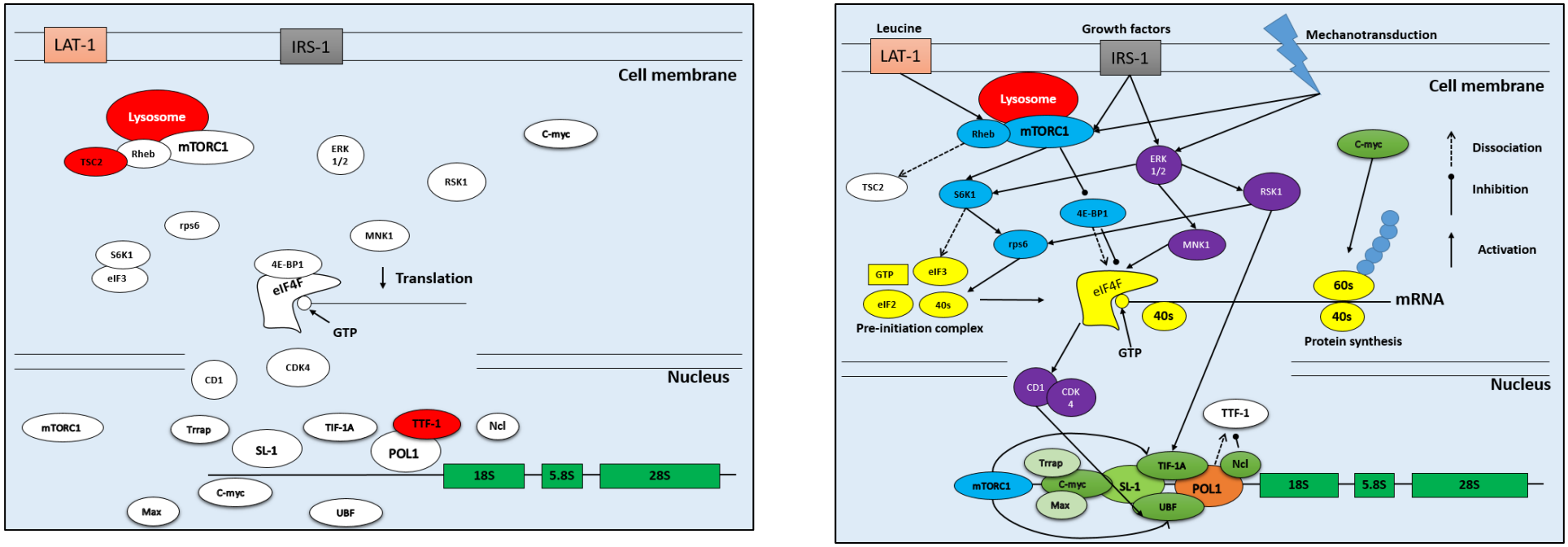


**Figure 1-1.** Two diagrams depicting the transition from an inactive muscle cell to an active cell (left to right) in relation to pathways involved in translational efficiency and rDNA transcription. The mTORC1 dependent pathway is activated in response to leucine, growth factors and mechanotransduction. This creates a variety of downstream effects which ultimately lead to an increased rate of protein synthesis. mTORC1 can also be found on the rDNA site and act on POL1 via TIF-1A and UBTF. The ERK 1/2, an mTORC1 independent pathway, can act on protein synthesis and POL1 activation. POL1 activation can be enhanced by transcription factors, such as MYC, TIF-1A, UBTF and SL-1. The white filled circles represent proteins that are inactive. Figure produced by the candidate.

## RIBOSOME BIOGENESIS

Another avenue in which muscle protein synthesis and therefore skeletal muscle hypertrophy can be increased is through translational capacity or ribosome concentration. Specifically, it has been theorised by authors that translational capacity is driving the chronic increases in basal muscle protein synthesis (Figueiredo, 2019), which is a typical response to continuous resistance training (Kim, Staron and Phillips, 2005). Secondly, an increased ribosome concentration would provide a greater capacity to increase translational efficiency as more ribosomes can engage in translation (Figueiredo, 2019). Thirdly, several studies have shown that translational capacity is highly correlated with skeletal muscle hypertrophy (Figueiredo *et al.*, 2015; Nakada *et al.*, 2016). Overall, ribosome concentration plays an integral role in regulating muscle protein synthesis rates and therefore skeletal muscle hypertrophy. Measuring ribosome concentration directly is a laborious and difficult procedure, therefore indirect methods must be utilised. As a consequence, a holistic approach must be taken by utilising several indirect measures of ribosome concentration to overcome this limitation. Some of the most common indirect measurements of translational capacity include, total RNA, RNA to DNA and 28S:18S ratio (Brook *et al.*, 2015, 2016; Figueiredo *et al.*, 2015; Kotani *et al.*, 2021).

The process in which new ribosomes are synthesised is termed ribosome biogenesis (figure 1-2). This is a highly detailed and complex process and many areas of ribosome biogenesis are yet to be explored. Since ribosome concentration plays a vital role in dictating muscle protein synthesis rates and therefore skeletal muscle hypertrophy, it is important to understand what mechanisms are driving this process. The next

subsequent sections will aim to display what is currently known and unknown about the regulation of ribosome biogenesis.

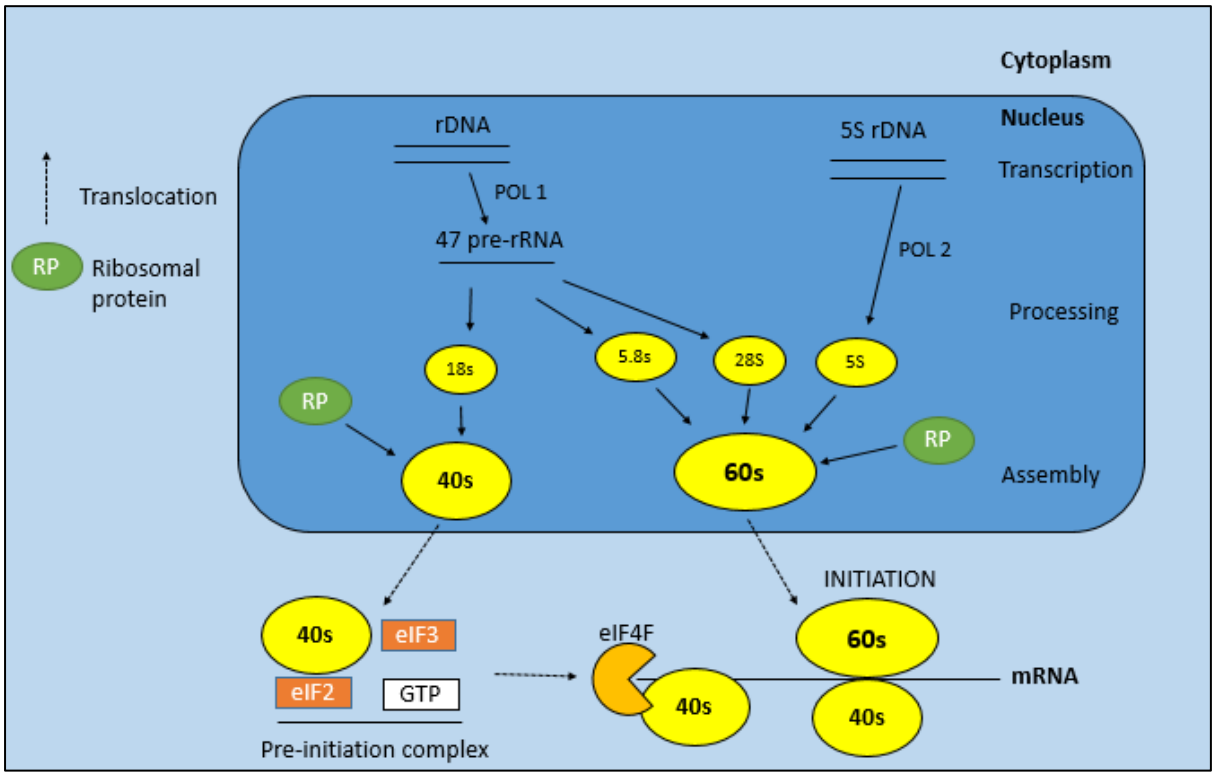


**Figure 1-2.** This figure illustrates the basic process of ribosome biogenesis and translation initiation. rDNA is transcribed to 47S pre-rRNA by the enzyme polymerase I. Parallel to this, 5S rDNA is transcribed to 5S rRNA by polymerase The 47S pre-rRNA is cleaved to produce 18S, 5.8S and 28S. The 40S and 60S subunit is made up of the mature transcripts and several ribosomal proteins. The 40S subunit forms part of the pre-initiation complex and the 60S large subunit is formed with the 40S subunit at the start of translation initiation. Figure produced by the candidate.

rDNA transcription

Ribosome biogenesis begins with ribosomal DNA (rDNA). rDNA is transcribed into 47S pre-rRNA through the enzyme polymerase I (POL1) and this step is regarded as the rate limiting step of ribosome biogenesis (Figueiredo and McCarthy, 2019). Therefore, this process requires further exploration. In order for rDNA transcription to occur, several transcription factors work in concert to activate POL1, each with a specific role but some are regarded more important than others. One transcription factor, MYC, has been shown to enhance rDNA transcription through several different mechanisms. But first, it co-recruits with transformation/transcription domain-associated protein (TRRAP) and the human histone acetyltransferase (HAT) GCN5 via direct physical interactions on its N-terminal activation domain with the human SPT3-TAF$_{II}$31-GCN5L acetylase (STAGA) complex (Liu *et al.*, 2003). Thereafter, MYC can directly promote transcription through POL1 activity by binding to the transcription initiation region, promoter and a region upstream of the promoter on rDNA (Grandori *et al.*, 2005). MYC can also work to activate rDNA transcription through indirect means, specifically, MYC has been shown to activate SL-1, an important regulator of POL1 (Grandori *et al.*, 2005). SL-1 is a transcriptional factor complex composed of TATA-binding protein and 4 TATA box-binding protein associated factors (TAF), TAF1A, TAF1B, TAF1C and TAF1D (Figueiredo and McCarthy, 2019). Additionally, MYC has been reported to interact with upstream binding factor (UBTF), which in turn increases the binding around the promoter region (Histone 1 and 42.9) in human rDNA (Grandori *et al.*, 2005). Lastly, in MYC overexpression animal models POl1 activity is markedly increased independent of resistance exercise like stimulation (Mori *et al.*, 2021).

As mentioned previously, mTOR plays a significant role in translational efficiency, however mTOR also has a pivotal role in enhancing rDNA transcription. mTORC1 binds directly to the rDNA promoter in human cells and dissociation of mTORC1 from the promoter region through rapamycin treatment has been shown to be correlated with a reduction in rRNA synthesis (Tsang, Liu and Zheng, 2011). Additionally, mTORC1 regulates rDNA transcription through indirect mechanisms. Firstly, inhibition of mTORC1 through rapamycin treatment inactivates Transcription Intermediary Factor 1-Alpha (TRIM24), which forms part of the pre-initiation complex responsible for rDNA transcription (Tsang, Liu and Zheng, 2011). Secondly, mTORC1 activates S6K1, which in turn phosphorylates the carboxy-terminal activation domain of UBTF, which allows it associate with the SL-1 complex (Hannan *et al.*, 2003).

Nucleolin, when located within the nucleolar, acts as a transcriptional factor for POl1 due to its abundance in the promoter and coding regions of rDNA. Nucleolin depletion leads to a decreased UBTF activity and the increased recruitment of Transcription Termination Factor 1 (TTF-1), which acts as a negative regulator of rDNA transcription (Cong *et al.*, 2012). Furthermore, knockdown of nucleolin *in vivo* by RNA interference results in inhibition of rDNA transcription (Rickards *et al.*, 2007).

Overall, MYC, mTOR and nucleolin are important direct regulators of rDNA transcription but also provide indirect activation of rDNA transcription through UBTF, TIF1A and SL-1. All the pathways regulating rDNA transcription can be found in figure 1-2.

The development of the ribosome subunits

The human 47S pre-rRNA is produced through rDNA transcription. It contains an External Transcribed Spacer at the 5 prime end (5'ETS), with two cleavage sites, A' and A0 (figure 1-3). The 18S rRNA is attached to this 5'ETS and contains a 1 cleavage site on the border between itself and the 5'ETS. On the 3 prime side of 18S is the Internal Transcribed Spacer (ITS) 1, which contains 3 cleavage sites. Cleavage site 3 is on the border between the 18S and the ITS1, whilst cleavage site E and 2 is solely on the ITS1. Following the ITS1 is the 5.8S and adjacent to that is the Internal Transcribed Spacer 2 . Next to the ITS2 is the 28S unit, with this being the largest unit in the 47S pre-rRNA. Lastly, there is the 3 primer External Transcribed Spacer (3'ETS) adjacent to the 28S, where the 02 cleavage site is situated on the border between the 3'ETS and the 28S unit. Once the 47S pre rRNA is generated, it undergoes many intricate and complicated steps to cleave the pre-rRNA into the separate 5.8S, 18S and 28S units, through the use of cleavage sites and exoRNases. To build the 40S small ribosome it requires the assembly of 18S rRNA and 33 ribosomal proteins, whilst the 60S large subunit requires the assembly of 5.8S, 28S and the 5S r-RNA and 47 ribosomal proteins, such as ribosomal protein L3 (RPL3) (Khatter *et al.*, 2015). With regards to location of this process, the formation of the ribosome subunits takes place within the nucleus, thereafter the subunits are exported to the cytoplasm and assembled with other proteins to form a mature 80S ribosome complex capable of manufacturing proteins.

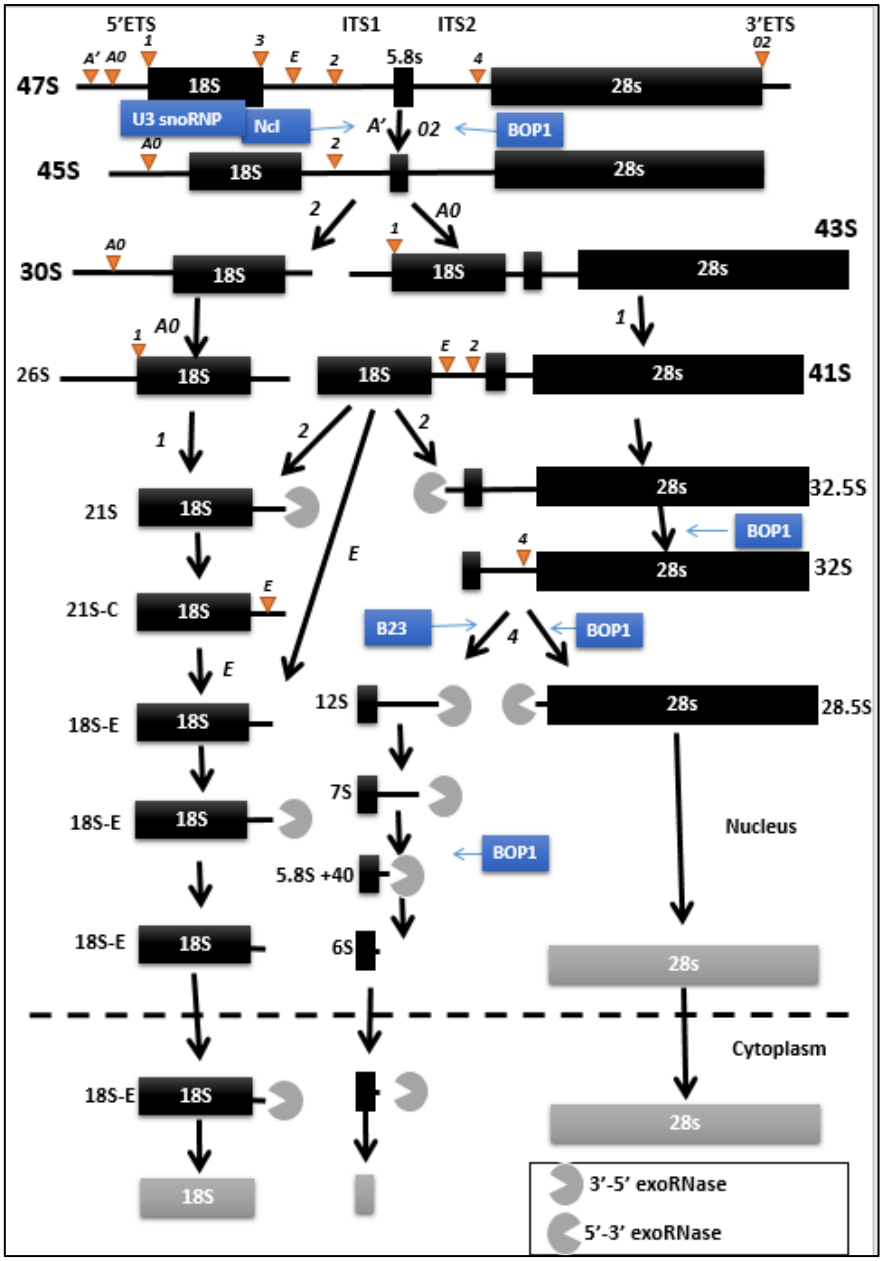


**Figure 1-3.** This figure displays a detailed look at the process of cleaving 47S pre-rRNA to form the 5.8S, 28S and 5S units. At the beginning of this process is the 47S pre-rRNA formed from rDNA transcription. At each sequential step, a site on the pre-rRNA is either cleaved (denoted from the orange triangles) or removed by ExoRNases (grey pacman). The ultimate goal being to remove the 5 prime Eternal Transcribed Spacer (5'ETS), Internal Transcribed Spacer 1 (ITS1), Internal Transcribed Spacer 2 (ITS2) and 3 prime External Transcribed Spacer (3'ETS). This figure was adapted from Aubert *et al.* (2018).

rRNA processing proteins

During ribosome biogenesis small nucleolar RNAs (snoRNAs) and trans-acting proteins form pre-ribosomal RNP (pre-rRNP) complexes, which play a role in aiding processing and modification of pre-rRNAs and assembly of rRNAs with ribosomal proteins (Hayano *et al.*, 2003). A class of snoRNAs are associated with four common core proteins, 15.5k, NOP56, NOP58 and fibrillarin that direct 2'-*O*-methylation of pre-rRNAs at specific sites. NOP56 specifically, is thought to function in pre-ribosomes during the early to middle stages of 60S subunit maturation. Conversely, fibrillarin is only thought to participate in the first step of processing pre-ribosomal RNA (Hayan*o et al*, 2003).

Block of proliferation 1 (BOP1) is one of three components of the PeBoW complex. The PeBow complex is a protein complex responsible for the maturation of large ribosomal subunits. Looking at BOP1 more closely, it is responsible for the cleavage of the 47S pre-rRNA through the targeting of the ITS1 and ITS2 the 3 primer Eternal Transcribed Spacer (3'-ETS) (figure 1-3). Therefore, BOP1 has an important influential role in the formation of the 5.8S and 28S unit due to the fact that an introduction of a BOP1 mutant decreased BOP1 levels and resulted in decreased levels of 28S and 5.8S (Aklina Strezoska, Pestov and Lau, 2002).

Nucleophosmin (NPM1), also known as protein B23, is an abundant phosphoprotein located in the nucleolus. It has been shown to have an important role in various stages of ribosome biogenesis. First, it is responsible for directing the nuclear export of both 40S and 60S ribosome subunits and modest increases in its expression can result in increased rates of protein synthesis. Therefore, it can be described as rate limiting in

its capacity to shuttle ribosomes to the cytosol (Maggi *et al*, 2008). Second, it can directly interact with MYC to form a NPM1-MYC binary complex which is recruited to the promoter of MYC target genes to induce the transcription of proteins required for transformation (Kim, Cho and Park, 2015).

Overall, rRNA processing proteins such as NPM1, BOP1, fibrillarin and NOP56 play pivotal roles in ribosome biogenesis and in some cases, depletion or knockout of specific proteins results in halting of specific ribosome subunit biosynthesis and ultimately, ribosome formation.

Impact of resistance exercise and diet

Resistance exercise and protein consumption have the ability to robustly stimulate muscle protein synthesis and as a consequence, skeletal muscle hypertrophy (Moore *et al.*, 2009; Antonio *et al.*, 2015; Damas *et al.*, 2016). Additionally, the manipulation of protein consumption, such as dose (Morton *et al.*, 2017), and resistance exercise, such as volume (Hammarström *et al.*, 2020), has a profound impact on the muscle protein synthetic response and degree of skeletal muscle hypertrophy. As established previously, ribosome biogenesis has a significant role in muscle protein synthesis and skeletal muscle hypertrophy. Therefore, the underpinnings of abovementioned processes may be attributed to changes in ribosome biogenesis. Indeed, the benefits of moderate volume versus low volume resistance exercise for skeletal muscle hypertrophy have been attributed to changes in ribosome biogenesis (Hammarström *et al.*, 2020). However, the impact of protein consumption on muscle protein synthesis and skeletal muscle hypertrophy has not be explored through the lens of ribosome biogenesis. Additionally, there is a lack of studies investigating the impact of different

types of protein (animal versus non-animal derived) on muscle protein synthesis and skeletal muscle hypertrophy. As a result, this raises some questions. Specifically, can consuming a different type of protein impact the muscle protein synthetic response and muscle mass accretion? Furthermore, if the response is differential between diets, is this underpinned by alterations in ribosome biogenesis? Both questions are still yet to be explored.

## BUILDING UPON EARLIER WORK OF RIBOSOME BIOGENESIS

The greatest rates of skeletal muscle hypertrophy occur usually between 0 and 3 weeks of resistance training (Brook *et al*, 2015). This would suggest that this period produces a great deal of molecular signalling and would be an area of interest in investigating ribosome biogenesis. Previous investigations of ribosome biogenesis with pre and post training measurements may have neglected alterations in ribosome biogenesis at intermediate time points, such as after 3 weeks of resistance training. To build upon the detailed work of Figueiredo *et al.* (2015), temporal measurements could be vital in understanding how ribosome biogenesis is altered throughout a resistance training program and its contribution to skeletal muscle hypertrophy.

## THESIS AIMS

In order to understand the regulation of ribosome biogenesis and its contribution to skeletal muscle hypertrophy in humans, a known model of skeletal muscle hypertrophy must be employed. Therefore, muscle biopsies of a previously conducted resistance training study (Monteyne et al., *unpublished*) were utilised. This study consisted of two phases, both of which had a significant diet and training intervention. The first phase consisted of 3 days of resistance training in conjunction with a controlled high protein

animal (omnivore) or non-animal derived (vegan) diet. The second phase consisted of 10 weeks of resistance training and in combination with a high protein vegan or omnivore diet. Both phases produced a robust metabolic (muscle protein synthesis) and physiological (muscle mass accretion) response to training and diet consumption, therefore providing an ideal environment to measure the regulation of the ribosome.

As established previously, the process of ribosome biogenesis is a highly intricate process and therefore, it is very difficult to measure all the components of ribosome biogenesis in a single study. However, measuring the expression of different genes through an easy reproducible approach is achievable. For example, mRNA microfluidic cards, such as TaqMan array card (ThermoFisher) have been utilised previously (Brook *et al.*, 2016) to understand transcriptional alterations in ribosome biogenesis. This provides an ideal approach to allow the measurement of expression of many different genes with high reproducibility. Although this is measuring transcription not protein expression, it provides the 'intent' of the muscle cell, which provides novel insight into ribosome biogenesis. Furthermore, the common protein expression measurement western blotting, can be laborious when measuring numerous proteins. Additionally, an omic approach to protein expression (proteomics) is a much specialised measurement and is not widely available in many laboratories.

As a result, the principle aims of the thesis are as follows:

1. Provide a temporal transcriptional regulation of ribosome biogenesis after 3 days and several weeks of resistance training and high protein consumption.

2. Investigate whether the type of protein consumed (animal or non-animal derived) differentially impacts the transcriptional regulation of ribosome biogenesis.

## CHAPTER 2

# GENERAL METHODS

# INTRODUCTION

In this chapter, the methods utilised in the experimental chapters will be discussed. Additionally, the fundamental principles of the instruments used and a description of the development of a DNA, RNA and protein extraction protocol from a single aliquot of muscle tissue will be discussed

# POLYMERASE CHAIN REACTION

Principles of PCR

Polymerase chain reaction (PCR) was invented by Kari Mullis (Mullis, 1990). For the first time it allowed the detection and production of a specific DNA fragment from a complex pool of DNA, which can be sourced from a variety of different tissues. Ultimately, it would provide an important tool for the sequencing of the human genome. The PCR method involves providing a pool of DNA with DNA-polymerase and a specific primer, which is specific to a certain sequence of DNA. The combination of these ingredients allows the amplification of a specific sequence of DNA that can be utilized for sequencing and genotyping. The amplification process is as follows. The DNA sequence is doubled at every cycle (when the required abovementioned components are added) and as the number of cycles increase so does the copies of the DNA sequence. This leads to an exponential and linear increase in copies but ultimately leads to a plateau phase whereby no more copies can be generated. This is stage where the abundance of the sequence of DNA is measured.

Development from PCR to RT- PCR

The use of PCR to measure DNA fragments in a variety of different tissues only provides what occurs on the gene level. To further build up on the principles of PCR, Reverse transcription PCR (RT-PCR) was introduced to understand what occurs on the transcription level, therefore, mRNA is utilised as a starting material. But more specifically, complementary DNA (cDNA) is used. CDNA is formed through the reverse transcription of mRNA, and is not subject to RNase (enzyme that degrades RNA) degradation, making it more stable than RNA and therefore a superior candidate for RT-PCR. As a consequence, the main advantage of RT-PCR is that mRNA is utilised as a starting ingredient rather than DNA, allowing the measurement of gene expression. Another important advantage is that the measurement is taken in the exponential phase when compared to the plateau phase in traditional PCR. This phase is very specific, precise and provides the least variability when compared to measurement in the linear and plateau phase, as it is assumed that this phase provides a 100% reaction efficiency or exact doubling in copies at every cycle.

As RT-PCR has grown in accessibility and reduced in cost, this method has begun to be adopted in the exercise and nutritional physiology field to understand how conditions such as exercise and nutrition can impact the transcriptional regulation of different genes involved in muscle metabolism.

RNA extraction protocol

The full RNA extraction and quantification and cDNA synthesis protocol for RT-PCR analysis utilized in the present thesis is as follows. First, 800 µL of TRIreagent/TRIzol solution was added to a 2 ml Eppendorf. This solution is most formally known as

Guanidinium thiocyanate or guanidinium isothiocyanate (GITC) and was first published in 1987 (Chomczynski and Sacchi, 1987). This solution works extremely well with chloroform and phenol to help extract RNA through phase separation, which will be explained in more detail later. Additionally, TRIzol can effectively maintain RNA integrity, whilst also disrupting and breaking down cellular components. Next, 20 µL of glycogen was added to the Eppendorf. Glycogen is typically used in RNA extraction as it can greatly enhance nucleic acid extraction. Glycogen is an inert carrier of nucleic acids and co-precipitates in isopropanol and ethanol. Isopropanol precipitation is used in this protocol and therefore the addition of glycogen (insoluble in alcohol) will further enhance the recovery of RNA. Around 20 – 30mg of wet muscle tissue is added to the Eppendorf and then briefly mixed through inversion. The muscle is then homogenized through the use of a Polytron mechanical homogenizer. This step is extremely important as it ensures that the contents inside the muscle cell, such as RNA, are fully available in the solution. Once the sample was full homogenized, it was incubated at room temperature for 5 minutes. Next, 160 µL of a choloform:iso-amyl alcohol (49:1) solution was added to the Eppendorf. Chloroform extraction first published in 1987 (Chomczynski and Sacchi, 1987) and as mentioned previously, interacts with the Guanidinium thiocyanate added at the beginning of the protocol, to produce three separate phases after centrifugation, aqueous, inter and organic phase (Rio *et al.*, 2010). The contents of these phases are RNA, DNA and protein, respectively. The Eppendorf was shaken vigorously by hand for 10 seconds and cooled on ice for 15 minutes, followed by centrifugation at 10,000 x g for 20 minutes at 4°C. 400 µL of the aqueous phase was transferred into a new RNase free Eppendorf tube and care was taken to ensure that the interphase or DNA was not present in pipette during transfer. This would heavily contaminate the RNA extraction with DNA. 400 µL of iso-propanol

was added to the aqueous phase sample. The addition of iso-propanol ensures the precipitation (the formation of a solid from a solution) of RNA after the overnight incubation period. The sample containing the aqueous phase and iso-propanol was then incubated at -20°C overnight. On the second day of RNA extraction, the sample was centrifuged at 10,000 x g for 20 minutes at 4°C to allow the formation of an RNA pellet. The supernatant was removed and the sample was drained by tipping the tube upside down over tissue paper for 5 minutes. The RNA pellet was washed once in 800 µL of 75% ethanol and 25% RNase free water. The samples were incubated for 10-15 minutes at room temperature. This is to ensure that possible residuals of TRIzol were dissolved. The samples were centrifuged at 10,000 x g for 5 minutes at 4°C and the supernatant was subsequently removed and the sample was air dried for 10 minutes. The RNA pellet was solubilised in 30 µL of RNase free water and the sample incubated for 10 minutes at 60°C. Once all samples were extracted for RNA, RNA concentration and purity was determined on a Nanodrop lite (Thermo Fisher). Measurements were taken in duplicates or triplicates depending on the consistency of measurements shown prior. Measurements were excluded from the overall mean RNA concentration mean value if they did not fall within the A260/A280 ratio range of 1.7-2.3.

Measurement of purity of samples

The purity of nucleic acids utilising the Nanodrop Lite instrument is regarded as the ratio between the absorbance at 260 nm and 280 nm (260/280). A ratio of ~1.8 is generally accepted as “pure” for DNA and a ratio of ~2.0 is generally accepted as “pure” for RNA. Any abnormality in this ratio could indicate reagent contamination from previous nucleic acid purification steps, such as phenol and/or guanidine. In this case,

the ratio is typically low but a high ratio is not usually indicative of a problem but if it is very high it may indicate a poor blank process when using the nanodrop. Importantly, changes in sample pH and ionic strength can alter the 260/280 ratio, thereby causing an over or underestimation. For example, using a Tris-EDTA (TE) buffer instead of RNase free water as a buffer would significantly decrease the 260/280 ratio (Wilfinger, Mackey and Chomczynski, 1997).

cDNA synthesis after RNA extraction

Once RNA was extracted and quantified, total RNA was diluted to 150 ng in 20 µL RNase free water. First, the concentration of RNA (explained previously) in buffer solution was divided by 150 (Step 2). This then gave the exact volume (known as 'Y') in which 150 ng of RNA would be present. However, this volume would be ~0.5-1.0 µL and therefore would provide a great deal of variability when pipetting out. In order to combat this, 'Y' was multiplied by 10 ('X'; step 3), which presented values around ~5-10 µL and would provide 1500 ng of RNA. This provided a far more achievable pipetting volume to maintain accuracy. Thereafter, RNase free water was added to the X volume to achieve a total volume of 200 µL (known as 'Q'; step 4). Lastly, 20 µL was pipetted from Q to another 0.5 ml tube to achieve a final concentration of 150 ng of RNA in 20 µL of RNase free water (Step 6). Overall, this method utilised greater pipetting volumes, which minimised sample and volume loss during pipetting.

Example:

1. 200 ng/µL concentration in 30 µL RNAse free water.
2. 150/200 = 0.75 µL, Y = 0.75
3. Y x 10 = 7.5 µL, 7.5 = X

4. 200 – X = 192.5 µL RNase free water
5. 200 µL volume = Q
6. 20 µL taken from Q, which achieved 150 ng of RNA in 20 µL of RNase free water.

Following this step, a master mix (Invitrogen superscript III supermix kit) was created on ice. For one sample, the sample mix was made up of 10 µL of 2xReverse Transcription (RT) reaction mix (containing oligo(dT)$_{20}$, random hexamers, $MgCl_2$ and dNTPs), 2 µL of RT enzyme (containing superscript® III RT and RNaseOUT™ and recombinant ribonuclease inhibitor) and 17 µL of DEPC-treated $H_2O$. Once the master mix was created and vortexed, 29 µL was pipetted into each sample. The sample was then gently vortexed and incubated within a thermocycler (Thermo Fisher), at the following temperatures, 25°C for 10 min, 50°C for 30 min, 85°C for 5 min. Each deviation in temperature provided a different role in promoting cDNA synthesis. Heating the sample to 25°C promotes the annealing or binding of the primer to the specific region of the RNA strand. A further heating to 50°C starts the DNA polymerization process whereby the RT enzyme promotes cDNA synthesis starting from the primer region. Finally, a further heating to 85°C deactivates the RT enzyme thereby halting cDNA synthesis. Once the thermocyler step was completed, 1 µL RNase H was added and then the sample was incubated at 37°C for 20 minutes. RNase H removes the RNA template that is attached to cDNA after cDNA synthesis. According to the manufacturer, this has been shown to increase sensitivity in RT-PCR. Following the incubation period, the samples were stored at -80°C, ready for RT-PCR analysis.

Sample preparation for PCR machine run

50 µL Taqman Fast Advanced Master Mix (4444557; Thermo Fisher Scientific) was added to 150 ng of RNA equivalent cDNA in an RNAse-free Eppendorf tube, and RNAse-free water was added to make the total reaction volume 100 µL. The reaction mixture was vortexed, centrifuged, and loaded into 1 of the fill reservoirs of the Micro Fluidic card, after which the cards were centrifuged (Hereaus 3 SRMicrofuge, Thermo Fisher Scientific) and run on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific).

Principles of the microfluidic card

The TaqMan array card by ThermoFisher was chosen for the present thesis. The TaqMan Array Card is a microfluidic card that allows the measurement of 12 to 384 (48 was chosen) gene targets and up to 8 samples on one card. This array card works by pipetting a small quantity of cDNA (30 ng – 1 µg) into a port, which then progresses to a main and feeder channel which leads to the reaction well or gene targets.

Strength and limitations of the microfluidic card approach

The microfluidic cards provide several advantages. It allows the standardization across multiple samples in multiple laboratories allowing reproducible results. As a result, authors can have confidence when comparing gene expression results between studies when using the microfluidic card approach. Another advantage is the streamline reaction set up saves time and reduces labor-intensive steps, which is the main disadvantage with current protein expression measurements such as western blotting and also northern blotting for measuring mRNA abundance. The microfluidic cards can allow many different gene targets to be run on a single card, which is far

greater than methods such as northern blotting for mRNA abundance. However, there are limitations to this measurement, one being the high cost associated with this product, when compared to methods such as northern blotting. Additionally, in some cases there is a disagreement between transcriptome and the proteome (Bathke *et al.*, 2019). Therefore, caution must be taken when translating transcription results to the protein level when protein expression has not been measured.

mRNA abundance

Each individual sample and gene on the Taqman cards provided an absolute Cycle Threshold (CT). The CT value or otherwise known as the Cq value is defined as the number of PCR cycles required for the samples reaction to reach the threshold line. The choice of threshold line could greatly affect this value and what is required is that the threshold needs to be above background noise, to indicate that the PCR cycle is not a consequence of background but true amplification of the sample. For each gene and sample, the PCR machine provided a Cq value which would be extracted to calculate mRNA expression. The Cq values were first expressed relative to the house keeping genes, this is known as a delta CT value. The choice of housekeeping genes is a highly contested area. The main premise of a house keeping gene is to ensure that there is equal expression between samples. In this case, the average of two housekeeping genes beta-2-microglobulin and Glyceraldehyde-3-phosphate dehydrogenase was used in order to ensure equal expression. These specific genes have been used in a previous study by our laboratory (Monteyne *et al.*, 2020). However, many different studies have utilised different housekeeping genes (Figueiredo *et al.*, 2020) and the stability (least amount variance in gene expression between samples) of housekeeping genes can be dependent on the source of tissue

and conditions utilised (Vandesompele *et al.*, 2002; Mahoney *et al.*, 2004). In the present thesis, the average of beta-2-microglobulin and Glyceraldehyde-3-phosphate dehydrogenase showed the greatest stability when compared to both genes alone and the geometric mean of both genes according to a RefFinder software (Xie *et al.*, 2012). Following this step, values were expressed relative the baseline measurement (pre-training or week 0), which gives the delta delta CT value (ΔΔCT). Expressing relative to a baseline measurement has been performed previously in similar studies investigating ribosome biogenesis (Figueiredo *et al.*, 2015, 2016) and therefore allows the appropriate comparison of results between studies. Next, the ΔΔCT values were transformed to 2 to the power of negative ΔΔCT or $2^{-\Delta\Delta CT}$. Finally the values were log2 transformed prior to statistical analysis to ensure normal distribution. Log2 transformation was applied to satisfy one assumption of an ANOVA, data is normally distributed. Additionally, previous resistance training studies have utilised log2 transformation of mRNA abundance data prior to statistical analysis (Hammarström *et al.*, 2020).

Coefficient of variation analysis

The present thesis utilised the QuantStudio 12K (Thermo Fisher) to measure mRNA abundance. To measure the coefficient of variation for this machine, one sample (Ham 16-3) was measured 3 times on two plates (3 and 4) to calculate the intra and interplate CV value and the individual values were grouped together to measure the overall sample CV value. The intra-sample CV was either 1.23 or 1.28%, whilst the overall sample CV with 6 measurements was 1.4% (figure 2-1). This shows that measuring mRNA expression with this machine and specific Taqman card is extremely accurate.

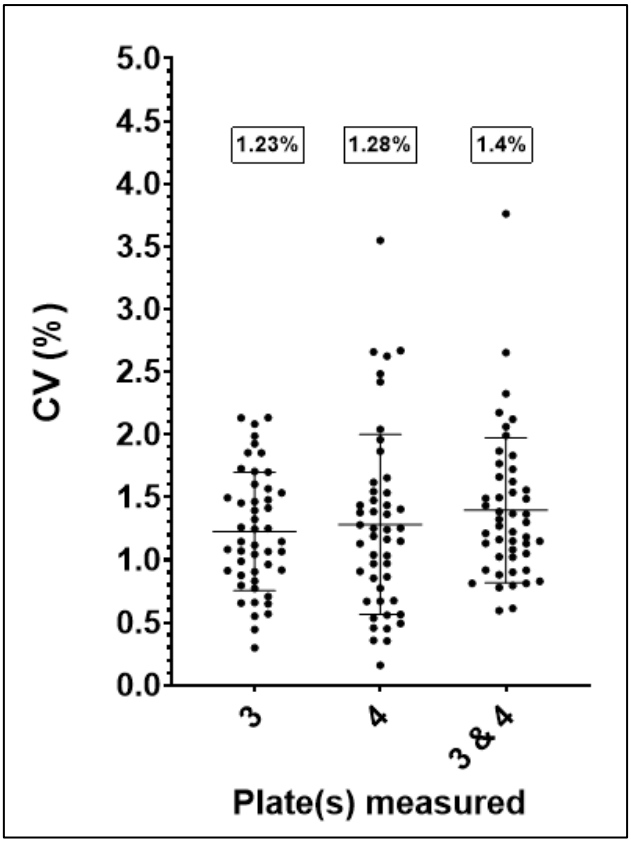


**Figure 2-1.** Coefficient of variation (CV) analysis for the PCR machine utilising sample Ham 16 – 3. Each dot represents a CV value for a single gene. Horizontal line is mean ± standard deviation

## QUANTIFICATION OF RNA

Nanodrop

A nanodrop lite spectrophotometry (Thermo Fisher) was utilised to quantify RNA concentration within the sample. The Nanodrop lite is an adapted version of the NanoDrop 2000 and 8000 created from the same company, but still provides the same rapid, accurate and reproducibly of micro volume measurements. The Nanodrop lite utilises the basic principles of UV photometry, whereby nucleic acids (DNA or RNA) can be measured due to their intrinsic absorptivity properties. RNA and DNA for example, on an absorption spectrum, absorbs light with a characteristic peak at 260

nm. This type of method differs from other methods of nucleic quantification such as fluorescence, which require the use of fluorescent dyes, which can bind to RNA and emit a signal when bound. The specific signal created by photometry can be captured using a spectrophotometer. Once the light is projected through the sample, the attenuation of the light that reaches the detector after passing through the sample indicates the absorbance value of the sample. In the case of the Nanodrop lite, light is projected vertically above the sample and is then captured on a column which the sample is placed on, this is depicted by figure 2-2. Importantly, the Nanodrop lite is unique because the sample can be suspended on a quartz column which relies on a sample retention system and ensures that the sample does not ‘flatten’ and cause inaccurate measurements.

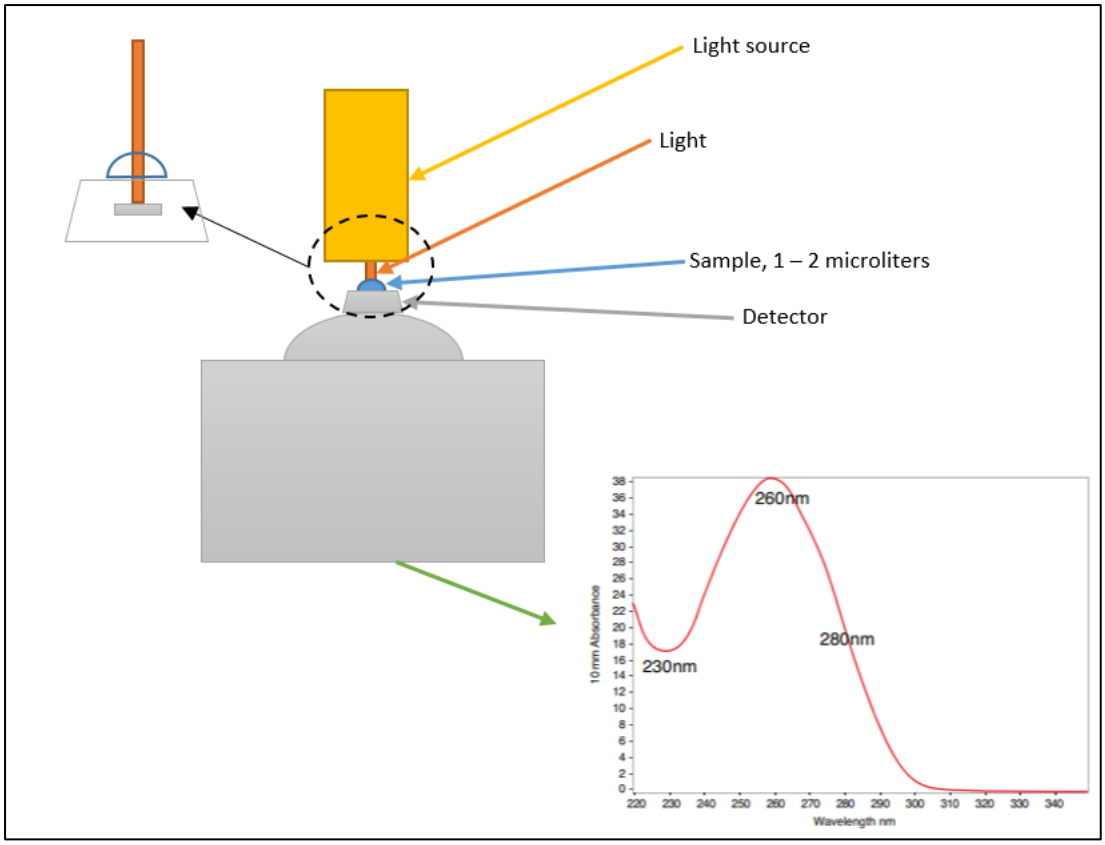


**Figure 2-2.** This figure depicts the core process of measuring nucleic acids by spectrophotometry but specifically by the Nanodrop Lite instrument. Light is project through the sample (usually 1 – 2 µl) and is measured by a detector. The pathlength or distance from light source to detector is 1 cm and this varies depending on the model used. The dashed circle indicates a blow up of this specific area, wherein it shows how light moves through the sample and into the detector. The green arrow is showing the typical nucleic acid spectrum output from this instrument. This graph depicts the absorbance value for different wavelengths, 260 nm being RNA and DNA, 280 nm being protein. The graph was taken from the 'Nanodrop Nucleic acid booklet, Thermo Fisher'.

Qubit

The Qubit instrument, manufactured by ThermoFisher, is utilised in the present thesis to quantify RNA and DNA. This instrument utilises fluorescent dyes to "tag" certain molecules of interest, such as RNA or DNA. Once the fluorescent dyes are bound to the target molecule they emit fluorescence of several orders of magnitude and reaches

a state of equilibrium at around two minutes. Additionally, according to the manufacturer the fluorescence remains stable for at least one hour. To validate, in the present work 47 samples were measured 5 times each with 30 seconds of rest in between measurements (to ensure sample was equilibrated to room temperature). This produced an average CV value of 1.25% (figure 2-3). This supports the claim that the fluorescent signal remains stable after 1 hour.

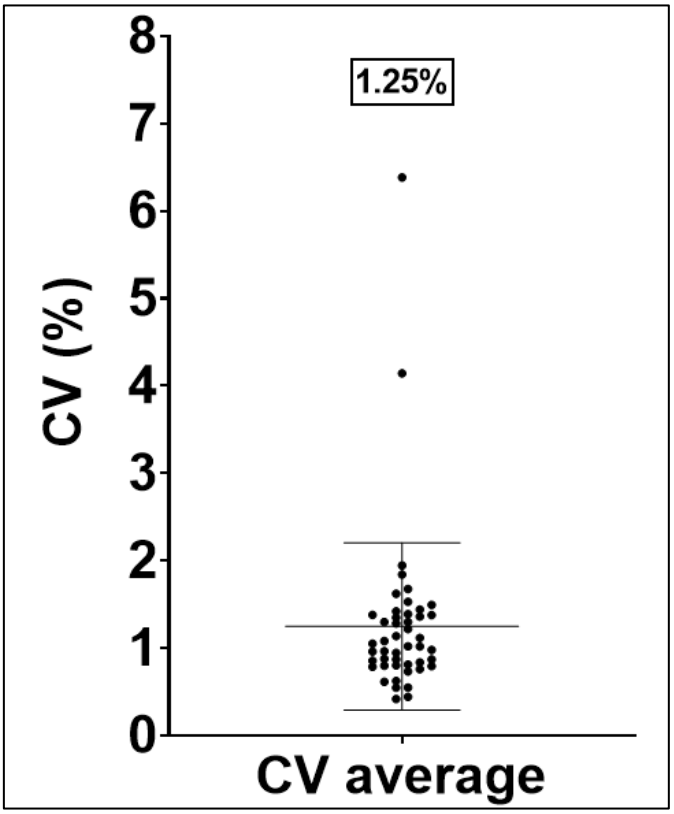


**Figure 2-3.** This figure depicts the coefficient of variation value for each RNA sample measured 5 times by the Quibit. The horizontal line presents the mean ± standard deviation.

Qubit vs Nanodrop

In this thesis two different instruments were utilised to measure RNA concentration, a fluorimeter (Qubit) and a spectrophotometer (Nanodrop Lite). The CV value for both

instruments are vastly different and show how the choice of instrument can greatly affect an outcome. The Qubit instrument produced an overall CV value of 2.9% whist the Nanodrop produced a CV value of 19% (figure 2-4). The primary reason for the increased accuracy of the Qubit versus the Nanodrop is due to the fact that the Qubit utilises dyes to bind to specific molecules of interest whilst the Nanodrop Lite measures the wavelength of the molecule of interest. In some cases for the Nanodrop, the presence of contaminants can be absorbed at the same wavelength of the molecule of interest. Therefore, the Qubit is not impacted by contaminants whilst the Nanodrop is. Consequently, the Qubit should be utilised for macro-molecule quantification where possible, whilst the Nanodrop can be used to indicate contamination of a sample.

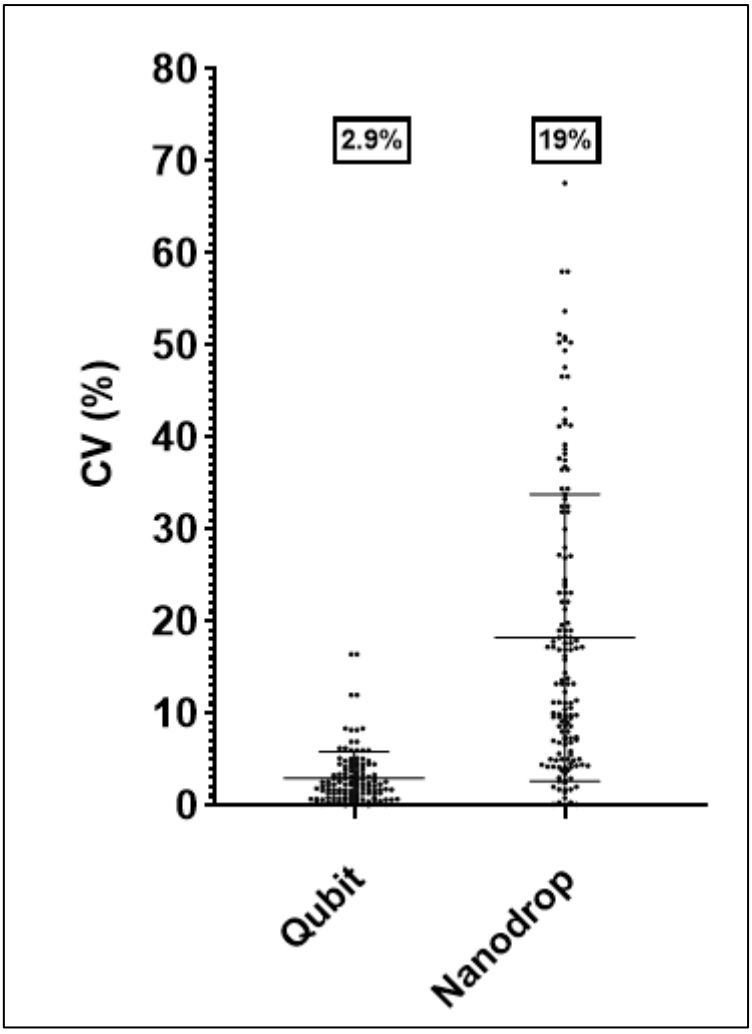


**Figure 2-4.** This figure compares the coefficient of variation (CV) values for the Qubit and Nanodrop instrument. Each individual dot represents a single CV value for an RNA extraction sample measured in duplicate. The horizontal line represents the mean ± standard deviation.

## DNA EXTRACTION

Notes on method development

To date, minimal information concerning methods to extract DNA from TRIzol-RNA extracted samples (thus allowing RNA and DNA extractions from the same piece of muscle) was available. Typically, DNA extractions according to standard procedures

are inconsistent with respect to extract quality (Vorreiter *et al.*, 2016) for a variety of reasons. For example, DNA pellets created through ethanol precipitation are insoluble and require extensive and laborious processes, likely compromising final sample quality. Indeed, an A260/A280 ratio between 1.6 - 1.7 was typically observed using this protocol and a ratio of 1.8 was rare. Muscle sample size at the onset of RNA extraction was also related to resultant DNA extraction quality (i.e. larger samples more effective), meaning excess tissue is required providing an additional challenge. Alternative DNA extraction protocols are therefore desirable to improve efficiency and effectiveness.

The back extraction buffer (BEB) method was first utilized by Triant and Whitehead (2009). This method provides several advantages over directly precipitating DNA in a TRIzol solution. The addition of a BEB solution to the interphase and organic phase changes the pH of the solution thereby shifting DNA into an aqueous phase whilst protein is located in the interphase. Thereby overcoming the abovementioned solubility issues. As a result of this manipulation of sample pH, DNA is readily available for precipitation, whilst also increasing sample quality by reducing TRIzol contamination. When utilizing this method, an average sample quality ratio of ~1.8 was achieved. Importantly, this method was far more consistent at obtaining this ratio when compared with traditional methods detailed above.

DNA extraction protocol

To extract DNA, the remainder of the aqueous phase from RNA extraction was removed. 280 µL of a modified Back Extraction Buffer (4M guanidine hydrochloride, 1M Tris (free base) and 50 mM sodium citrate) from Triant and Whitehead (2009) was

added to the inter and organic phases. Samples were mixed constantly by inversion for 3 minutes. This inevitably changes the pH of the solution and after centrifugation DNA is shifted into an aqueous phase, whilst protein is situated in the interphase. ~250 µL of the aqueous phase was transferred to a new tube and ~250 µL of isopropanol was added. Samples were left overnight at -20°C to aid in precipitation. After centrifugation to pellet the DNA, the addition of 800 µL of 75% ethanol and 25% RNAse free water and an incubation of 15 minutes was utilized to wash the DNA pellet. This was repeated twice, for a total of 3 washes. After the final wash, the wash solution was removed and pellet was left to air dry until all the ethanol was removed and then dissolved in 30 µL of TE buffer. To improve solubilisation the sample was incubated at 60°C for 1 hour. Samples were quantified in duplicates using the Qubit fluorimeter with a dsDNA HS assay kit (Thermo Fisher) and produced a CV score of 4.1% (figure 2-5).

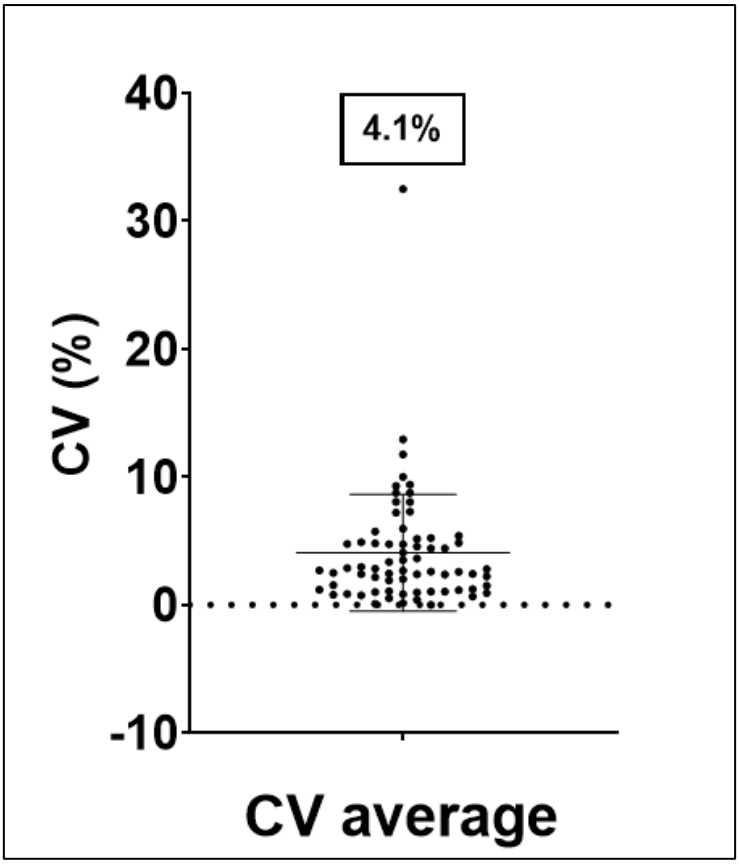


**Figure 2-5.** This figure depicts the coefficient of variation value for each of the 69 DNA samples measured in duplicates. Each dot on the graph represents a single sample. The long horizontal line represents mean ± standard deviation

## PROTEIN EXTRACTION

Notes on method development

The main challenge with protein extraction, again from the same piece of muscle, is how to solubilise the protein pellet. The manufacturer protocol suggests using 1% sodium dodecyl sulfate (SDS). However, this provides a low protein yield, already an issue with small sample sizes previously extracted for RNA/DNA. Further, the protein pellet created is extremely tough and SDS alone is ineffective. Simões *et al.* (2013) compared various methods of extracting protein from different TRIzol samples and the

study found that solubilisation with an 8M urea and 1% SDS buffer and sonication provided the greatest protein yield. As a result of these findings, this was the method chosen but one key point is that no method was able to completely solubilise the protein.

Protein extraction protocol

To extract protein, a modified protocol from Simões *et al.* (2013) and Wen *et al.* (2020) was utilised. The remaining aqueous phase from the DNA extraction was removed. 650 µL of 100% ethanol was added to the inter- and organic phase. After vortexing, 100 µL of bromo-chloro-propane was added and vortexed again. 600 µL distilled water was then added to the sample, followed by vortexing and centrifugation. The upper aqueous layer was removed and then 700 µL of 100% ethanol was added to the remaining interphase and BCP phase. Samples were once again vortexed and centrifuged. The supernatant was removed and the pellet was washed three times in 1 ml of 0.3 M guanidine hydrochloride in 95% ethanol and incubated for 20 minutes at room temperature. Thereafter, the pellet was washed in 1.6 ml of 100% ethanol and incubated for 20 minutes. The protein pellet was solubilised in 300 µL of 8M Urea, 1% SDS and 1M Tris-HCL and 3 µl of an inhibitor cocktail was also added (78440; Thermo Fisher). To aid in solubilisation the sample was sonicated (VCX 130, Vibra-cell). Specifically, 3 cycles of 10 seconds at 30% amplitude was completed with the sample constantly on ice. After the 3 cycles the sample was left on ice for 30 seconds to minimise overheating. This process was completed 5 times. Following sonication, the sample was left overnight to further solubilise. Next day, the sample was centrifuged and the supernatant was transferred to a new fresh Eppendorf to be quantified. Prior to quantification the sample was diluted 5 fold in distilled water. Samples were quantified with a Bio-rad DC protein assay and with a bovine serum albumin standard

curve. This specific assay produced an average CV value (intra-variability) of 9.3% (figure 2-7).

Bio-rad DC protein assay

In the present thesis, protein was quantified using a Bio-rad detergent compatible (DC) protein assay (5000111, Bio-rad laboratories). This method is modified from the Lowry method published in 1951 (Lowry *et al.*, 1951) and is based on the reaction of protein with alkaline copper tartrate solution and folin reagent. Two steps result in a colour development, reaction between protein and copper in an alkaline medium and reduction of folin reagent by the copper treated protein. The result is a distinctive blue colour. In order for samples to be quantified with the DC protein assay, a standard curve needs to be generated. A standard curve allows a range of known standards against different absorbance values. Once a standard curve is plotted, it can be used as a reference for the samples with unknown values. An example of a typical standard curve for this assay is shown in figure 2-6.

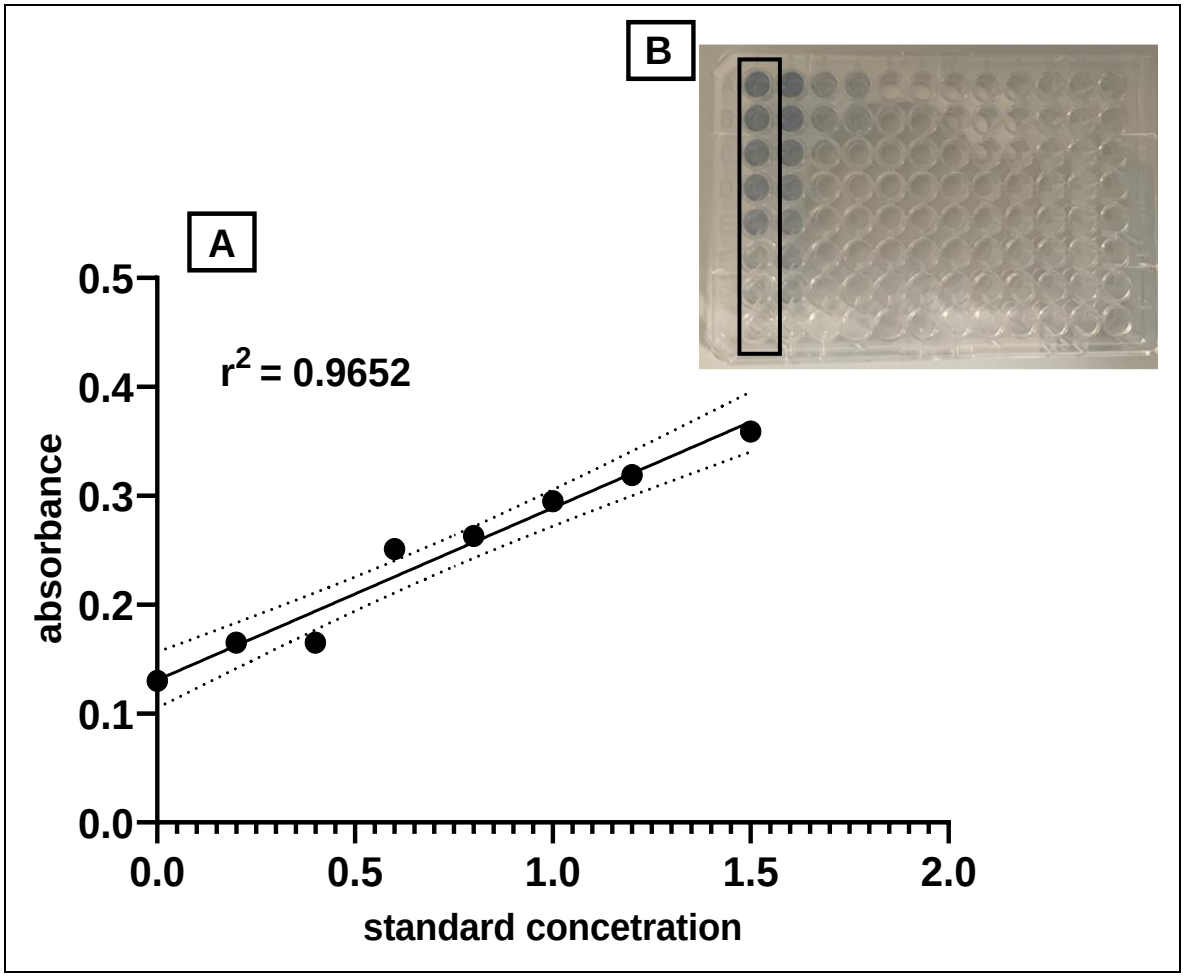


**Figure 2-6.** Figure A displays a typical standard curve required for the quantification of samples with unknown concentrations. 8 standards of BSA ranging from 0 mg/ml to 1.5 mg/ml were plotted against their absorbance. The standard curve is required to produce an $r^2$ value of more than 0.95, to provide accurate results. Figure B displays what is seen on the plate prior to quantification.

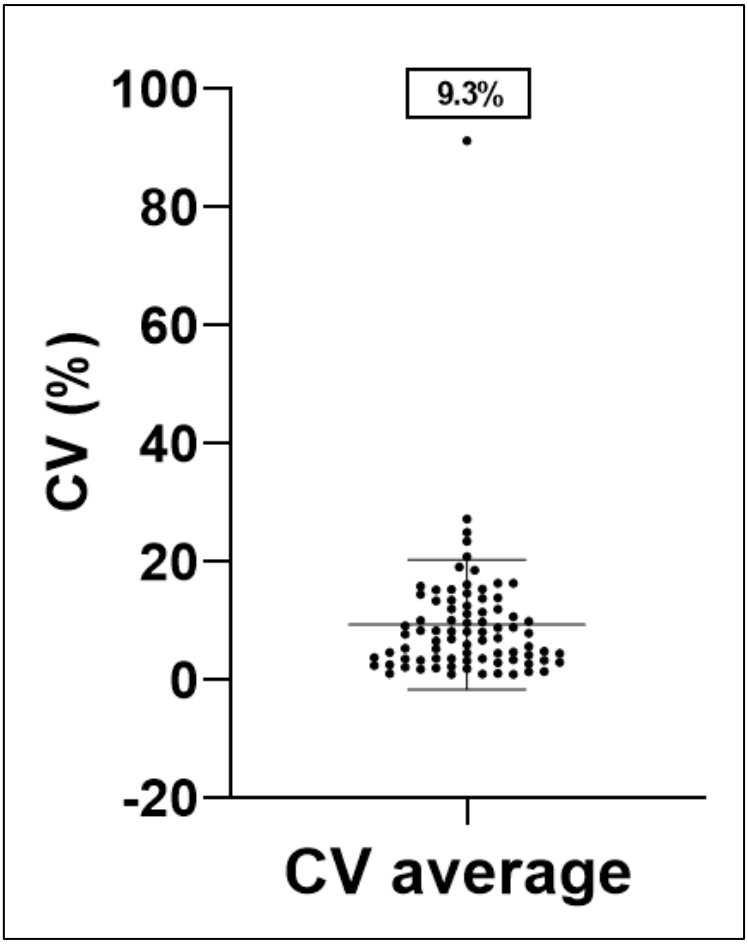


**Figure 2-7**. This figure displays the coefficient of variation average for each of the 82 protein samples measured in duplicates. The horizontal line represents mean ± standard deviation.

# CHAPTER 3

# TRANSCRIPTIONAL REGULATION OF RIBOSOME BIOGENESIS AFTER 3 DAYS OF RESISTANCE EXERCISE AND HIGH PROTEIN CONSUMPTION

# ABSTRACT

*Introduction.* A single bout of resistance exercise and high protein ingestion stimulates ribosome biogenesis. However, little is known about the impact of multiple bouts of resistance exercise or the type of protein consumed during high protein consumption. Therefore, the present study aimed to display the transcriptional characterisation of ribosome biogenesis after 3 days of resistance exercise and high protein consumption (animal or vegan derived protein sources). *Methods.* Twenty-one resistance training experienced participants were recruited and were randomized to either consume a high protein (1.8 g/kg of bodyweight per day) diet from vegan (VEG; n=11) or omnivorous (OMN; n=10) sources for 3 days. On each day, participants completed 5 x 30 repetitions of leg extensions. Muscle biopsies of the *vastus lateralis* were taken 24 hours after the last bout of exercise. Muscle samples were utilised to measure quantities of RNA (indirect measure of translational capacity), DNA, protein and mRNA expression of 48 genes using real-time polymerase chain reaction. *Results.* 3 days of resistance exercise did not significantly increase one indirect measure of muscle ribosome concentration. mRNA expression of genes regulating the synthesis of the 60S ribosome subunit were upregulated whereas the regulation of the 40S subunit did not change. The abovementioned results were unaffected by the type of diet (OMN or VEG) consumed. *Conclusion.* Three days of resistance exercise was unable to increase indirect measures of translational capacity but provides a robust stimulation in ribosome biogenesis. Specifically, there is a diverse regulation of the 60S and 40S ribosome subunit, which suggests that the 60S subunit but not the 40S subunit is in high demand at the commencement of a resistance training program.

# INTRODUCTION

Skeletal muscle mass is dictated by the balance between muscle protein synthesis and muscle protein breakdown. A shift in this balance will lead to skeletal muscle gain or loss. In the case of muscle gain (skeletal muscle hypertrophy), this is thought to be predominately driven by increases in muscle protein synthesis rather than decreases in muscle protein breakdown (Phillips *et al.*, 1997). Resistance exercise and protein are two potent stimulators of muscle protein synthesis (Monteyne *et al.*, 2020). Consistent consumption of protein and resistance lead to increases in skeletal muscle hypertrophy (Morton *et al.*, 2017). Therefore, any alterations in resistance training, such as load, and protein, such as dose, can impact the degree of muscle protein synthesis (Moore *et al.*, 2009; Burd *et al.*, 2010) and therefore skeletal muscle hypertrophy (Morton *et al.*, 2017; Hammarström *et al.*, 2020).

Non-animal derived sources of protein have increased in popularity due to their ethical and environmental benefits (Pimentel and Pimentel, 2003). Previous evidence has shown that non-animal derived protein can stimulate hourly muscle protein synthesis to a lesser extent than animal-derived sources (Tang *et al.*, 2009). However, this response can be rescued through increasing the amount of protein consumed (Monteyne *et al*., 2020). Indeed, two recent studies have shown that by consuming a high amount daily protein and resistance exercise, the degree of daily muscle protein synthesis (Monteyne *et al.*, 2020) and skeletal muscle hypertrophy (Hevia-Larraín *et al.*, 2021) is unaffected by the type of protein consumed.

Muscle protein synthesis is modulated through sub cellular processes at the level of the ribosome, through either increasing ribosome efficiency (translational efficiency) or ribosomal capacity (translational capacity). Recently, it has been shown in rats that the early (1 day) muscle protein synthetic response to resistance exercise training is primarily driven by increases in translational efficiency rather than capacity (Kotani *et al*., 2021). Furthermore, translational capacity was only increased after 3 days of resistance exercise, where it likely provides a more significant role in driving muscle protein synthesis. Similarly, in humans, translational capacity was found to only be increased after 2 days of resistance exercise (Bickel *et al*., 2005). However, both studies utilised neuromuscular stimulation as a form of resistance exercise, whereas gym based resistance exercise is usually selected in resistance training studies (Brook *et al.*, 2015, 2016; Hammarström *et al.*, 2020). Therefore, it is unclear what the contribution of translational capacity to muscle protein synthesis in response to multiple bouts of gym-based resistance exercise. Additionally, the regulation of translational capacity was not explored in both studies, as a result, it is unclear which pathways were contributing to the early increases in translational capacity.

The process of translational capacity (ribosome biogenesis) is a multi-step and complex process. Ribosome biogenesis begins at the ribosomal DNA (rDNA), which is transcribed through the activation of polymerase 1 via transcriptional factors, such as MYC, UBTF. TRIM24, NCL (Grandori *et al.*, 2005; Tsang, Liu and Zheng, 2011; Cong *et al.*, 2012). Once rDNA transcription occurs, a 47S ribosomal RNA (rRNA) is formed and undergoes remodelling. Specifically, the 47S rRNA is cleaved in several steps to form a 5.8S, 18S and 28S rRNA unit. To build the 40S small ribosome subunit it requires the assembly of 18S rRNA and 33 ribosomal proteins, whilst the 60S large

subunit requires the assembly of 5.8S, 28S and the 5S r-RNA and 47 ribosomal proteins.

The present study aimed to measure changes in indirect measures of translational capacity after 3 bouts of resistance exercise consumption and how this is modulated by animal and non-animal derived protein consumption.

## METHODS

The experimental methods for the human work required for conducting this chapter were conducted prior to commencement of this project as part of a PhD thesis submitted to the University of Exeter by Dr Alistair Monteyne. The novel data, therefore, are a post-hoc secondary analyses of the tissue samples obtained in that work. Human method are briefly described below but can be found in greater detail in the PhD thesis of Dr Alistair Monteyne. Additionally, the measurement of muscle volume can be found in greater detail in the PhD thesis of Dr Alistair Monteyne.

Participants

Participants who were recreationally active and had resistance training experience were recruited for the present study. An initial screening took place to record the participant's blood pressure, height and body mass. Furthermore, participants completed a general medical questionnaire to assess their eligibility for participation. Participants were excluded if their BMI exceeded the range of 18-30, had any diagnosed metabolic impairment, cardiovascular disease or motor disorders. All participants were informed of the nature and possible risks of the experimental procedure before providing written informed consent. The present study was

conducted in accordance with the Declaration of Helsinki. This study was registered as a clinical trial (NCT04325178) and was approved by the NHS Health Research Authority Ethics Committee (18/LO/0374).

Experimental Overview

Participants were randomised to either consume a controlled high protein omnivore (OMN; n = 10) or vegan (VEG; n = 11) diet in conjunction with a 3 day uni-lateral resistance exercise program. Muscle biopsies were taken pre and post dietary and exercise intervention.

**Table 3-1.** Baseline participant characteristics for OMN and VEG diet conditions before undertaking the present study

| | OMN (n=10) | VEG (n=11)[1] |
|---|---|---|
| Age (years) | 25.6 ± 8.2 | 23.7 ± 5.2 |
| Height (cm) | 176.2 ± 11.7 | 172.1 ± 9.0 |
| Weight (kg) | 73.7 ± 9.3 | 67.9 ± 8.8 |
| BMI (kg/m$^2$) | 23.8 ± 2.6 | 22.9 ± 1.4 |

[1], denotes no significant differences between OMN and VEG conditions. Data is presented as mean ± standard deviation.

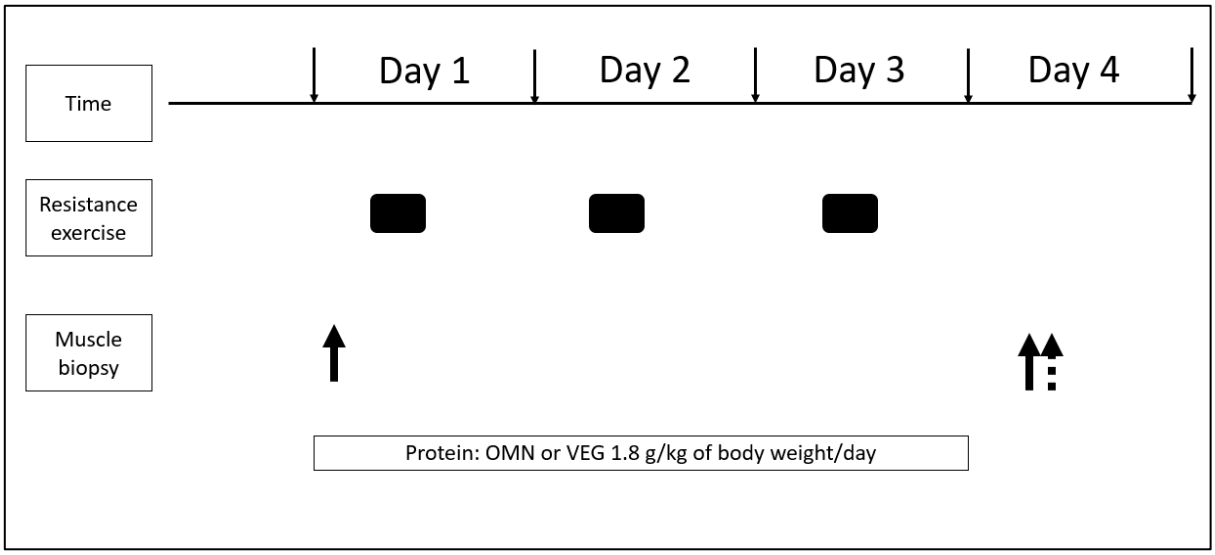


**Figure 3-1.** This figure depicts the experimental design of the present study. Participants underwent 3 days or 3 days of uni-lateral resistance exercise (black boxes) whilst consuming a high protein animal based (OMN) or vegan based (VEG) diet. One muscle biopsy (arrows) were taken on day 1. Bi-lateral biopsies were taken on day 4, therefore one leg was exposed to the exercise intervention whilst the other leg was not (rested leg). The rested leg tissue was utilised as a replacement for the baseline biopsy, in some cases (participant 20 and 26).

Muscle sampling

Muscle biopsies of the *vastus lateralis* were obtained at day 1 and day 4 (bilateral) with the modified Bergstrom (Tarnopolsky *et al.*, 2011) technique under local anaesthesia (2% lidocaine). Muscle samples were quickly frozen in liquid nitrogen and stored at -80 °C until further analyses. Muscle biopsies were taken 24 hours after the last bout of resistance exercise which reflects the muscle's "active" state.

Resistance exercise training program

Participants completed 3 days of unilateral leg extensions on a biodex machine (Biodex Medical Systems, Shirley, New York, USA). On each training day participants

completed 5 sets of 30 repetitions of maximal concentric isokinetic leg extension contractions.

Diet manipulation

Participants were given a controlled diet to consume for 3 days and participants were asked to refrain from eating any food not prescribed by the principle researchers. The diet contained 1.8 g of protein per kg of body weight per day (g/kg of bw/day) with 24-27% of energy being provided by fat and 50-55% from carbohydrates in OMN, and with 22-27% and 48-58% of energy being provided by fat and carbohydrates, respectively, in VEG. The OMN group utilised meat products and a milk supplement to meet protein requirements whilst the VEG group utilised Quorn™ products and a mycoprotein supplement.

RNA extraction and mRNA abundance

RNA extraction and mRNA abundance were described previously (Monteyne *et al*., 2020) but can be found in greater detail in chapter 2 (general methods).

DNA and protein extraction and quantification

A novel method was introduced to extract and quantify DNA and protein from RNA-TRIzol samples and can be found in detail in chapter 2 (general methods).

Statistics

mRNA expression data was $\log_2$ transformed prior to statistical analysis to ensure equal distribution of data. mRNA expression and macromolecules were analysed via a two-way mixed effects model 2 x 2 (OMN and VEG x baseline and Fw3 days). A

Bonferroni post-hoc test was used to detect significant differences between time points and diet groups. If there was a significant main time effect but no effect of diet or/and interaction effect, groups were collapsed together and then a two-tailed paired t-test was performed. For correlational analysis, a Pearson's two-tailed test was utilised. Results are reported as mean ± standard error of the mean (SEM), unless otherwise stated. Statistical significance was set at 0.05 and all statistical analysis was completed using GraphPad Prism 9.0.0.

## RESULTS

For molecular analysis, in both OMN (n=10) and VEG (n=11) groups, samples were missing due to tissue availability or measurement error. The number of samples that were available for full analysis can be found in table 2. When groups were collated together and a paired t-test was performed, an n of 17 was utilized due to missing samples. For participant 20 and 26, baseline muscle tissue was unavailable for analyses therefore the rested biopsy at day 4 (see figure 3-1) was utilised as a replacement.

**Table 3-2.** This table represents the number of samples analysed for macromolecules and mRNA expression.

| | **Baseline** | | **3 days** | |
|---|---|---|---|---|
| | **OMN** | **VEG** | **OMN** | **VEG** |
| **RNA** | 9 | 8 | 8 | 8 |
| **DNA** | 8 | 9 | 8 | 7 |
| **Protein** | 8 | 9 | 8 | 7 |
| **mRNA expression** | 8 | 10 | 8 | 8 |

Macromolecules

All macromolecules, muscle protein and DNA concentration and ratios, protein: DNA and protein: RNA ratio, did not produce a time, diet or interaction effect ($P > 0.1$; figure 3-2).

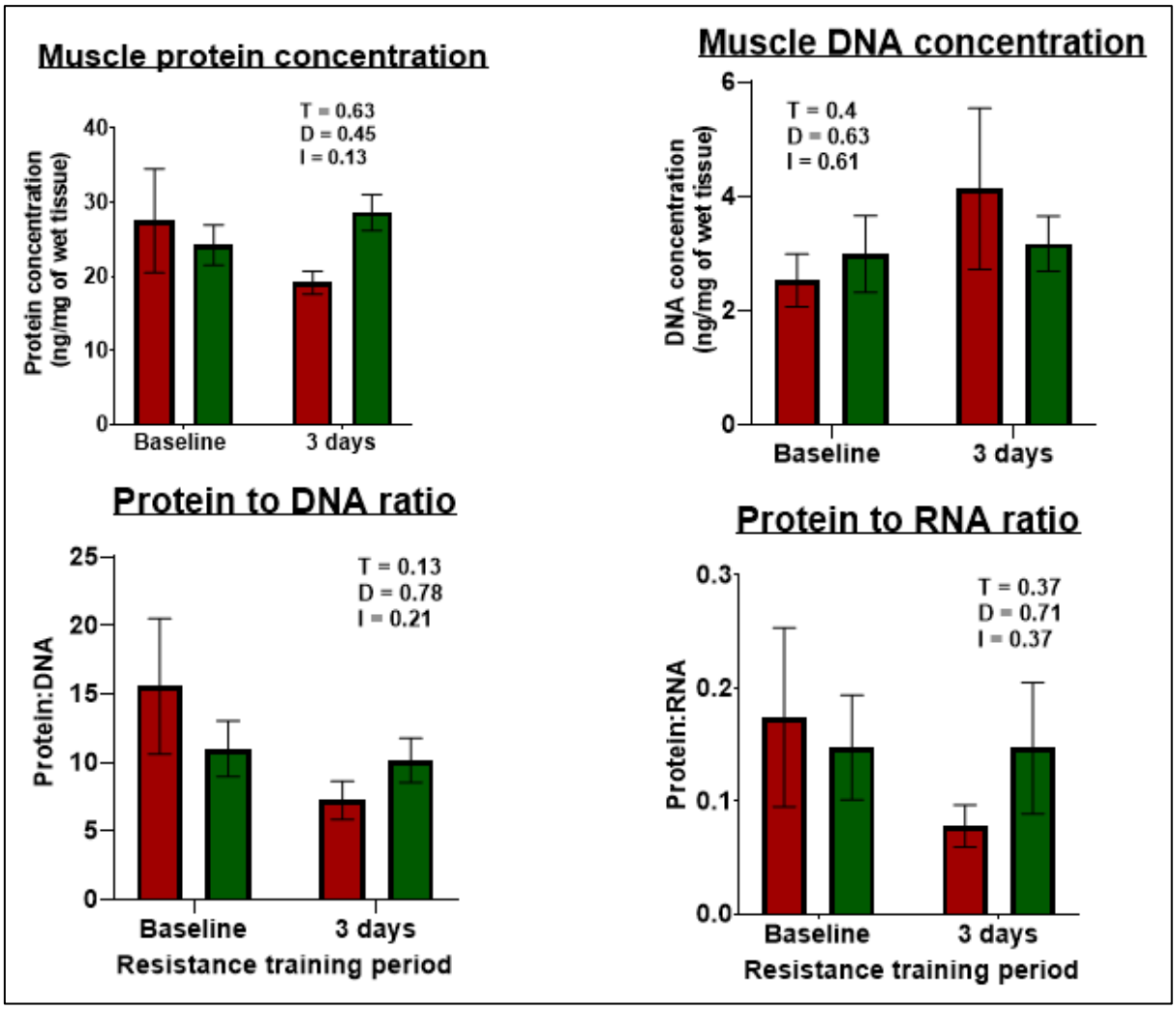


**Figure 3-2.** This figure depicts the quantification of muscle protein and DNA concentration and RNA:DNA, protein:DNA and protein:RNA ratios at baseline and after 3 days of resistance training. Bars are mean ± SEM. Green bar, VEG. Red bard, OMN. T, time effect. D, diet effect. I, interaction effect.

Indirect markers of translational capacity

Indirect markers of translational capacity, muscle RNA concentration and RNA: DNA ratio, did not produce a time, diet or interaction effect ($P > 0.1$; figure 3-3).

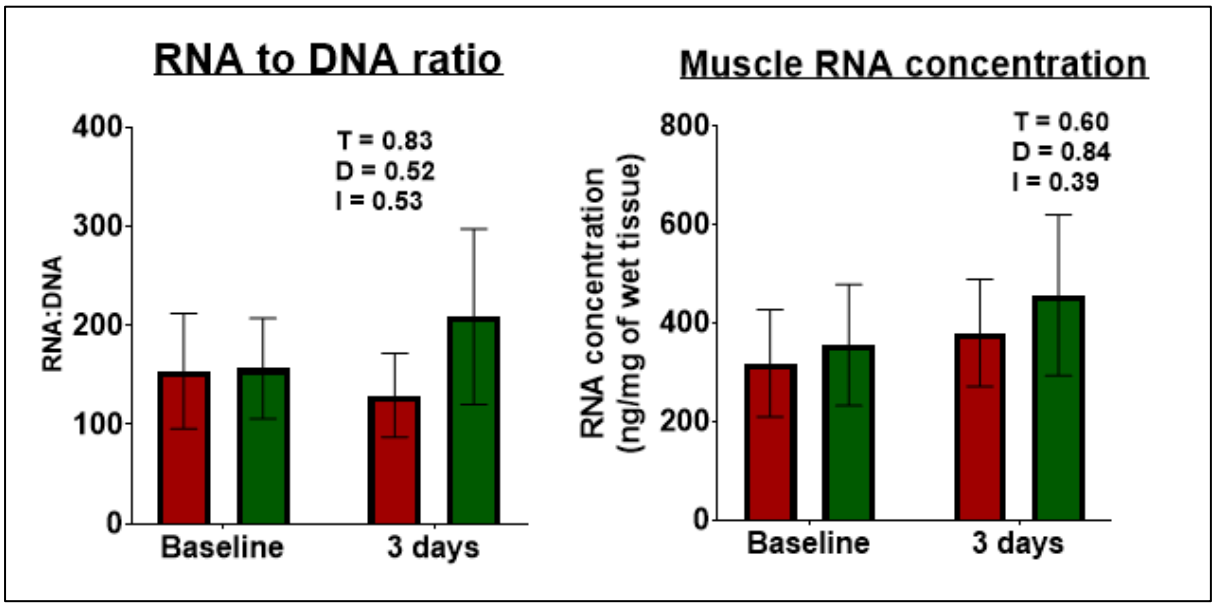


**Figure 3-3.** This figure depicts the quantification of markers of translational capacity, RNA:DNA ratio and muscle RNA concentration at baseline and after 3 days resistance training. Bars are mean ± SEM. Green bar, VEG. Red bard, OMN. T, time effect. D, diet effect. I, interaction effect.

rRNA transcripts

45S RNA did not produce a time ($P$ = 0.4), diet ($P$ = 0.6) or interaction ($P$ = 0.6) effect. 28S RNA expression produced a time effect ($P$ = 0.002) but not a diet ($P$ = 0.15) or interaction ($P$ = 0.15) effect. A paired t-test revealed a significant increase from baseline ((0 ± 0 relative expression ($log_2$ transformed)) to after 3 days ((0.199 ± 0.07 relative expression ($log_2$ transformed)). Conversely, there was trend for a diet ($P$ = 0.088) and interaction ($P$ = 0.088) effect for 18S RNA but no time effect was found ($P$ = 0.15; table 3-3 and figure 3-4).

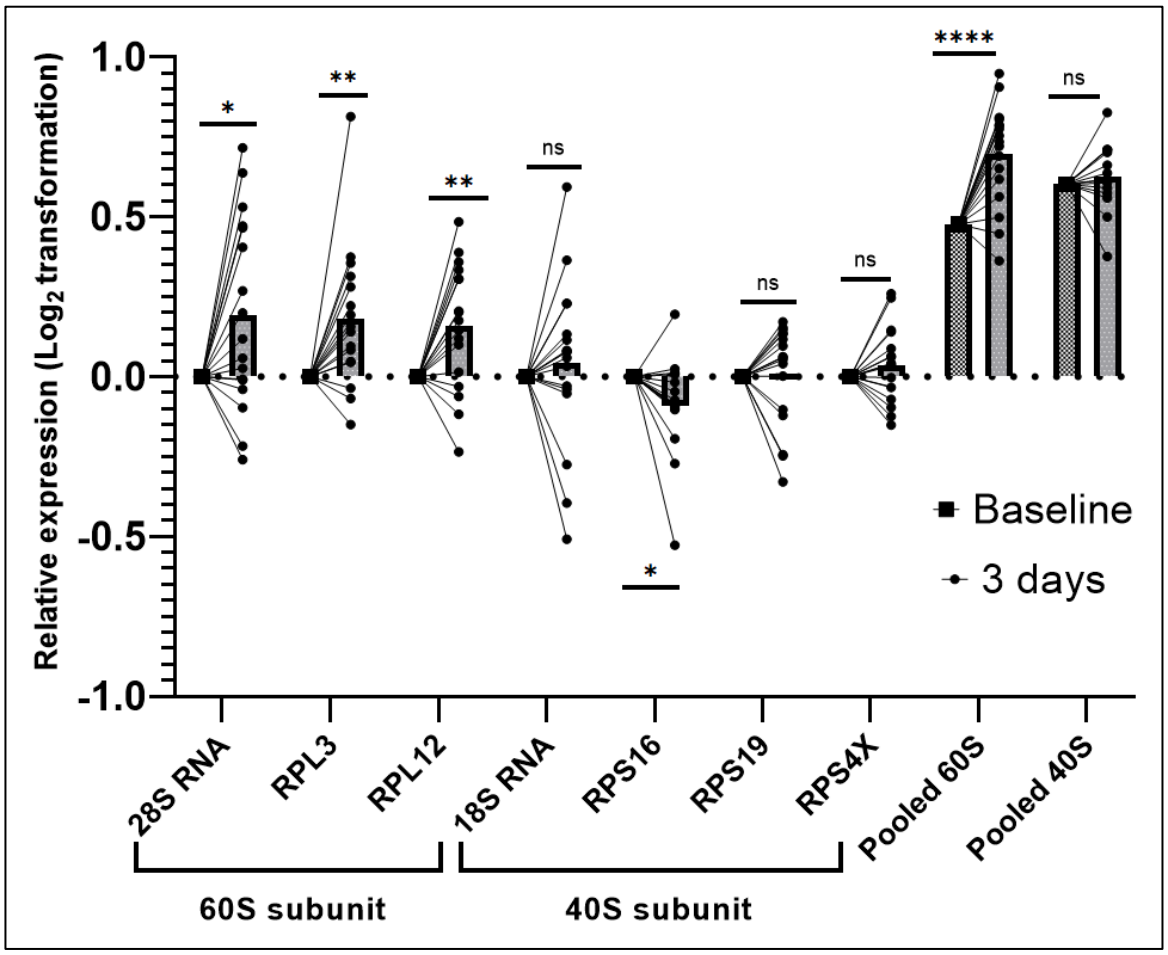


**Figure 3-4.** This figure depicts the mRNA abundance of genes involved in the formation and the pooled expression of the 60S and 40S subunit after 3 days of resistance exercise when both diet groups are collapsed together. Bars represent the mean. Circles or squares represent individual points. *, *P* <0.05. **, *P* <0.01, ****, *P* <0.0001. n = 17 for statistical analyses. ns = not significant

The remainder of the mRNA expression data can be found in more detail in table 3 and figure 3-5-9.

Pooled expression of 60S and 40S subunit

Genes involved in the 60S and 40S ribosome subunit biogenesis were pooled together to create a pooled expression of the 60S and 40S ribosome subunit. This was subjected to a paired t-test to detect significant differences between time points. The pooled expression of the 60S subunit significantly increased from baseline to after 3

days (*P* <0.0001). Conversely, the 40S subunit did not significantly change between baseline and after 3 days (*P* = 0.41; figure 3-4).

Correlational analysis

POl1RE was positively correlated with NCL (r = 0.45, *P* = 0.0697) and there was trend for a significant correlation with POL1RC and NCL (r= 0.47, *P* = 0.055). Finally, POl1RB was not correlated with NCL (r = 0.4, *P* = 0.11; figure 3-10).

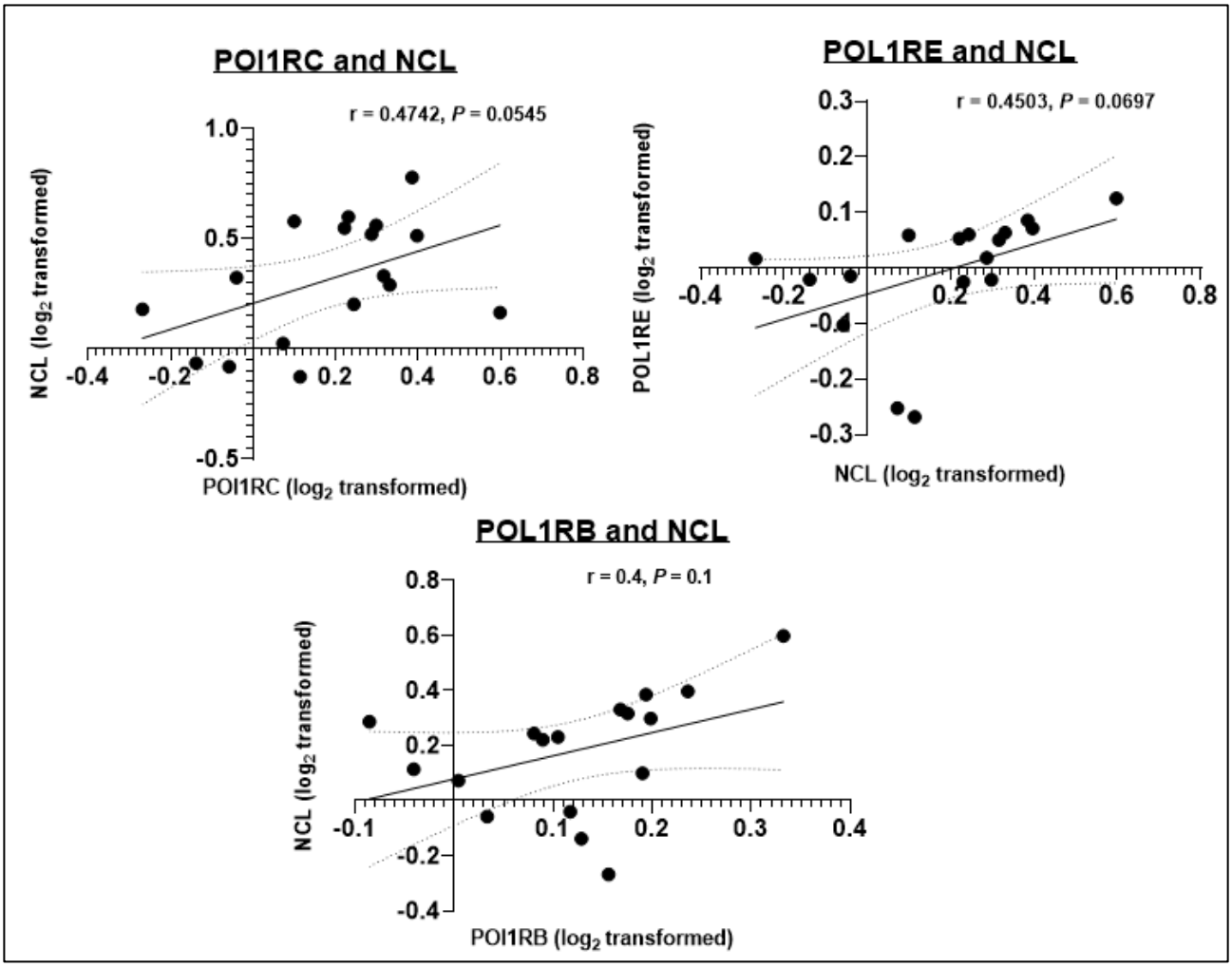


**Figure 3-10.** This figure displays the results of correlational analysis of POl1RC, POL1RB, POL1RE and NCL. Each dot on the graph represents a different sample from 3 days. The r and *P* values are presented on the graph. The linear regression line is presented on the graph with 95% confidence intervals either side.

# DISCUSSION

The present study is the first study to characterise the transcriptional regulation of genes regulating ribosome biogenesis after 3 days of resistance exercise. Additionally, it is the first study to investigate the effects of consuming a high protein vegan or omnivore diet on transcriptional markers of ribosome biogenesis. The findings of the present study are as follows, first, macromolecules, DNA, ribosome and protein concentration did not significantly increase after 3 days of resistance exercise. Second, transcription of genes involved in the formation of the 60S ribosome subunit are upregulated whereas the 40S subunit shows no clear pattern of regulation, suggesting a diverse regulation of the 60S and 40S ribosome subunits. Third, transcription of genes regulating rDNA transcription are highly upregulated. Fourth, there was no clear impact of diet consumption on transcriptional regulation of ribosome biogenesis and markers of muscle turnover (table 3-3 and figure 3-9).

The present study found that macromolecules, DNA, ribosome and protein were not increased following 3 days of resistance exercise. The synthesis of large molecules typically requires several bouts of a stimulus, such as resistance exercise, to be increased (Roberts *et al.*, 2010; Brook *et al.*, 2016). Contrastingly, total RNA, an indirect marker of ribosome concentration, has been previously shown to increase after two days of resistance exercise (Bickel *et al.*, 2005). However, Bickel *et al.* (2005) utilised bouts of 5 seconds of neuromuscular stimulation for 30 minutes whilst the present study utilised 5 x 30 repetitions of leg extension. As such, the present study may have lacked the required exercise duration to increase the ribosome pool.

The present study was part of a bigger study (Monteyne et al., unpublished) and it was reported that muscle protein synthesis did increase after 3 days of resistance exercise. Muscle protein synthesis is regulated through the balance of translational capacity and efficiency and the present study found strong evidence to suggest that translational capacity (based upon indirect markers) was unchanged after training. This would suggest that translational efficiency is primarily driving the muscle protein synthetic response found in the present study, which follows in line with a recent study highlighting that increases in translational efficiency occur prior to increases in capacity (Kotani *et al.*, 2021).

The ribosome comprises a small (40S) and large subunit (60S). The 60S subunit contains 5.8s, 28s and the 5s mature rRNA transcripts and 47 ribosomal proteins, whilst the 40S subunit of the 18S mature rRNA transcript and 33 ribosomal proteins. The present study aimed to elucidate the transcriptional regulation of rRNA transcripts and ribosomal proteins that comprise the 40S and 60S subunit. 28S RNA, RPL12 and RPL3 were highly upregulated in both diet conditions indicating an increased synthesis of the 60S subunit. In contrast to the 60S subunit, the 40S subunit was unclear in its regulation. The 18S RNA was only upregulated in the VEG condition but the OMN reported no change from baseline values. The transcriptional regulation of RPS16, RPS19, RPS4X was found to be unaltered or was downregulated. This diverse regulation of both subunits is surprising considering that both subunits are required to make a functional ribosome. The findings would suggest that 40S subunit is not in great “demand” but the 60S subunit is. Figueired*o et al*. (2016) reported increases in all mature rRNA transcripts, 28S, 18S and 5.8S, 24 hours after resistance exercise. However, the ribosomal proteins were not measured. Chaillou, Kirby and McCarthy

(2014) reported that RPS16, RPS19, RPS4X, RPL12 and RPL3 were all upregulated during a synergist ablation model. As a result, it is unclear why the present study found an upregulation in RPL-coding genes and a downregulation or lack of regulation in RPS-coding genes. Clearly, there is a lack of human work investigating both the ribosomal protein coding genes and mRNA transcripts. Therefore, more studies should aim to investigate this finding by incorporating a detailed measurement of the synthesis of both the 60S and 40S subunit.

The transcription of rDNA to rRNA is known to be a rate limiting step of ribosome biogenesis (Figueiredo and McCarthy, 2019). The enzyme that is responsible for transcribing rDNA is polymerase I (POL1). This study measured three subunits of POL1, POL1RB, POL1RC and POL1RE. Only POL1RC and B were upregulated after 3 days of resistance exercise but POL1RE did not change. Figueired*o et al*. (2016) supports this finding as it was found that POL1RB expression increased 24 hours post resistance exercise. However, POl1RC and POL1RE subunits were not measured. Additionally, Figueiredo *et al.* (2021) reported no changes in POL1RE expression 24 hours after resistance exercise. Importantly, no other studies have measured all three of POl1 subunits 24 hours after exercise. Previous studies have neglected subunits or have taken measurements less than 24 hours post exercise (Brook *et al.*, 2016; Fyfe *et al.*, 2016; Hansson *et al.*, 2019) and the ability to make comparisons between other studies is difficult as a result. However, overall the findings suggest that POL1RC and POL1RB are driving POl1 activity acutely after resistance exercise. The present study would expect to see an increase in 45S RNA based upon the findings of POl1 subunits and previous studies (Figueiredo *et al.*, 2016), however this was not the case. When measuring 45S RNA and other mature transcripts, previous studies have utilised

specialised primers to highlight a certain region of the 45S RNA, which best represents 45S RNA expression. The specialised primer were not available for us to use, therefore the divergent 45S RNA results between the present study and previous studies could be as a result of this (Figueiredo *et al.*, 2015, 2016, 2021).

The activity of POL1 is dictated through transcriptional factors. The present study found a selective upregulation in transcriptional factors. TRIM24 and NCL were upregulated in both groups, UBTF, TRRAP, TAF1D and TBP did not change and MYC was only upregulated in the OMN condition. Previously, Figueiredo *et al*. (2016) has shown an increase TRIM24 but not UBTF 24 hours after resistance exercise, which supports the findings of the present study. NCL, when located within the nucleolar acts as a transcriptional factor for POL1 due to its abundance in the promoter and coding regions of rDNA. It has been shown previously that nucleolin is a requirement for POl1 transcription *in vivo* (Rickards *et al.*, 2007). The present study and Figueiredo *et al*. (2021) found an upregulation in NCL after resistance exercise, which is likely required to support increased POl1 activity and therefore rDNA transcription. To investigate this further, NCL and every POl1 subunit was subjected to correlational analysis. Interestingly, there was only a trend for a significant correlation with POl1RC (r=0.472, P = 0.0545) and POl1RE (r=0.4503, P = 0.0697). As a result, this only displays weak evidence that POl1 and NCL transcription are linked in the present study. Future studies should aim to investigate this on the protein level to elucidate this mechanism. TAF1D and TBP form the SL-1 complex, which acts a transcriptional factor for rDNA transcription (Grandori *et al*, 2005; Figueiredo and McCarthy, 2019). TBP has been utilised previously as a housekeeping gene (Chaillou *et al.*, 2012; Fyfe *et al.*, 2018; Mitchell *et al.*, 2018), hence it is unsurprising that the present study found no changes

in TBP expression. This is the first study in humans to measure the expression of TAF1D, therefore a comparison to animal models is warranted. At the beginning of a synergist ablation model it was found that TAF1D was highly upregulated (Chaillou, Kirby and McCarthy, 2014), which goes against the findings of the present study. However, a synergist ablation model creates a supraphysiological condition which is likely unachievable to a human resistance exercise model, so the present study may have either lacked the required resistance exercise intensity or further bouts of resistance exercise are required or this result is likely not physiologically possible to achieve in humans.

An interesting finding is that MYC expression was differentially expressed between groups (table 3-3). MYC overexpression animal models show that MYC is causally linked to POL1 activity and rDNA transcription (Mori *et al*., 2021). This is the first study to investigate MYC expression after a dietary intervention and it is not clear why there is a difference MYC expression between consumption of OMN and VEG diets. Chan *et al.* (2019) measured MYC expression 2 and 4 hours after resistance exercise and the ingestion of a milk protein concentrate, casein or a modified milk protein concentrate. Proteins were matched on protein content but their digestion and absorption kinetic altered. It was found that MYC expression was increased in all conditions but there were no significant differences between conditions. There have been no other studies investigating the effects of dietary protein intake and MYC expression. Since protein and leucine were matched between conditions, it is unclear why there was a difference between conditions and is possibly the result of an unknown mechanism and requires further research. However, since most mechanisms driving ribosome biogenesis and MYC's co activator TRRAP was

unaltered by diet, it is likely that this would not lead to differential changes in ribosome concentration. Furthermore, an investigation of MYC protein expression is warranted as transcription does not always equal protein expression.

In order for the pre-rRNA to be processed into mature rRNAs transcripts it requires the use of rRNA processing proteins. The present study found that gene expression of BOP1, FBL, NPM1 but not NOP56 were upregulated after resistance training (table 3-3 and figure 3-6). Previously, Figueired*o et al*. (2021) found an upregulation in BOP1 and NOP56 expression 24 hours after resistance exercise, which partly supports the findings of the present study. Additionally, in a synergist ablation model BOP1, NOP56, FBL and NPM1 were upregulated (Chaillou, and McCarthy, 2014). This would suggest that rRNA processing proteins are in high demand and are an important requirement of ribosome biogenesis. Moreover, it is surprising that NOP56 did not increase in the present study as it is involved in the early to late phases of 60S subunit biogenesis (Hayano *et al.*, 2003). Interestingly, components of the 60S subunit, 28S RNA, RPL12 and RPL3 were all upregulated, which suggests increased 60S subunit biogenesis. As a result, it is unclear why NOP56 expression was unchanged in the present study.

The ERK 1/2 pathway has an important role in the regulation of transcriptional factors, UBF and TRIM24. Specifically, ERK 1/2 or MAPK3 activates UBTF via a MKNK1-EIF4E-CDK4/CCND1 mechanism, whilst TRIM24 is activated through RPS6KA1 (Figueiredo and McCarthy, 2019). The MAPK3 to UBTF pathway showed a robust upregulation (figure 3-8 and table 3-3), however UBTF, the end product of the pathway is not upregulated. As mentioned previously, Figueiredo *et al*. (2016) showed that UBTF expression peaks at 48 hours post exercise, therefore the present study may

have missed the regulation of UBTF by utilising a 24 hour post exercise measurement. Surprisingly, in the same study CCND1 was not upregulated on the transcriptional but was on the protein level. However, the present study showed an upregulation in CCND1 and also CDK4. CCND1 forms a dimeric active complex with CDK4 in order to activate UBTF (Voit, Hoffmann and Grummt, 1999), therefore the upregulation of CDK4 and CCND1 supports the notion that this complex is upregulated on the transcriptional level post resistance exercise. An important point is that in the present study, participants completed 3 days of resistance exercise whilst participants in Figueiredo *et al*. (2016) completed one bout of resistance exercise. As a result, CCND1 on the transcriptional level may require multiple bouts of resistance exercise in order to be upregulated.

The mTOR pathway has a causal role in the regulating muscle protein synthesis (Drummon*d et al*, 2009). Previous work by Monteyne *et al*. (2020) has shown that mycoprotein ingestion and resistance exercise increased the upregulation of the mTOR pathway to the same extent as milk protein ingestion. The present study built upon those findings by investigating the effects of 3 days of VEG (predominately mycoprotein) and OMN (predominately animal protein) diet consumption. As predicted, the type of protein had no impact on the mTOR pathway. Additionally, the resistance exercise stimulus in combination with a high protein diet produced a robust increase in the transcriptional regulation of the mTOR pathway (figure 3-7).

The main limitation of the present study is that protein expression was not measured. An upregulation in transcription can only give an indication of what occurs on the protein level. Areas of ribosome biogenesis such as rRNA processing proteins are not

characterised in human participants on the protein level and should be a focus of future research. A second possible limitation is that muscle biopsies were taken 24 hours after resistance exercise. It is possible that the transcriptional regulation of genes may have been missed as they 'peak' before or after 24 hours. However, work by Figueiredo *et al.* (2016) showed that the majority of genes regulating ribosome biogenesis 'peak' in their activity at and around 24 hours. This shows that the use of 24 hours as a measurement was suitable when investigating transcriptional regulation of ribosome biogenesis. The present study measured the transcriptional regulation of genes regulating ribosome biogenesis, yet, the overall process of ribosome biogenesis (synthesis) can be quantified through stable isotope methodology (Brook *et al.*, 2017). Indeed, several studies have utilised this measurement in many long term training studies (Sieljacks *et al.*, 2019; Hammarström *et al.*, 2020) but has not been utilised acutely (less than one week). It is clear from the results of this study that many areas of ribosome biogenesis were increased on the transcriptional level, however with the lack of measurement of ribosome synthesis it is unclear if the whole process of ribosome biogenesis was increased. Therefore, future studies should utilize stable isotope methodology to establish whether ribosome biogenesis is increased after an acute period (~ 3 exercise bouts) of resistance training.

Translational capacity is an important driver of skeletal muscle hypertrophy, but its regulation is not well understood. The present study displayed the transcriptional regulation of every step of ribosome biogenesis from indirect markers of translational capacity to rDNA transcription to rDNA transcription related signalling. We have shown that the transcriptional regulation of ribosome biogenesis is highly upregulated after 3 days of resistance training irrespective of the type of protein (OMN or VEG) consumed.

Furthermore, this is the first study to show a diverse regulation of the 60S and 40S subunit, which suggests that the 60S but not the 40S subunit is in high demand at the commencement of a resistance training program. Further studies should aim to elucidate this mechanism with more markers of 60S and 40S synthesis and longer periods of resistance training.

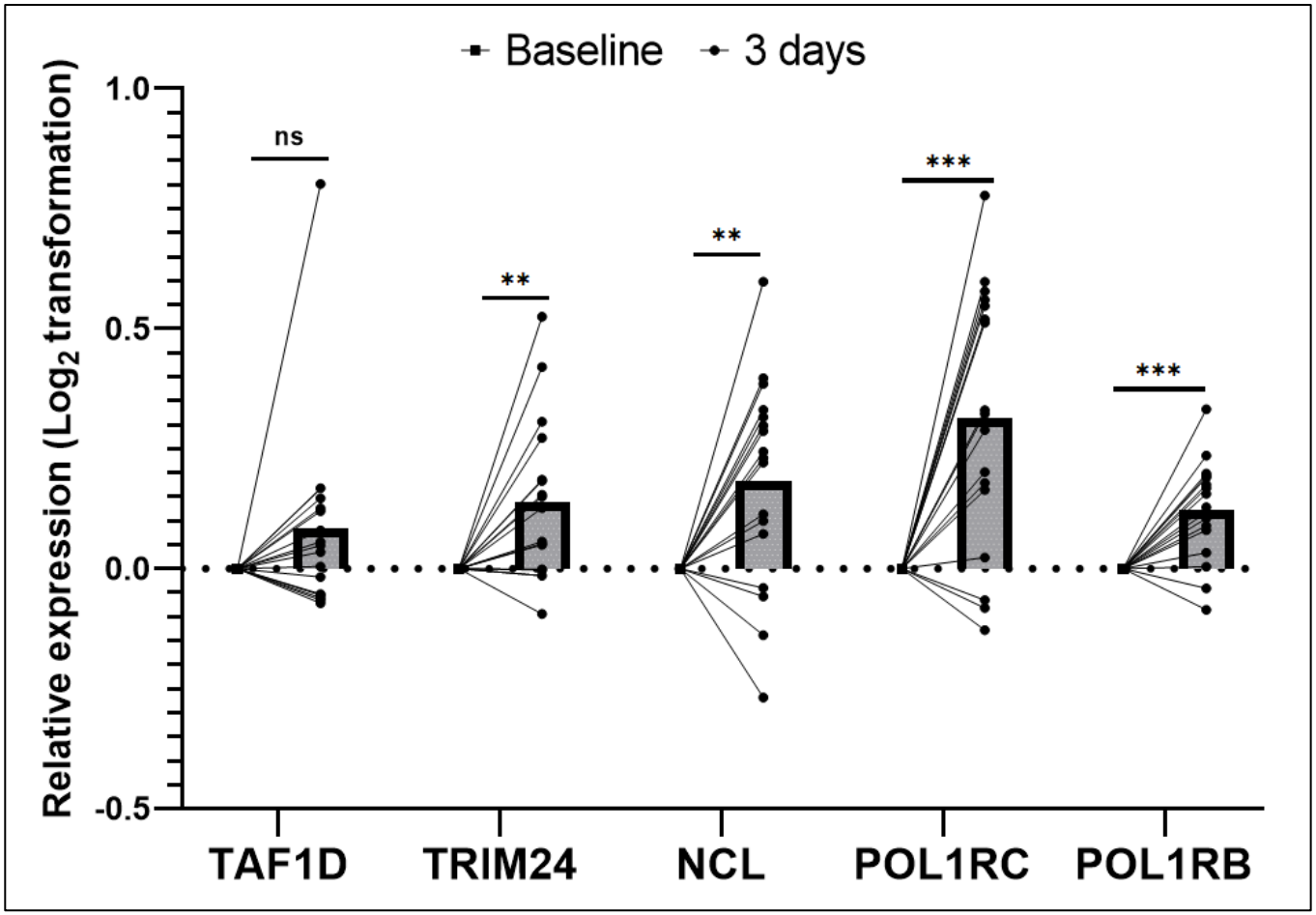


**Figure 3-5.** This figure depicts the mRNA abundance of genes involved in rDNA transcription after 3 days of resistance exercise when both diet groups are collapsed together. Bars are mean. Circles or squares represent individual points. **, *P* <0.01, ***, *P* <0.001. ns = not significant. n = 17 for statistical analyses.

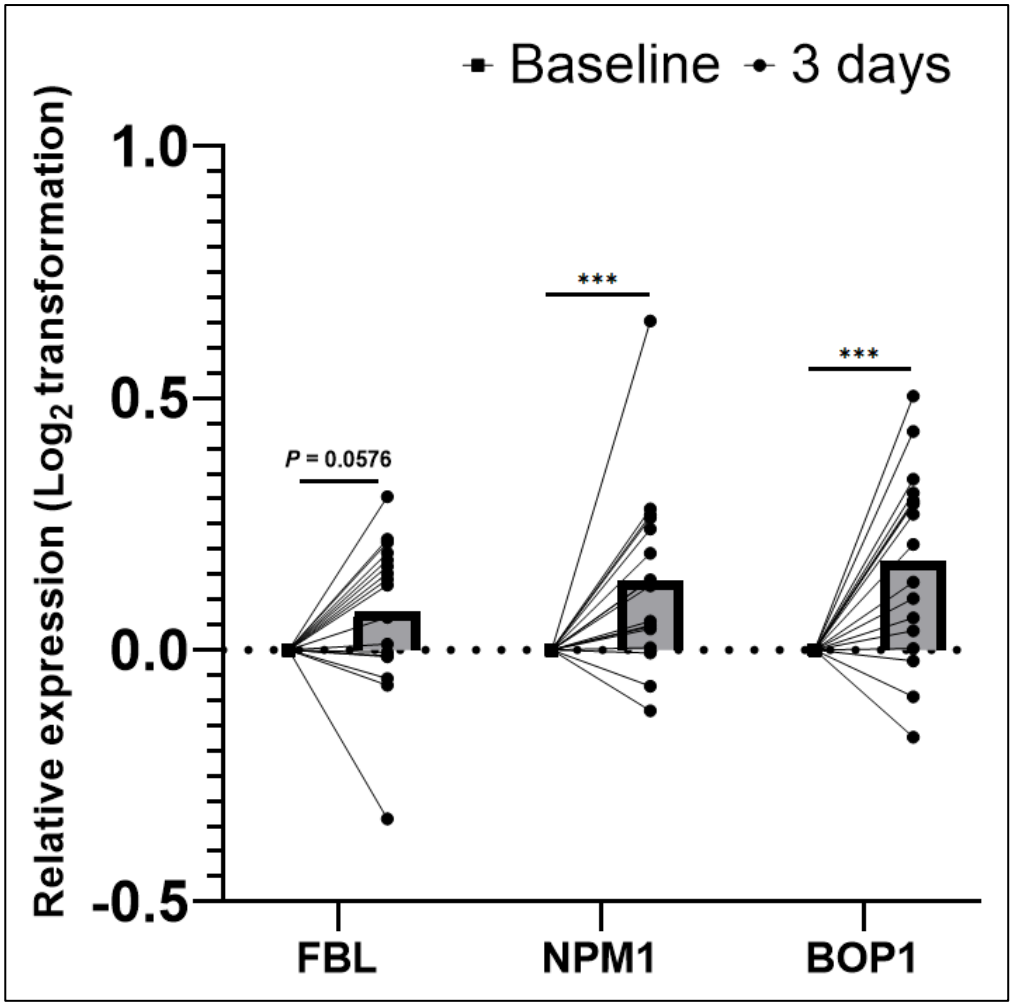


**Figure 3-6.** This figure depicts the mRNA abundance for rRNA processing factors after 3 days of resistance exercise when both diet groups are collapsed together. Bars are mean. Circles or squares represent individual points. ***, *P* <0.001. n =17 for statistical analyses.

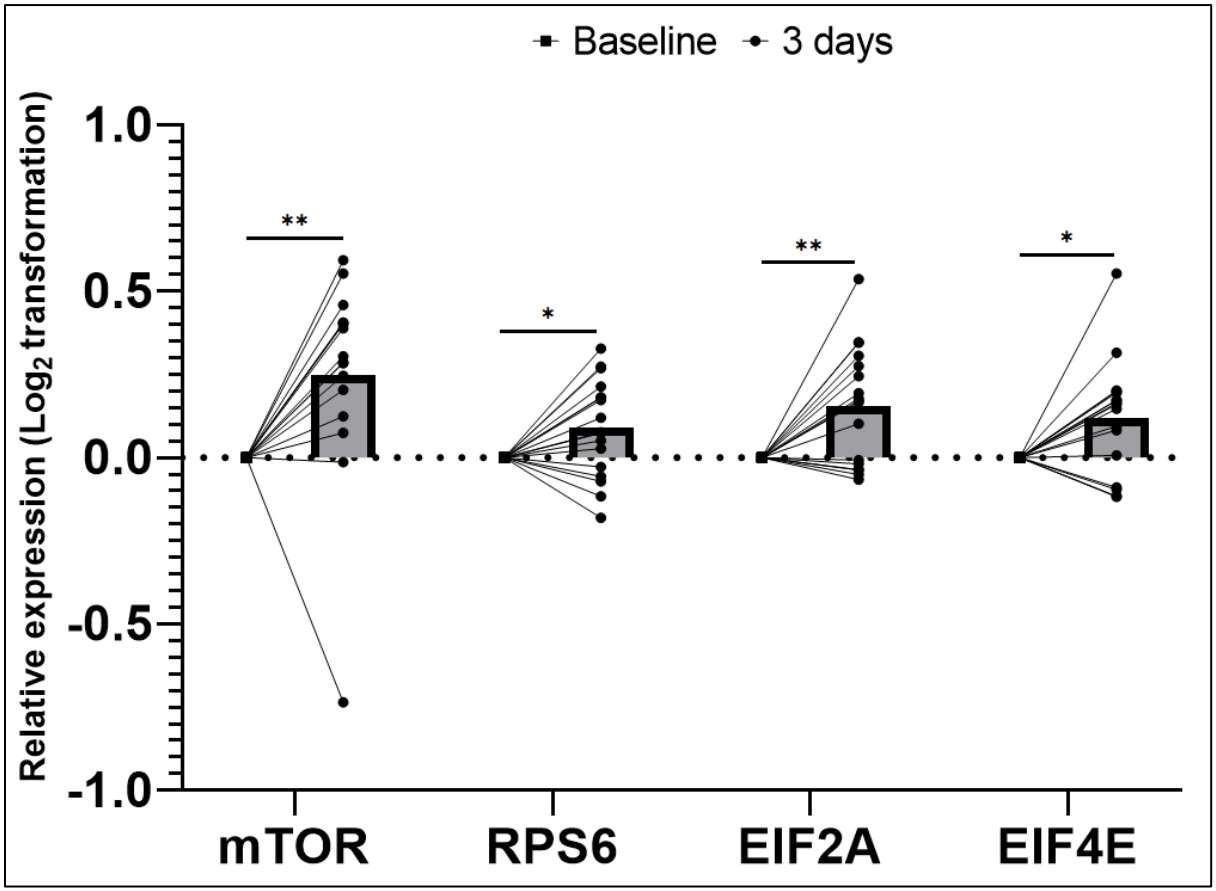


**Figure 3-7.** This figure depicts the mRNA abundance for the mTOR pathway after 3 days of resistance exercise when both diet groups are collapsed together. Bars are mean. Circles or squares represent individual points. *, *P* <0.05. **, *P* <0.01. n = 16 for statistical analyses

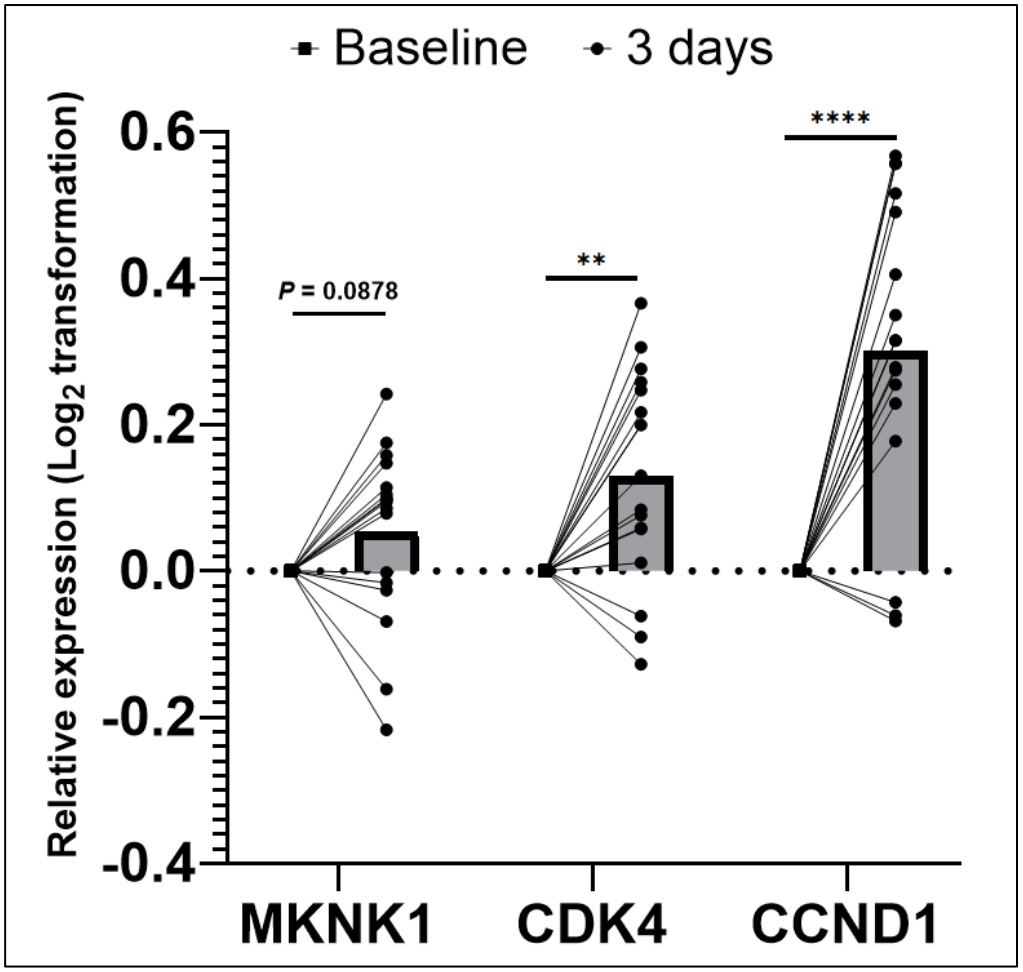


**Figure 3-8.** This figure depicts the mRNA abundance for the ERK 1/2 pathway after 3 days of resistance exercise when both diet groups are collapsed together. Bars are mean. Circles or squares represent individual points. **, *P* <0.01, ****, *P* <0.0001 n =17 for statistical analyses.

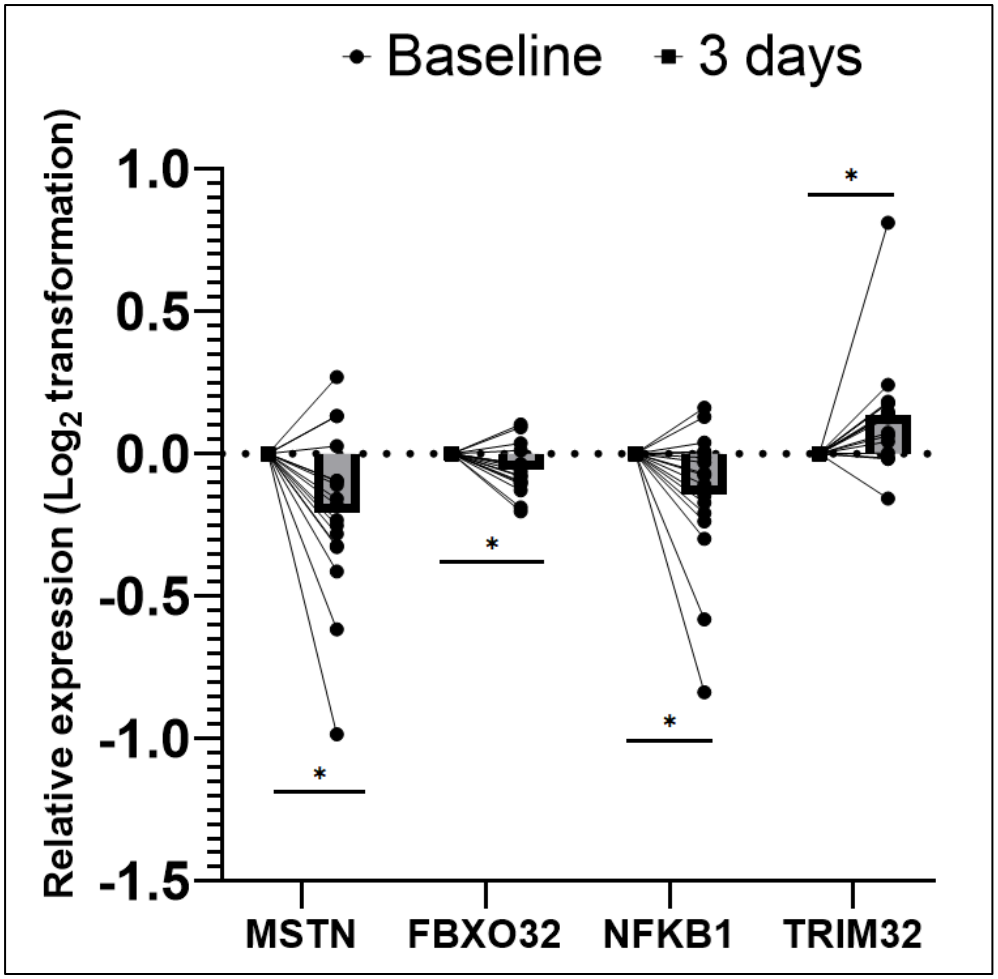


**Figure 3-9.** This figure depicts the mRNA abundance for MSTN, FBXO32, NFKB1 and TRIM32 after 3 days of resistance exercise when both diet groups are collapsed together. Bars are mean. Circles or squares represent individual points. *, *P* <0.05. n =17 for statistical analyses.

**Table 3-3.** This table outlines the mRNA expression results for genes not described in the full text.

| | Gene | Baseline | | 3 days | | Time | Diet | Interaction |
|---|---|---|---|---|---|---|---|---|
| | | OMN | VEG | OMN | VEG | | | |
| **Macromolecules** | Protein (mg/mg of wt tissue) | 27.5 ± 7.0 | 24 ± 2.7 | 19.1 ± 1.5 | 19.1 ± 1.5 | 0.6 | 0.5 | 0.1 |
| | RNA (ng/mg of wt tissue) | 318 ± 109 | 355 ± 123 | 379.7 ± 109 | 456 ± 164 | 0.6 | 0.8 | 0.39 |
| | DNA (ng/mg of wt tissue) | 2.5 ± 0.4 | 2.99 ± 0.67 | 4.1 ± 1.4 | 3.2 ± 0.48 | 0.4 | 0.6 | 0.6 |
| **Ratio of macromolecules** | Protein: RNA | 0.15 ± 0.05 | 0.17 ± 0.08 | 0.15 ±0.06 | 0.08 ± 0.02 | 0.37 | 0.7 | 0.4 |
| | Protein: DNA | 15.6 ± 4.9 | 11.0 ± 2.0 | 7.2 ± 1.4 | 10.2 ± 1.6 | 0.13 | 0.78 | 0.2 |
| | RNA: DNA | 154.1 ± 58 | 156.6 ± 50.7 | 129.6 ± 42 | 208.8 ± 89 | 0.8 | 0.5 | 0.5 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Ribosomal proteins** | RPL3 | 0 | 0.178 ± 0.05** | | **0.0020** | 0.65 | 0.65 |
| | RPL12 | 0 | 0.158 ± 0.05** | | **0.002** | 0.85 | 0.85 |
| | RPS16 | 0 | -0.09 ± 0.04* | | **0.01** | 0.14 | 0.14 |
| | RPS19 | 0 | -0.01 ± 0.05 | 0.029 ± 0.05 | 0.82 | 0.6 | 0.6 |
| | RPS4X | 0 | 0.047 ± 0.04 | 0.023 ± 0.04 | 0.23 | 0.67 | 0.67 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **rDNA transcription** | POl1RB | 0 | 0.12 ± 0.03*** | | **<0.0001** | 0.75 | 0.75 |
| | POL1RC | 0 | 0.3 ± 0.07*** | | **<0.0001** | 0.8 | 0.8 |
| | POl1RE | 0 | 0.04 ± 0.02 | 0.004 ± 0.02 | 0.14 | 0.4 | 0.2 |
| | UBTF | 0 | 0.01 ± 0.05 | -0.02 ± 0.05 | 0.94 | 0.66 | 0.66 |
| | TAF1D | 0 | 0.08 ± 0.05 | | 0.098 | 0.19 | 0.19 |
| | TBP | 0 | 0.04 ± 0.05 | 0.06 ± 0.04 | 0.1 | 0.87 | 0.87 |
| | MYC | 0 | 0.17 ± 0.07#* | -0.005 ± 0.07 | 0.1 | 0.08 | 0.08 |
| | TRRAP | 0 | 0.04 ± 0.03 | 0.03 ± 0.03 | 0.1 | 0.7 | 0.7 |
| | TRIM24 | 0 | 0.14 ± 0.04** | | **0.0008** | 0.14 | 0.14 |
| | NCL | 0 | 0.25 ± 0.07 | 0.1 ± 0.07 | 0.0013 | 0.16 | 0.16 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **rRNA processing** | NPM1 | 0 | 0.12 ± 0.05* | **0.0028** | 0.12 | 0.12 |
| | BOP1 | 0 | 0.2 ± 0.05*** | **0.0005** | 0.32 | 0.32 |
| | NOP56 | 0 | 0.03 ± 0.2 0.027 ± 0.2 | 0.85 | 0.99 | 0.99 |
| | FBL | 0 | 0.07 ± 0.04$^{\$}$ | **0.047** | 0.2 | 0.2 |
| **ERK 1/2 pathway** | MAPK3 | 0 | -0.04 ± 0.05 0.03 ± 0.05 | 0.86 | 0.28 | 0.28 |
| | MKNK1 | 0 | 0.05 ± 0.03$ | 0.077 | 0.79 | 0.79 |
| | CDK4 | 0 | 0.13 ± 0.04** | **0.0007** | 0.5 | 0.5 |
| | CCND1 | 0 | 0.3 ± 0.05**** | **<0.0001** | 0.13 | 0.13 |
| | RPS6KA1 | 0 | 0.07 ± 0.11 0.09 ± 0.11 | 0.3 | 0.9 | 0.9 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **mTOR pathway** | MTOR[%] | 0 | 0.25 ± 0.08* | | **0.0002** | 0.39 | 0.75 |
| | RPS6KB1 | 0 | 0.14 ± 0.07 | -0.02 ± 0.07 | 0.23 | 0.14 | 0.14 |
| | RPS6 | 0 | 0.089 ± 0.04* | | **0.0189** | 0.6 | 0.6 |
| | 4E-BP1 | 0 | -0.03 ± 0.04 | 0.007 ± 0.04 | 0.7 | 0.6 | 0.6 |
| | EIF2A | 0 | 0.16 ± 0.04** | | **0.0009** | 0.5 | 0.5 |
| | EIF4E | 0 | 0.12 ± 0.04* | | **0.0074** | 0.6 | 0.6 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Growth factors** | IRS-1 | 0 | -0.02 ± 0.07 | 0.04 ± 0.07 | 0.86 | 0.54 | 0.54 |
| | IGF-1 | 0 | -0.09 ± 0.09 | 0.02 ± 0.09 | 0.6 | 0.4 | 0.4 |
| | MSTN | 0 | -0.2 ± 0.07* | | **0.0072** | 0.4 | 0.4 |
| **Protein degradation** | TRIM63 | 0 | 0.057 ± 0.06 | 0.09 ± 0.06 | 0.11 | 0.7 | 0.7 |
| | TRIM32 | 0 | 0.13 ± 0.05* | | **0.01** | 0.7 | 0.7 |
| | FBXO32 | 0 | -0.06 ± 0.02* | | **0.0089** | 0.7 | 0.7 |
| | NFKB1 | 0 | -0.14 ± 0.06* | | **0.017** | 0.07 | 0.07 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Amino acid transporters** | SLC7A5 | 0 | 0.05 ± 0.3 | -0.27 ± 0.3 | 0.6 | 0.46 | 0.46 |
| | SLC38A2 | 0 | 0.01 ± 0.06 | -0.03 ± 0.06 | 0.8 | 0.6 | 0.6 |
| | SLC7A1 | 0 | 0.006 ± 0.08 | -0.07 ± 0.08 | 0.6 | 0.5 | 0.5 |
| | SLC36A1 | 0 | 0.14 ± 0.07# | -0.046 ± 0.07 | 0.36 | <u>0.08</u> | <u>0.08</u> |
| | ATF4 | 0 | 0.08 ± 0.06 | -2.132e-007 ± 0.06 | 0.3 | 0.3 | 0.3 |
| | LARS | 0 | 0.14 ± 0.07 | 0.03 ± 0.07 | 0.12 | 0.3 | 0.3 |

Data is presented as mean ± SEM and units are relative expression ($log_2$ transformed) unless otherwise stated. If a time effect was found, the mean ± SEM presented when VEG and OMN are collated together. Numbers in bold represent a significant effect. Numbers underlined represent a trend for an effect or a forced post hoc test. *, significant difference from baseline. *, significant difference from baseline, #, significant difference between groups. $, trend for significance ($P < 0.1$). * or #, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$. ****, $P < 0.0001$. %, one data point was excluded due to being greater than 2 standard deviations from the mean.

## CHAPTER 4

# TEMPORAL TRANSCRIPTIONAL REGULATION OF RIBOSOME BIOGENESIS IN RESISTANCE TRAINING INDUCED-SKELETAL MUSCLE HYPERTROPHY

# ABSTRACT

*Introduction.* Ribosome biogenesis plays a significant role in the process skeletal muscle hypertrophy. However, the mechanisms driving this process are not well understood. The aim of the present study was to characterise the transcriptional regulation of ribosome biogenesis during a period of resistance training-induced muscle hypertrophy. *Methods.* Eighteen resistance training experienced participants were recruited and were randomized to consume a high protein (~1.8 g/kg of bodyweight per day) diet from with vegan (VEG; n=9) or omnivorous (OMN; n=9) sources. All participants underwent a 5 day a week resistance training program for 10 weeks. Temporal muscle biopsies of the *vastus lateralis* were taken at baseline (0 week) and after 2 weeks, 5 weeks and 10 weeks of resistance training. Muscle samples were utilised to measure quantities of RNA, DNA, protein and mRNA expression of 48 genes using real-time polymerase chain reaction. *Results.* Markers of muscle ribosome content, 28S RNA and muscle total RNA concentration, increased from baseline to 2 weeks and remained elevated for the remainder of the 10-week resistance training period. Muscle mRNA expression of rRNA processing proteins, NOP56, NPM1 and FBL significantly increased from 0 to 2 weeks. Pooled analysis of genes regulating ribosome biogenesis showed the greatest transcriptional regulation occurs at 2 weeks of resistance training. *Conclusion.* Ribosome biogenesis on the transcriptional level is highly upregulated during 10 weeks of resistance training and shows the greatest activation at and around 2 weeks of training. Additionally, we characterised the transcriptional regulation of novel areas (rRNA processing proteins) of ribosome biogenesis.

# INTRODUCTION

Refer to chapter 3- introduction, for the background information for this study.

Muscle protein synthesis modulated through sub cellular processes at the level of the ribosome, through either increasing ribosome efficiency (translational efficiency) or ribosomal capacity (translational capacity). Translational capacity has thought to underpin the longer term changes in muscle protein synthesis (Kim, Staron and Phillips, 2005; Figueiredo, 2019) and skeletal muscle hypertrophy (Figueiredo *et al.*, 2015, 2020; Nakada *et al.*, 2016), however little is known about the time course of translational capacity and its transcriptional regulation during skeletal muscle hypertrophy.

This present study aimed to elucidate the time course and transcriptional regulation of indirect markers of translational capacity during 10 weeks of resistance training and high protein consumption, aimed at producing a high degree of skeletal muscle hypertrophy, and how this is modulated by animal and non-animal derived protein consumption.

# METHODS

The experimental methods for the human work required for conducting this chapter were conducted prior to commencement of this project as part of a PhD thesis submitted to the University of Exeter by Dr Alistair Monteyne. The novel data, therefore, are a post-hoc secondary analyses of the tissue samples obtained in that work. Human methods are briefly described below but can be found in greater detail in the PhD thesis of Dr Alistair Monteyne.

Participants

Participants who were recreationally active and had resistance training experience were recruited for the present study. An initial screening took place to record the participant's blood pressure, height and body mass. Furthermore, participants completed a general medical questionnaire to assess their eligibility for participation. Participants were excluded if their BMI exceeded the range of 18-30, had any diagnosed metabolic impairment, cardiovascular diseases or motor disorders. All participants were informed of the nature and possible risks of the experimental procedure before providing written informed consent. The present study was conducted in accordance with the Declaration of Helsinki. This study was registered as a clinical trial (NCT04325178) and was approved by the NHS Health Research Authority Ethics Committee (18/LO/0374).

Experimental Overview

Participants were randomised to either consume a high protein animal derived, omnivore (OMN) or primarily non-animal derived, vegan (VEG) diet. Additionally, both groups underwent a 10-week high volume and intensity progressive resistance training program. The participant's characteristics can be found in table 1 and a schematic diagram of the protocol can be found in figure 4-1.

**Table 4-1.** Baseline participant characteristics for OMN and VEG diet conditions before undertaking a 10-week resistance training program

| | OMN (n=9) | VEG (n=9)[1] |
|---|---|---|
| Age (years) | 25.6 ± 8.2 | 24.1 ± 5.7 |
| Height (cm) | 176.2 ± 11.7 | 171.5 ± 9.6 |
| Weight (kg) | 73.7 ± 9.3 | 67.2 ± 8.6 |
| BMI (kg/m$^2$) | 23.8 ± 2.6 | 22.8 ± 1.4 |

[1], denotes no significant differences between OMN and VEG conditions. Data is presented as mean ± standard deviation.

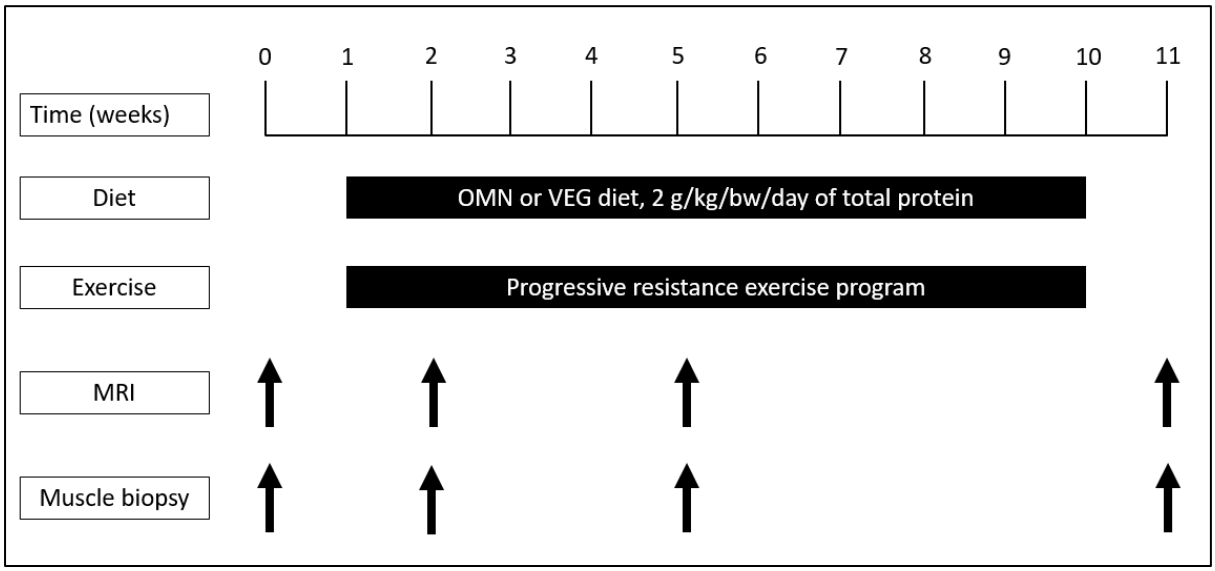


**Figure 4-1.** This figure displays an overview of the experimental design of the present study. Participants underwent 10 weeks of resistance training whilst consuming either a high protein omnivore or vegan diet. Muscle biopsies and MRI scans were taken at baseline and after 2, 5 and 10 weeks of resistance training.

Muscle sampling

Muscle biopsies of the *vastus lateralis* were obtained at baseline (week 0) and after 2, 5 and 10 weeks of resistance training using the modified Bergstrom (Tarnopolsky *et al.*, 2011) technique under local anaesthesia (2% lidocaine). Muscle samples were quickly frozen in liquid nitrogen and stored at -80°C until further analyses. Furthermore, muscle biopsies were taken in rested state (~72 hours after exercise).

Resistance exercise training program

Each day of the training program focused to progressively overload a single compound exercise (table 4-2). In order to maintain compliance of training, participants were instructed to complete a training log which was inspected every week by the principle researcher.

**Table 4-2.** An example week of training for subjects, including the type and specific exercises implemented.

| | Type of exercise | Specific exercises |
|---|---|---|
| Day 1 | Upper body pull exercises | Rope straight arm pulldown, deadlift, bent over dumbbell row, seated cable row, seated cable row, prone rear deltoid fly, hammer curl, seated dumbbell curl, plank |
| Day 2 | Upper body push exercises | Neutral cable fly, low incline Barbell press, arnold press, bench lateral raise, seated lateral raise, press up, rope pushdown, decline sit up |
| Day 3 | Lower body exercise | Unilateral leg curl, Barbell squat, dumbbell Romanian deadlift, leg press, leg extension, calf raise, hanging leg raise |
| Day 4 | Upper body Pull exercises | Face pull, one arm dumbbell row, pull up lat pulldown, dumbbell shrug, dumbbell curl, hammer curl, bicycle crunch |
| Day 5 | Lower body and push exercises | Heel elevated dumbbell goblet squat, dumbbell split squat, single leg glute bridge, unilateral leg extension, incline dumbbell press, machine chest press, seated lateral, seated front raise, unilateral pushdown |

Diet manipulation

Participants were instructed to consume either a high protein OMN or VEG diet (predominately mycoprotein). Participants aimed to consume ~2 g/kg of bodyweight of daily protein as this has been shown to exceed the recommended dose to maximally stimulate skeletal muscle hypertrophy (Morton *et al.*, 2018). Participants were instructed to complete a weekly food diary in order quantify protein intake and other macronutrients. To help reach the protein targets, the VEG group were given Quorn™ products and a mycoprotein supplement on a weekly basis. Similarly, the OMN group were given a milk protein supplement (Quorn™) and a weekly food stipend for animal products.

RNA extraction and mRNA abundance

RNA extraction and mRNA abundance were described previously (Monteyne *et al*., 2020) but can be found in greater detail in Chapter 2 (general methods).

Indirect markers of translational capacity

28S RNA levels transiently increase and decrease shortly after an exercise stimulus (Figueiredo *et al.*, 2015) and when muscle biopsies are taken in “active” state 28S, RNA is an unsuitable indirect marker of translational capacity. However, since muscle biopsies were taken in the rested state (~72 hrs after exercise) in the rested state, 28S RNA is a justified indirect marker of translational capacity and was unlikely impacted by acute resistance exercise.

DNA and protein extraction and quantification

A novel method was introduced to extract and quantify DNA and protein from RNA-TRIzol samples and can be found in detail in Chapter 2 (general methods).

MRI data

Muscle volume was taken from the PhD thesis of Dr Alistair Monteyne and used to express novel data not found in any other publication or thesis submission.

Statistics

mRNA expression data was $log_2$ transformed prior to statistical analysis to ensure equal distribution of data. If sphericity was not assumed ($P < 0.05$) a Geisser-Greenhouse correction was deployed. mRNA expression and macromolecules was analysed via a two way mixed effects model 2 x 4 (OMN and VEG x week 0, 2, 5 and 10). A Bonferroni post hoc test was used to detect significant differences between time points and diet groups. For correlational analysis a Pearson's two-tailed test was utilised. Results are reported as mean ± standard error of the mean (SEM) or mean difference (MD), unless otherwise stated. Statistical significance was set at 0.05 and all statistical analysis was completed using GraphPad Prism 9.0.0.

# RESULTS

For all biological analysis, both OMN (n=9) and VEG (n=9) groups have missing samples due to tissue availability. This led to some statistics appearing possibly underpowered, and therefore some forced post-hoc test was reported. The total number of samples per time point are shown in Table 4-3.

**Table 4-3.** This table represents the number of samples analysed for macromolecules and mRNA expression.

| | Week 0 | | Week 2 | | Week 5 | | Week 10 | |
|---|---|---|---|---|---|---|---|---|
| | **OMN** | **VEG** | **OMN** | **VEG** | **OMN** | **VEG** | **OMN** | **VEG** |
| **RNA** | 8 | 9 | 7 | 8 | 8 | 9 | 8 | 8 |
| **DNA** | 8 | 9 | 7 | 7 | 8 | 9 | 7 | 8 |
| **Protein** | 8 | 9 | 7 | 7 | 8 | 9 | 8 | 9 |
| **mRNA expression** | 8 | 9 | 7 | 8 | 8 | 9 | 8 | 9 |

Macromolecules

There was no time, diet or time x diet interaction for muscle protein, Protein: DNA ratio and Protein: RNA ratio. There was a trend for a main time effect for muscle DNA concentration ($P$ = 0.057). A forced post hoc test revealed no significant differences between time points. However, there was an increase in muscle DNA concentration of 47% from week 0 (2.8 ± 0.4 ng/mg of wt tissue) to 2 (4.1 ± 0.7 ng/mg of wt tissue). Finally, there was no diet or time x diet interaction for muscle DNA concentration (figure 4-2).

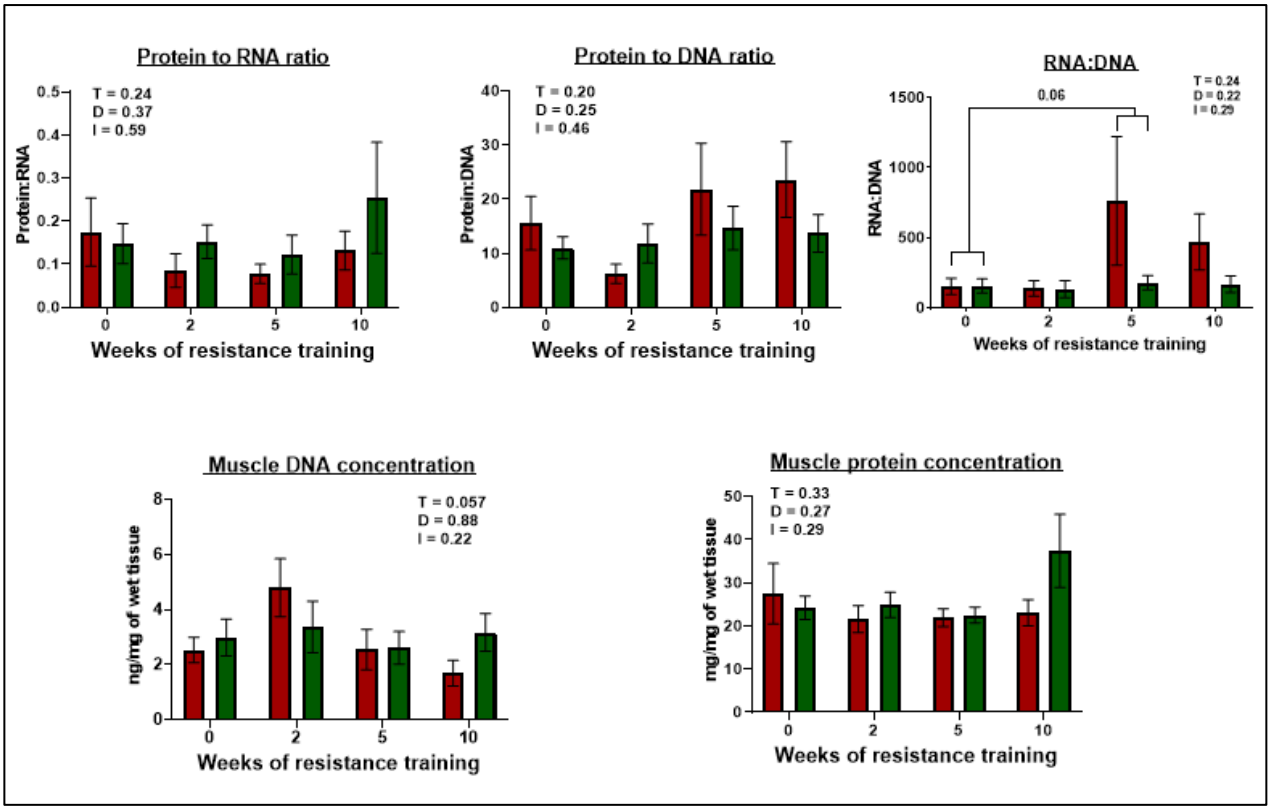


**Figure 4-2.** This figure displays the temporal response of muscle DNA, protein, protein:RNA, protein:RNA and RNA:DNA during 10 weeks of resistance training. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect.

Indirect markers of translational capacity

There was a trend for a main time effect for muscle total RNA concentration (*P* = 0.088). A forced post-hoc test revealed a significant increase in muscle RNA concentration from baseline to week 5 (337.6 ± 80.4 to 458.8 ± 104.8 ng/mg of wt tissue, *P* = 0.037). Although, there was a 27% numerical increase in RNA concentration from week 0 to week 2 (431.2 ± 120.7 ng/mg of wt tissue), this was found to be not significant (*P* = 0.25).

There was a significant main time effect for muscle 28S RNA expression ($P$ = 0.011). A post hoc analysis revealed significant increase from week 0 to week 2 (MD = 0.22 ± 0.068 relative expression ($log_2$ transformed), $P$ = 0.0379), week 5 (MD = 0.22 ± 0.064 relative expression ($log_2$ transformed), $P$ = 0.021) and week 10 (MD = 0.248 ± 0.079 relative expression ($log_2$ transformed), $P$ = 0.0412). There was no main time effect for RNA: DNA ratio. ($P$ = 0.24). However, a forced post-hoc analysis revealed a trend for an increase in RNA: DNA ratio from week 0 to week 5 (155.5 ± 37.1 to 454.8 ± 222, $P$ = 0.06). Finally, there was no diet or time x diet interactions for muscle RNA concentration, 28S RNA and RNA: DNA ratio (figure 4-3).

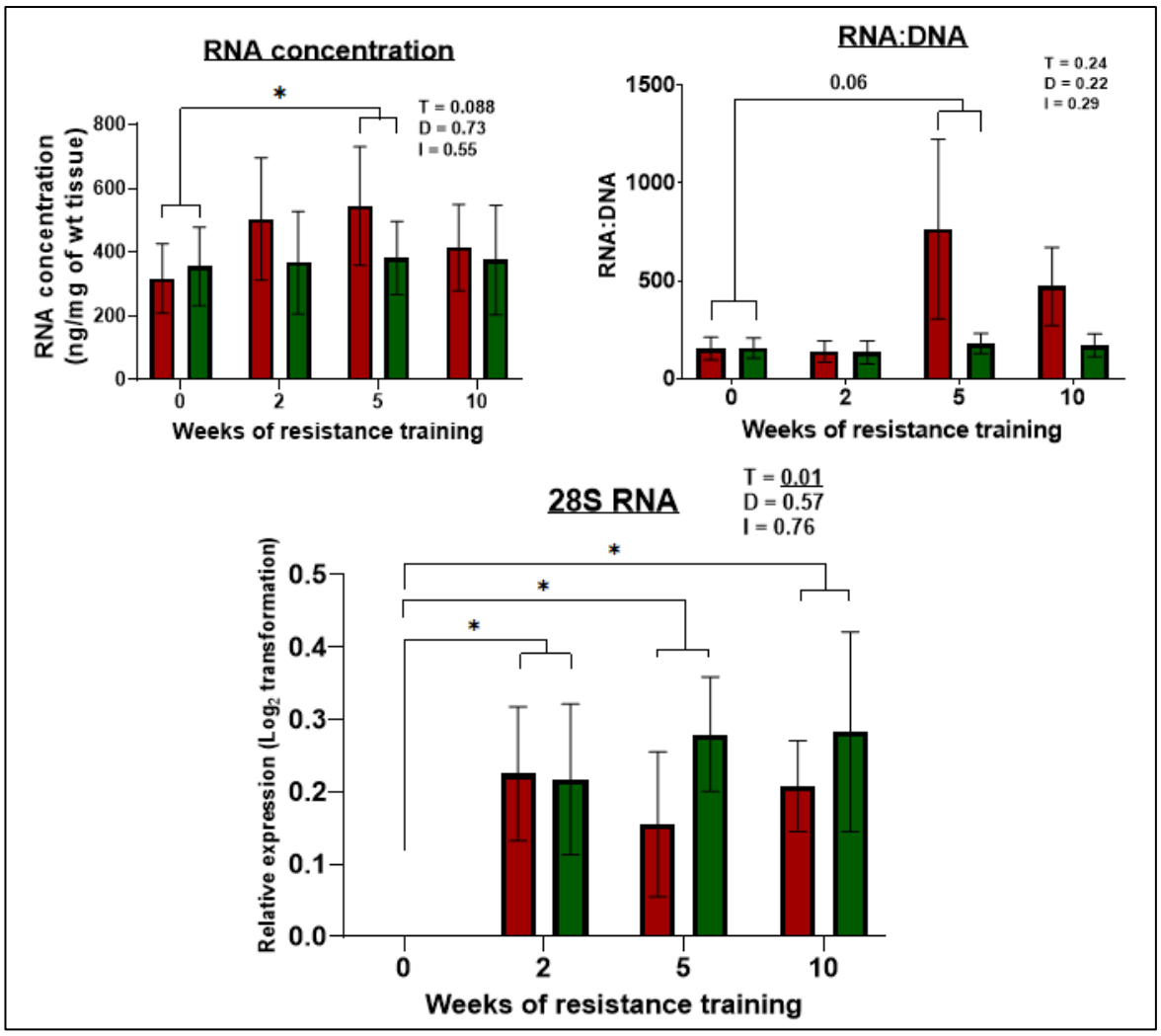


**Figure 4-3.** This figure displays the temporal response of 28S RNA, RNA:DNA and RNA concentration during 10 weeks of resistance training. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect. *, $P$ < 0.05.

Pooled expression of 60S and 40S ribosome subunit

Genes involved in the 60S and 40S ribosome subunit biogenesis were pooled together to create a pooled expression of the 60S and 40S ribosome subunit. The 60S subunit, which was created through the pooling of 28S RNA, RPL3 and RPL12, produced a time effect ($P$ = 0.0032) but no diet ($P$ = 0.26) or time x diet interaction ($P$ = 0.36). A post-hoc test revealed a significant increase in expression of this subunit from 0 to 2 ($P$ = 0.0468), 5 ($P$ = 0.0012) and 10 ($P$ = 0.0065) weeks. The 40S subunit which was created through the pooling of 18S RNA, RPS19, RPS16, RPS4X produced a time effect ($P$ = 0.03) and no diet ($P$ = 0.17) or time x diet interaction ($P$ = 0.13). A post-hoc test revealed no significant differences between time points (figure 4-4).

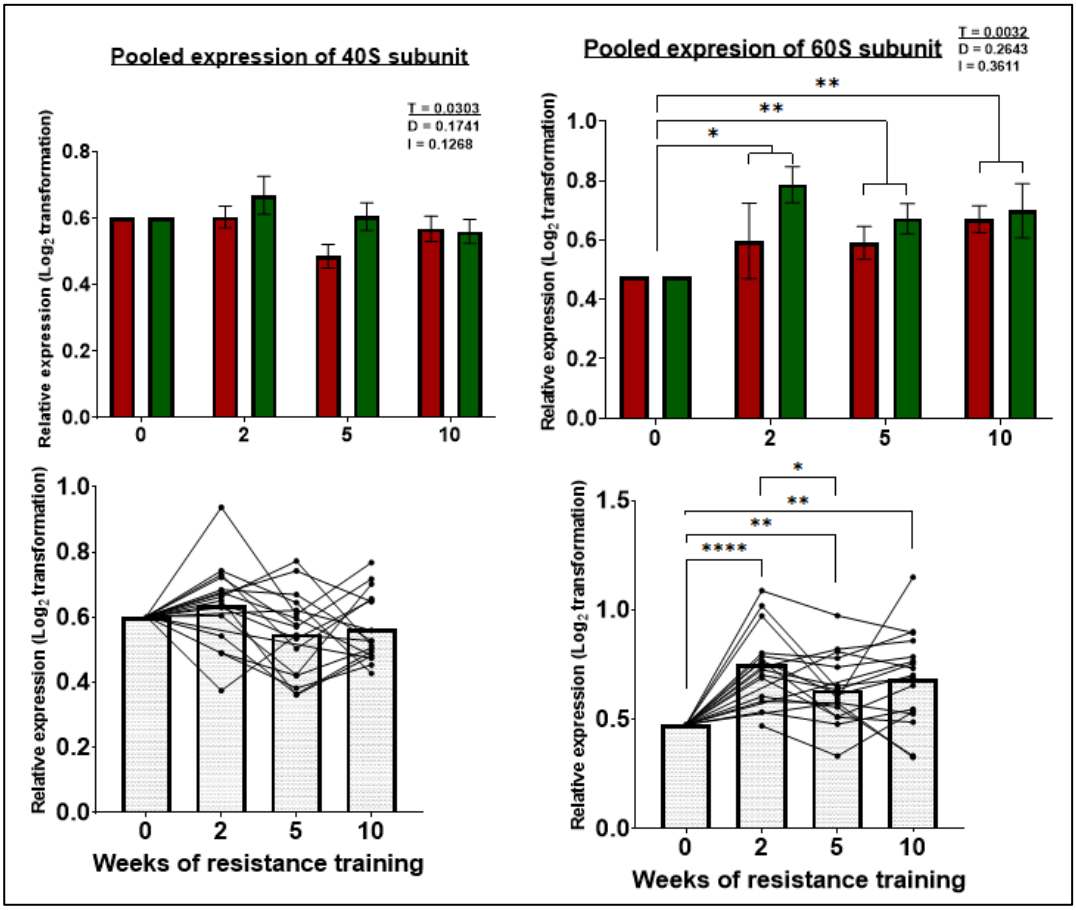


**Figure 4-4.** This figure displays the temporal response of the pooled expression of the 40S and 60S ribosome subunits during 10 weeks of resistance training. Both groups for each subunit are collated at the bottom of the figure. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect. *, $P$ < 0.05.

rRNA transcripts

There was no time effect (*P* = 0.43) for 18S RNA but there was a significant diet and time x diet interaction (*P* = 0.003 and 0.0047, respectively). A post-hoc analysis revealed a significant difference from OMN and VEG at week 2 (*P* = 0.0175) and week 5 (*P* = 0.0001). There was a significant time x diet interaction for 45S RNA (*P* = 0.0147). A post-hoc test revealed a significant increase in the OMN condition only from week 2 to week 10 (*P* = 0.0121). No main time (*P* = 0.59) or diet (*P* = 0.35) effect was found for 45S RNA (figure 4-5).

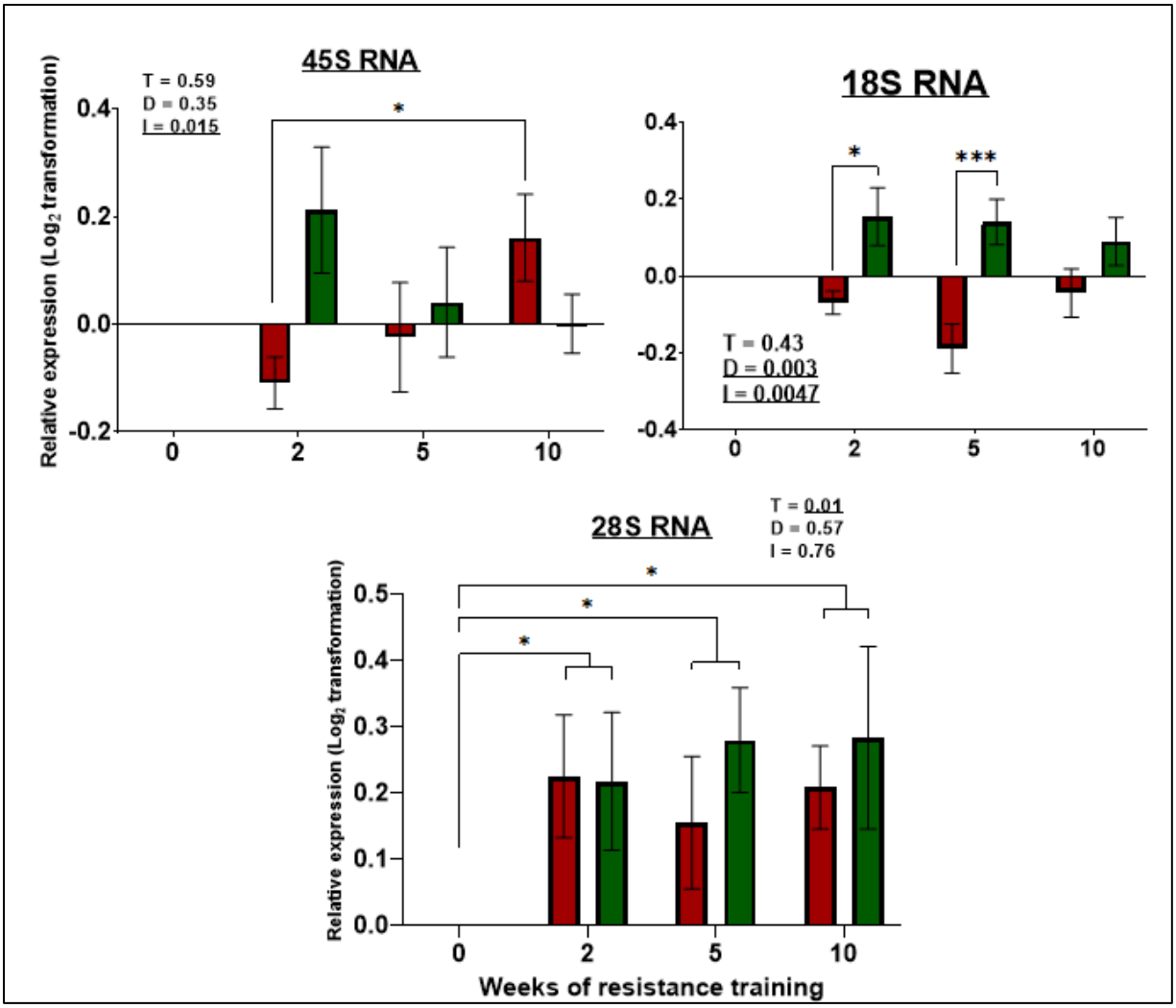


**Figure 4-5.** This figure displays results of rRNA mature transcripts, 28S RNA, 45S RNA and 18S RNA during 10 weeks of resistance training. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect. *, *P* < 0.05. **, *P* < 0.01. ***, *P* < 0.01.

The remainder of the mRNA expression data can be found in great detail in table 4-4.

Pooled mRNA expression of all genes

mRNA expression of genes reported in the present study were pooled together (figure 4-6) and subjected to a two-way repeated measures ANOVA. There was significant time effect (P <0.0001) but no diet (*P* = 0.45) or time x diet interaction (*P* = 0.14). A post-hoc test revealed a significant increase in expression from 0 to 2 (*P* <0.0001), 5 (*P* <0.0001) and 10 (*P* <0.0001) weeks. Additionally, there was a significant decrease in expression from 2 to 5 (*P* <0.0001) and 10 (*P* = 0.0013) weeks.

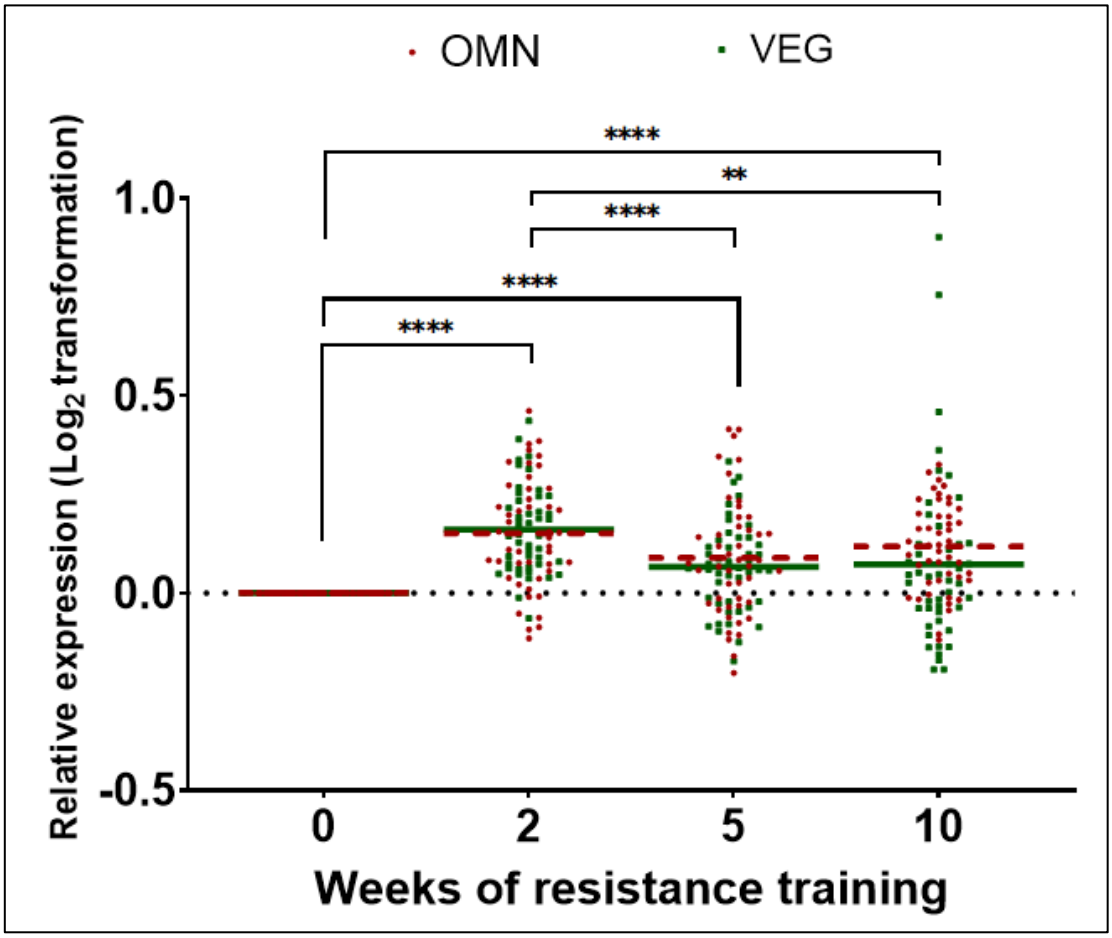


**Figure 4-6.** This graph depicts the temporal transcriptional regulation of genes regulating muscle metabolism. Each dot represents the mean relative expression for each gene presented in this study, excluding the housekeeping genes. The green solid bar represents the mean for the VEG group. The dashed red bar represents the mean for the OMN group. **, P <0.01. ****, P <0.0001.

Pooled mRNA expression of genes regulating ribosome biogenesis

Similar to the previous paragraph, mRNA expression of genes involved in ribosome biogenesis were pooled together (figure 4-7) and subjected to a two-way repeated measures ANOVA. There was a significant time effect ($P$ <0.0001) but no diet effect ($P$ = 0.94) or time x diet interaction ($P$ = 0.57). A post-hoc test revealed a significant increase in mRNA expression from week 0 to week 2 ($P$ <0.0001), 5 ($P$ <0.0001) and 10 ($P$ <0.0001). Additionally, there was significant decrease in expression from week 2 to week 5 ($P$ <0.0001).

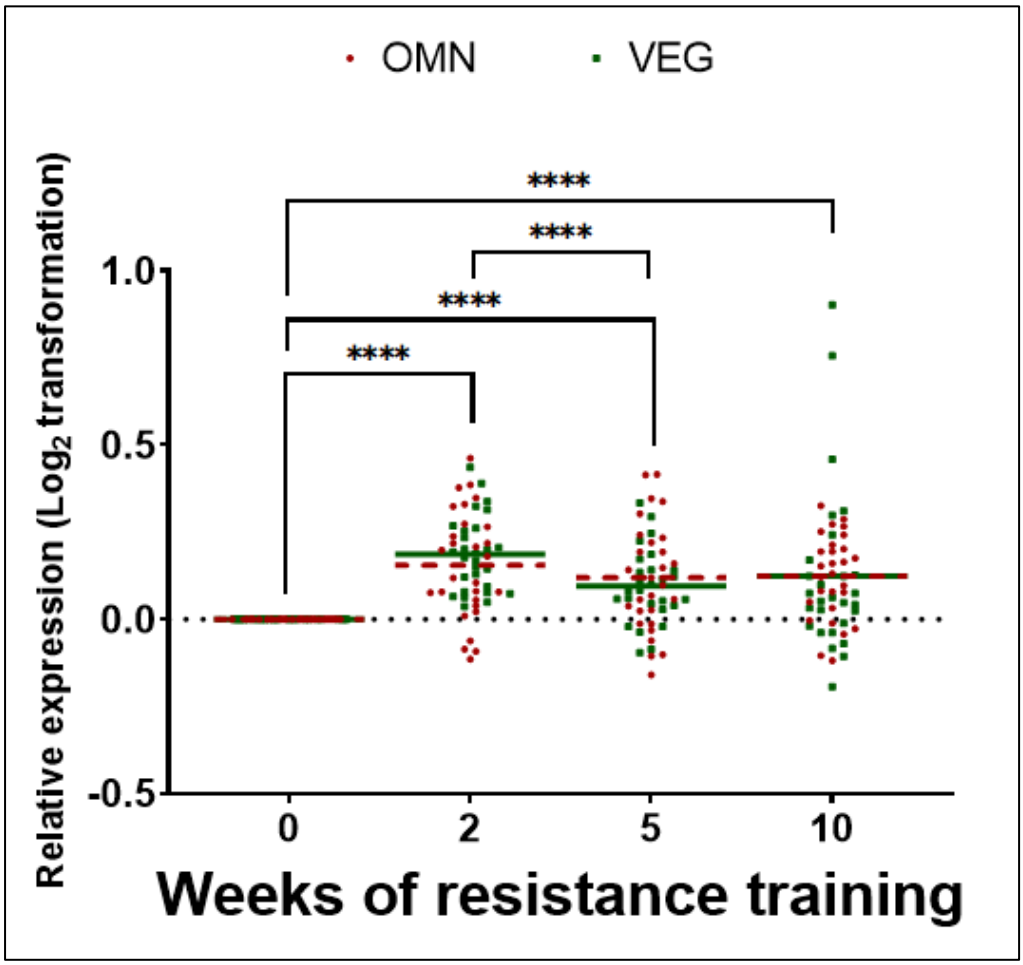


**Figure 4-7.** This graph depicts the temporal transcriptional regulation of genes regulating ribosome biogenesis. Each dot represents the mean relative expression for each gene presented in this study, excluding the housekeeping genes. The green solid bar represents the mean for the VEG group. The dashed red bar represents the mean for the OMN group. ****, P<0.0001.

Correlational analysis

For the correlational analysis all time points were pooled together. NCL was positively correlated with POl1RB (r = 0.68, *P* <0.0001), POL1RC (r = 0.33, *P* = 0.022) and POL1RE (r = 0.49, *P* = 0.0004; figure 4-8). 45S RNA was positively correlated with POl1RE (r = 0.36, *P* = 0.01) but not POl1RB (*P* = 0.3354, r = 0.14) or POL1RC (*P* = 0.24, r = 0.17; figure 4-9). Additionally, POl1RE was positively correlated with UBTF (r = 0.35, *P* = 0.015; figure 4-9). There was trend for a negative correlation with muscle protein concentration and quadriceps muscle volume (r = -0.22, *P* = 0.089). Furthermore, quadriceps muscle volume was negatively correlated with muscle DNA concentration (r = -0.28, *P* = 0.037). Lastly, protein: DNA ratio was positively correlated with quadriceps muscle volume (r = 0.27, *P* = 0.04; figure 4-10).

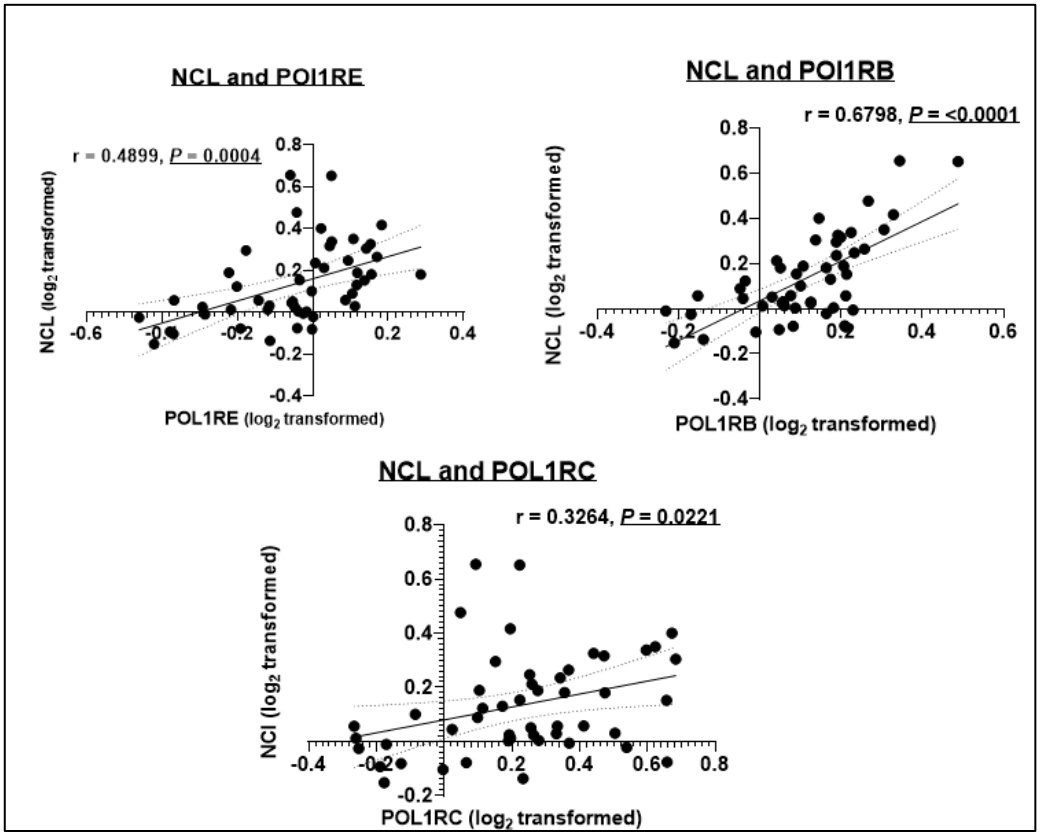


**Figure 4-8.** This figure displays the results of correlational analysis of NCL and POl1RB, POL1RE and POL1RC. Each dot on the graph represents a different sample from each group and time point. The r and p values are presented on the graph. The linear regression line is presented on the graph with 95% confidence intervals either side.

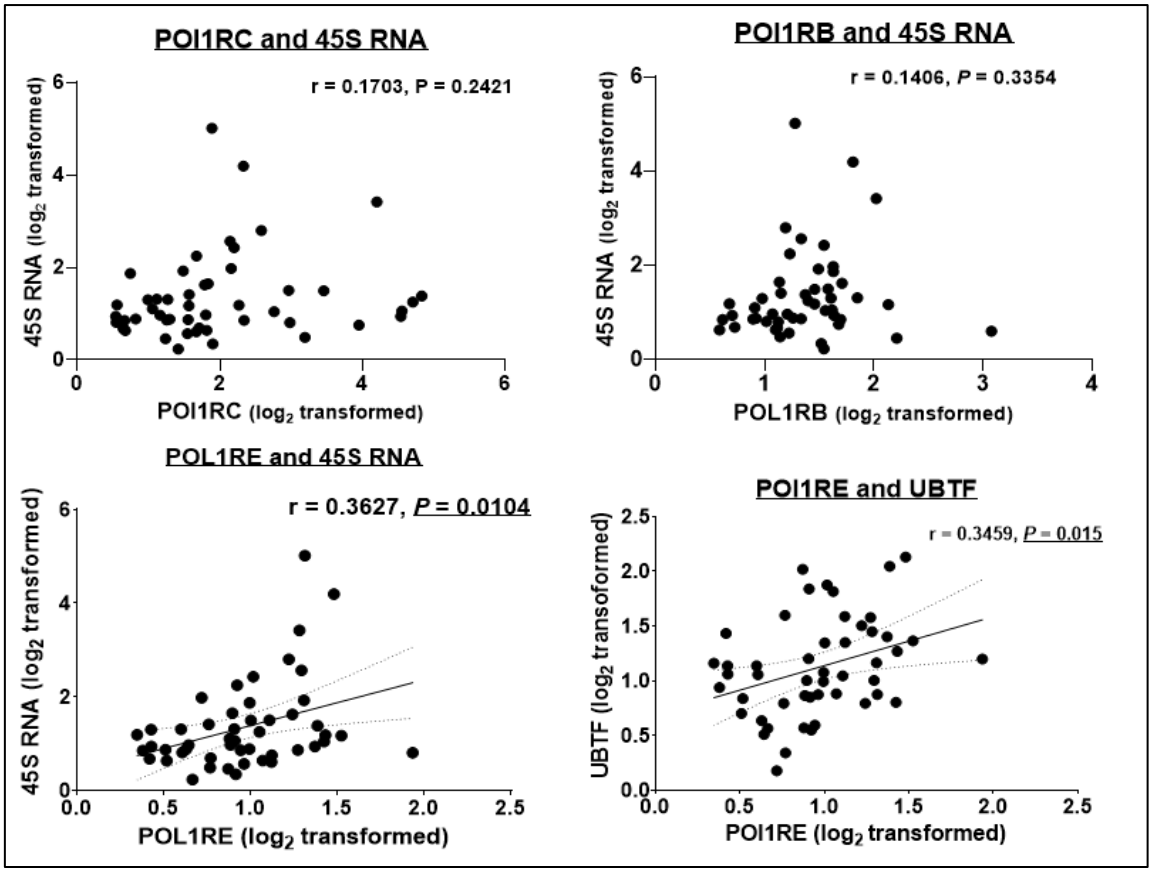


**Figure 4-9.** This figure displays the results of correlational analysis of 45S RNA and POL1RC, POL1RE and POL1RB and POL1RE and UBTF. Each dot on the graph represents a different sample from each group and time point. The r and p values are presented on the graph. The linear regression line is presented on the graph with 95% confidence intervals either side.

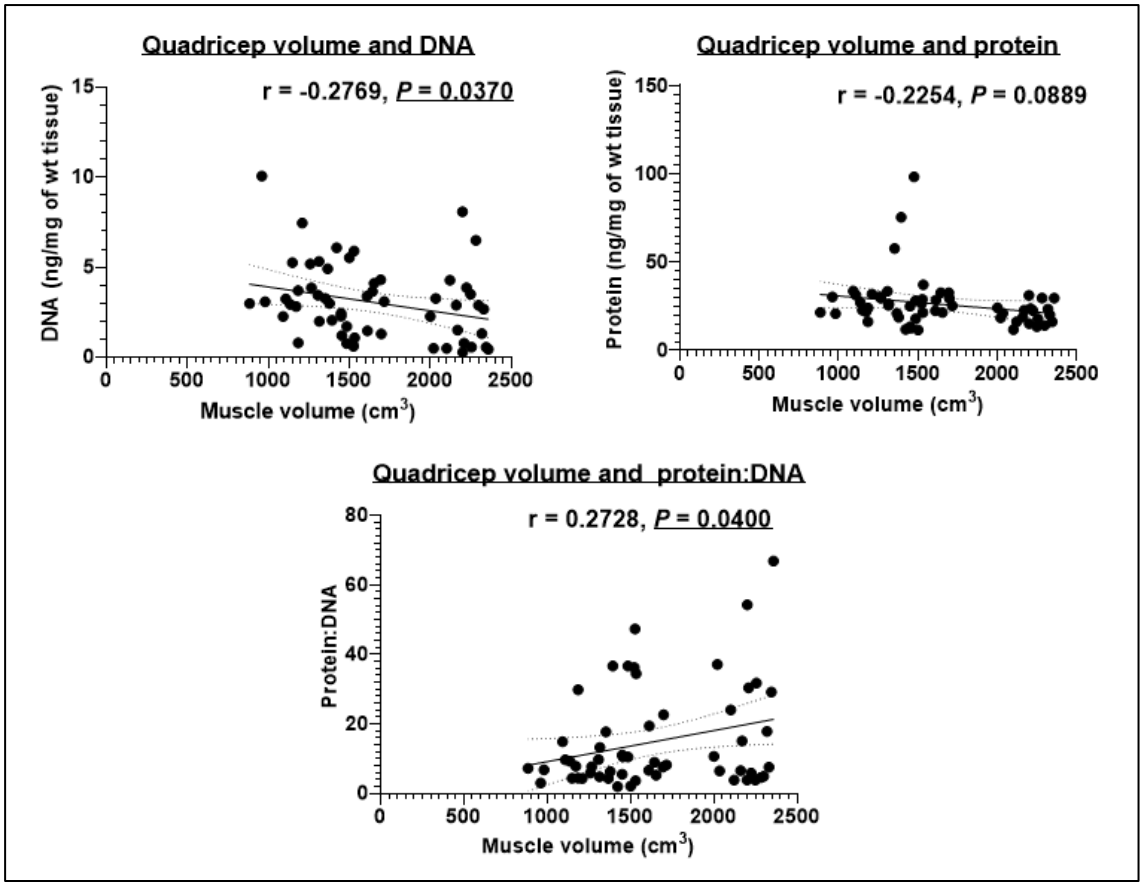


**Figure 4-10.** This figure displays the results of correlational analysis of quadricep volume and DNA, protein and protein:DNA. Each dot on the graph represents a different sample from each group and time point. The r and p values are presented on the graph. The linear regression line is presented on the graph with 95% confidence intervals either side.

# DISCUSSION

The present study established detailed temporal alterations in indirect markers of translational capacity and temporal transcriptional regulation of ribosome biogenesis during 10 weeks of resistance training under a high protein diet. Additionally, the present study aimed to establish the impact of the type of protein (animal vs non-animal derived) consumed on ribosomal capacity and its transcriptional regulation. The principle findings is that indirect markers of translational capacity increase at and around 2 weeks of training and remained elevated throughout the training program. A second main finding is that the large 60S ribosome subunit pathway was highly upregulated whilst the small 40S ribosome subunit showed minimal changes and may in fact decrease in the latter part of training. Third, the greatest transcriptional regulation of ribosome biogenesis occurs at and around 2 weeks of resistance training. Lastly, consuming an animal or non-animal derived diet for 10 weeks with resistance training had no significant impact on the abovementioned processes.

The present study measured the concentration of DNA, ribosomes and protein to provide an overall regulation of skeletal muscle hypertrophy. DNA showed a trend towards a main time effect of training. From the data, there was a 47% increase in DNA concentration at and around 2 weeks, which was returned to baseline values thereafter (figure 4-2). However, post-hoc tests did not reveal any significant findings between time points. Two studies by Brook *et al.* (2015 and 2016) have shown no significant changes in DNA concentration at and around 2 weeks of resistance training, although the P value of the main time effect was not reported. DNA, like ribosome levels, could be rate limiting to skeletal muscle hypertrophy if they are not increased.

It is known that the DNA pool does in fact increase with resistance training but this is regarded a long-term effect of training (Brook *et al.*, 2019) and has not been shown previously to be increased after 2 weeks of resistance training (Brook *et al.*, 2016). Interestingly, the increase in DNA occurs at a time where the greatest transcriptional regulation occurs (figure 4-6). Therefore, it is plausible that DNA levels increased to support the increased transcriptional regulation. This has not been previously reported because first, a novel method of extracting and quantifying DNA compared to other studies was utilised. Second, an instrument which produces smaller (fluorimeter) CV value compared to traditional method (spectrophotometer) was utilised. Specifically, the fluorimeter (Qubit, ThermoFisher) produced CV value of 4.1% when measured in duplicates, whilst previous reports have shown a CV value of 12.5% utilising spectrophotometers (Roberts *et al.*, 2010). Additionally, comparison of instruments for RNA quantification found a similar result (see general method, Chapter 2).

Protein and DNA can be expressed as a ratio to give an indirect marker of cell size. The present study found that the protein: DNA ratio did not change with training. This is a similar finding to Brook *et al.* (2016), which found no changes after 6 weeks of resistance training. However, another study by Brook *et al.* (2015) did find a trend for an increase in protein: DNA ratio at and around 6 weeks of training. The present study did employ a novel method of DNA and protein extraction, one that has not been used as a quantification method previously, therefore it is difficult to make comparisons between studies. Since protein to DNA ratio is reported as an marker of cell size, it may follow a similar pattern to a physiological measure of cell size, such as muscle volume. The present study subjected the measurement of protein: DNA and muscle volume to correlational analysis (figure 4-10). It was found that protein: DNA was

weakly but significantly correlated to muscle volume when all time points were brought together. This would suggest that the protein: DNA measurement employed in the present study could provide a suitable predictor of skeletal muscle hypertrophy size.

It has been well established that several weeks of continuous resistance training is required to elevate translational capacity. The present study utilised a variety of measures, 28S RNA, RNA concentration and RNA: DNA ratio to indirectly measure translational capacity. The present study found strong evidence to show that indirect markers of translational capacity is elevated after 2 weeks of resistance training and remains elevated throughout the 10-week training period. The results of the present study are supported by several studies (Brook *et al.*, 2015, 2016; Sieljacks *et al.*, 2019; Hammarström *et al.*, 2020). Therefore, resistance training program with high protein consumption leads to a robust increase in indirect markers of translational capacity. This response is likely produced to support further increases in skeletal muscle hypertrophy.

The 80S ribosome comprises a large 60S and a small 40S subunit. The present study measured the expression of rRNA transcripts and ribosomal proteins that are incorporated into both subunits, thereby highlighting regulation of each subunit. For the 60S subunit, 28S RNA, RPL3 and RPL12 were measured and all were upregulated throughout the training period (figure 4-11 and 4-5). Conversely, for the 40S subunit, 18S RNA, RPS16, RPS19 and RPS4X were either down regulated, did not change and/or were differentially regulated between groups (figure 4-11 and 4-5). As a result of this observation, data were collated together to display a pooled expression of the 60S and 40S subunits. Both subunits produced a main time effect, however the 60S

was clearly upregulated throughout 10 weeks of training whilst the 40S subunit showed a small increase at and around 2 weeks and then a small decrease to below basal values at 5 and 10 weeks (figure 4-4). Since both subunits are required for a functional ribosome it would suggest that in healthy tissue there exists a 1:1 ratio of both subunits. However, our data suggest two theories. Firstly, if the ratio of both subunits exists as 1:1 at the beginning of training, then our data suggest that this ratio is unbalance on the side of the 60S subunit (>1:1). The second theory is that 40S subunit maybe in high numbers at the start of training, whilst the opposite is the case of 60S. During resistance training, the ratio unbalanced ratio corrects itself, to gradually move towards a 1:1 ratio. Evidence from the literature suggests that a healthy individual possesses a 1:1 ratio of 60S to 40S subunits (Burwic*k et al*, 2012). However, this is reported in bone marrow cells and this has yet to be tested in human skeletal muscle tissue (Yoshikawa *et al.*, 2018, 2021).

rRNA processing proteins play an important role in the processing of rRNA transcripts which are later incorporated into the 40S and 60S ribosome. The present study is the first human study to temporally characterise the transcriptional regulation of rRNA processing genes during a prolonged resistance training program. NOP56, FBL and NPM1, with the exception of BOP1, showed a robust upregulation in mRNA expression after 2 weeks of resistance training, suggesting that rRNA processing proteins is an important requirement for ribosome biogenesis (figure 4-12).

The present study also investigated the temporal regulation of transcriptional factors involved in rDNA transcription on the transcription level. rDNA transcription is known as a rate-limiting step in ribosome biogenesis and has not been previously investigated

temporally. The transcriptional factors did show diverse regulation during 10 weeks of training. TAF1D, a component of the SL-1 complex, and NCL was shown to be highly upregulated at and around 2 weeks of training and then returned to baseline values (figure 4-13). Conversely, MYC and TRRAP (a co-activator of MYC), was highly upregulated at and around 10 weeks of training (figure 4-14), which has been reported previously (Hammarström *et al.*, 2020). In the present study, there was no upregulation in the expression of MYC after 2 or even 5 weeks of training. Previous studies have shown that on the protein level there was no changes in MYC protein expression after 2 and 5 weeks of training (Brook *et al.*, 2016). This would suggest that basal MYC protein levels are sufficient to increase rDNA transcription but at and around 10 weeks of training, the requirement for more MYC protein levels is increased, which requires increased MYC transcription. The measurement of MYC protein expression during a prolonged resistance training program is lacking, however one study by Mobley *et al.* (2018) reported no change in resting MYC protein expression after 12 weeks of resistance training, which does not support this theory. Clearly, more prolonged training studies are required to understand what occurs on the protein level.

TRIM24, also known as TIF-1A, did not change with training, whilst UBTF produced a time effect with a visible increase at and around 2 and 5 weeks of training and then a sudden decrease at 10 weeks of training but none of these changes were found to be significant with post-hoc analysis (figure 4-13 and 4-14). Fyfe *et al.* (2018) reported no differences in UBTF expression after 8 weeks of resistance but did not utilize temporal measurements. Conversely, Figueiredo *et al.* (2015) found an upregulation UBTF expression in rested muscle tissue after 8 weeks of resistance training. The increased UBTF expression after 2 and 5 weeks of training in the present study would suggest

an increased UBTF protein content, however Figueiredo *et al.* (2015) found no increases in resting UBTF protein content after 8 weeks of resistance training. This would suggest that protein content of UBTF is in sufficient quantities in the trained state. With regards to TRIM24, transcriptional expression and protein content was unchanged after 8 weeks and of resistance training (Figueiredo *et al.*, 2015; Fyfe *et al.*, 2018), which supports the results of the present study. Overall the findings support the notion that a selective number of transcriptional factors (UBTF, MYC and TRIM24) are in sufficient quantities at 0-5 weeks of resistance training to stimulate rDNA transcription. Indeed, this theory was proposed and supported by Figueiredo *et al.* (2015). However, to add to this previous study, transcription of NCL (figure 4-13) and one part of the SL-1 complex (TAF1D) was upregulated during the early phase of training (0-2 weeks). This suggests that both of these transcriptional factors are not in sufficient quantities at the early phase of training to support rDNA transcription, however data on the protein level are required to support this theory.

The primary role of the transcriptional factors is to activate POL1, an enzyme responsible for the transcription of rDNA. The present study measured different subunits of POL1, POL1RB, POL1RC and POL1RE. POL1RB and POL1RC showed a robust upregulation from 0-5 weeks of resistance training, but at and around 10 weeks this regulation was markedly reduced (figure 4-15). Synergist ablation models have supported these findings by showing a large upregulation in POL1RB and POL1RC at the beginning period of synergist ablation (von Walde*n et al*, 2012; Chaillou, Tyler J. Kirby and McCarthy, 2014). In humans, the measurement of POL1 subunits are scarce. Figueiredo *et al.* (2015) found no changes in POL1RB mRNA expression after 8 weeks of resistance training, however Fyf*e et al*. (2018) reported a

decrease in expression compared to baseline values. Interestingly between 5-10 weeks POL1RB expression was not elevated above baseline values in the present study. This certainly supports the findings of Figueiredo *et al.* (2015) but not Fyf*e et al*. (2018). Importantly, both studies did not utilize temporal measurements, thereby missing the regulation of this subunit at earlier time points but overall the data suggest that POl1RB and POL1RC are in high demand in the early phase of skeletal muscle hypertrophy.

POL1RE displayed no changes during 0-5 weeks of resistance training, but there was a large downregulation at and around 10 weeks of training. During 7 days of a synergist ablation model, POL1RE was highly upregulated, which is contrast to the results of the present study. However, in humans, up to 24 hours after a bout of resistance exercise in the untrained state, POL1RE expression was unaltered. This suggests that in humans, POl1RE expression likely does not change in response to an acute stimulus and likely alters in response to multiple bouts of exercise in the rested state. Additionally, in the context of POl1RE, the model of synergist ablation provides a supraphysiological condition which is incomparable to a human model of skeletal muscle hypertrophy.

NCL, when located within the nucleolar acts as a transcriptional factor for POL1 due to its abundance in the promoter and coding regions of rDNA. It has been shown previously that NCL is a requirement for POl1 transcription *in vivo* (Rickards *et al.*, 2007). As a result, the present study submitted NCL to correlational analysis with every POl1 subunit. Interestingly, every subunit was strongly correlated with NCL (figure 4-

8), which supports the previous mechanistic data and further highlights the importance of NCL for POl1 activity.

As mentioned previously, POl1 is integral to the transcription of rDNA and therefore the production of 45S pre-rRNA. This would suggest that POl1 activity follows a similar trend to 45S RNA abundance. We subjected 45S rRNA and each POl1 subunit to correlational analysis. Only POL1RE showed a significant correlation with 45S RNA. This is a surprising finding because 45S RNA showed a time x diet interaction but POl1RE only displayed a time effect. Additionally, by looking at figure 4-16 it is clear that both genes do not follow a similar transcriptional regulation during 10 week training period. Another interesting finding is that POl1RE was found to be correlated with UBTF (figure 4-10). There is some evidence to suggest that POl1RE (also known as PAF53) facilitates the recruitment of POl1 to the rDNA promoter region by interacting with POl1 and UBTF (Chen *et al.*, 2013). Although the present study cannot provide mechanistic evidence of this link, it is certainly interesting that POL1RE and UBTF are correlated with each other but also POL1RE is correlated with the product of rDNA transcription, 45S RNA. This provides an associated link between POL1RE, UBTF and 45S RNA and should be further explored in knock out animal models utilising synergist ablation interventions (muscle hypertrophy model).

Transcriptional factors UBTF and TRIM24 are activated via the ERK 1/2 pathway. ERK 1/2 or MAPK3 activates UBTF via a MKNK1-EIF4E-CDK4/CCND1 mechanism, whilst TRIM24 is activated through RPS6KA1 (Figueiredo and McCarthy, 2019). In the present study, it is clear that the ERK1/2-UBTF pathway is highly upregulated from 0-2 weeks (figure 4-16), with the exception of UBTF, which only slightly increases from

0-2 and 0-5 weeks. This would suggest that this pathway is in great demand at the early phase of training (0-2 weeks) and the protein levels that exist at basal levels are limiting. However, a study by Figueiredo *et al.* (2015) does not support this theory. After 8 weeks of resistance training there was no significant increases in protein expression of this pathway. The present study did not measure protein expression so it is unclear if the large transcriptional increase would amount to detectable increases in protein level. Overall, the present study characterises the transcriptional regulation of this pathway during a 10 week training program but more work should be completed on the protein level to fully build upon the findings of the present study.

Looking at the RPS6KA1-TRIM24 pathway (figure 4-16 and 4-13), there is a diverse transcriptional regulation. One possible reason for this is that RPS6KA1 can also activate RPS6 and interestingly both genes follow a similar transcriptional regulation. This would suggest that RPS6KA1 is acting upon RPS6 rather than TRIM24. However, RPS6KA1 was not correlated with either TRIM24 or RPS6 (data not shown). Another reason for this diverse regulation is that TRIM24 protein levels are not limiting for rDNA transcription. This is supported by Figueiredo *et al.* (2015) who found TRIM24 protein levels to be unchanged after 8 weeks of training and this was supported by an unchanged transcriptional response. Therefore, this further supports the theory laid out by Figueiredo *et al.* (2015) that a selective number of proteins are limiting for rDNA transcription, therefore they require upregulation on the transcriptional level.

The mTOR pathway has a causal role in the regulating muscle protein synthesis (Drummond *et al*, 2009). The translational initiation pathway mTOR-RPS6KB1-RPS6-EIF2A follows a similar transcriptional regulation. Specifically, there is a large

upregulation in this pathway from 0-2 weeks and thereafter the pathway begins to return to baseline values (figure 4-17). This suggests that there is a large requirement in this pathway at and around 2 weeks of training, presumably to accommodate the large increases in muscle protein synthesis and skeletal muscle hypertrophy at the early phase of training (Brook *et al.*, 2015).

One advantage of the present study is the utilization of temporal molecular measures. A predominant theme of the data is that the greatest “activity” of mRNA expression occurred at and around 2 weeks (figure 4-6). This coincides with the physiological data, which shows the greatest hypertrophic effect occurs between 0 and 2 weeks of resistance training. This provides a clear recommendation for measuring molecular markers of muscle growth and atrophy. Molecular measures taken as pre and post measures during a prolonged training program may miss vital temporal molecular information. Additionally, at and around 2 weeks provides a suitable time point to make temporal measures and would greatly add to the findings of a pre and post study design.

The present study provided a detailed transcriptional regulation of ribosome biogenesis. However, it is unclear what occurs on the protein level. Increased transcription does not always equal increased protein expression (Figueiredo *et al.*, 2015). Therefore future studies should aim to build upon the findings of the present study and understand what occurs on the protein level by the use of western blotting or even proteomics.

To conclude, this study is the first study characterise the temporal transcriptional regulation of ribosome biogenesis during a 10 week resistance training program. Furthermore, this study provided a detailed regulation of every step of ribosome biogenesis, starting from the regulation of rDNA transcription up to indirect markers of ribosome concentration. Secondly, this study shows that the type of protein consumed (non-animal vs animal derived) has no significant impact on the regulation of ribosome biogenesis, mTOR pathway, amino acid transporters, protein degradation and growth factors. Thirdly, this study has highlighted that at and around 2 weeks of resistance training there is a robust transcriptional activity, which likely coincides with the large increase in skeletal muscle hypertrophy when compared to later time points

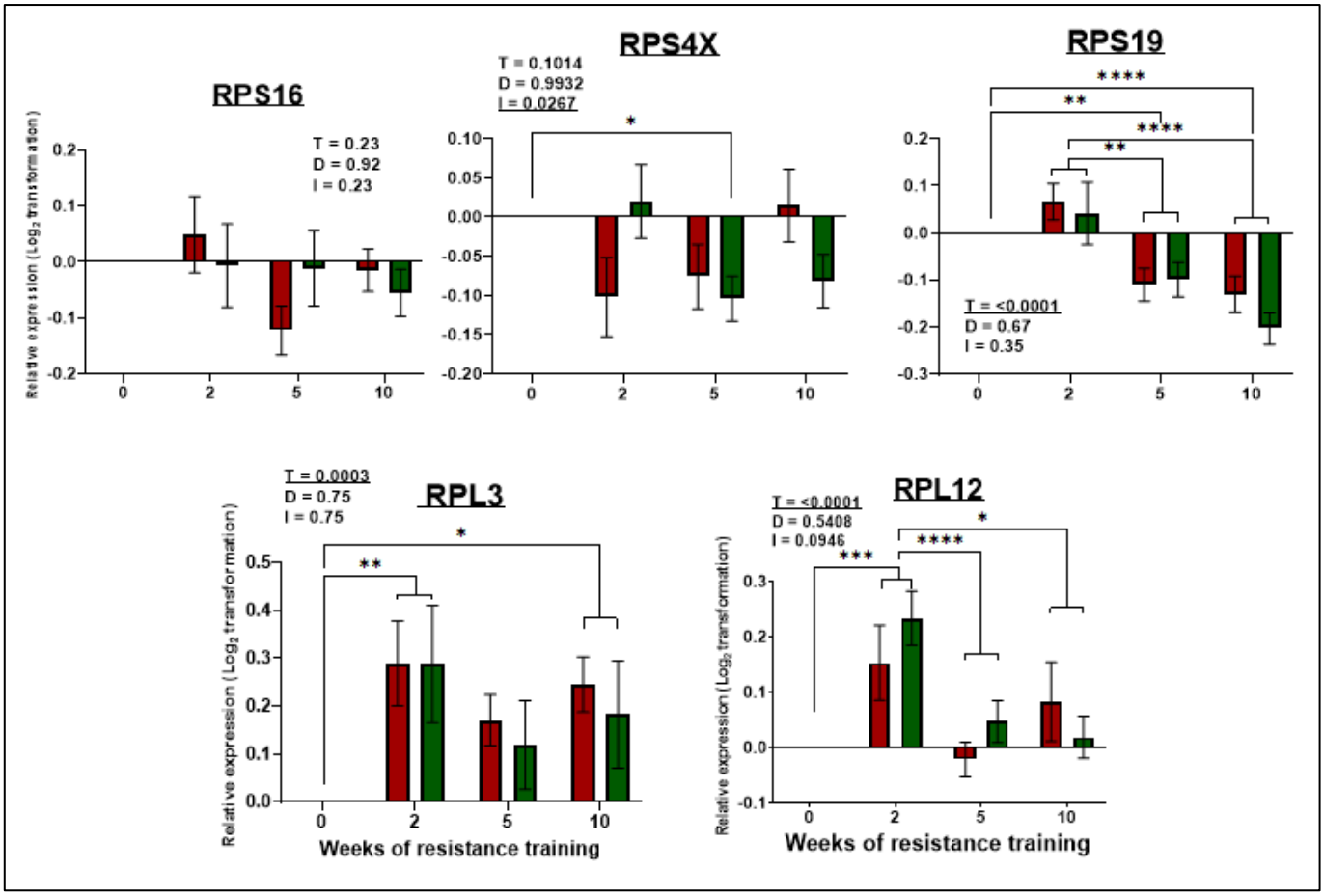


**Figure 4-11.** This figure displays the temporal response of ribosomal proteins, RPS16, RPS4X, RPS19, RPL3 and RPL12 during 10 weeks of resistance training. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$, ****, $P < 0.0001$

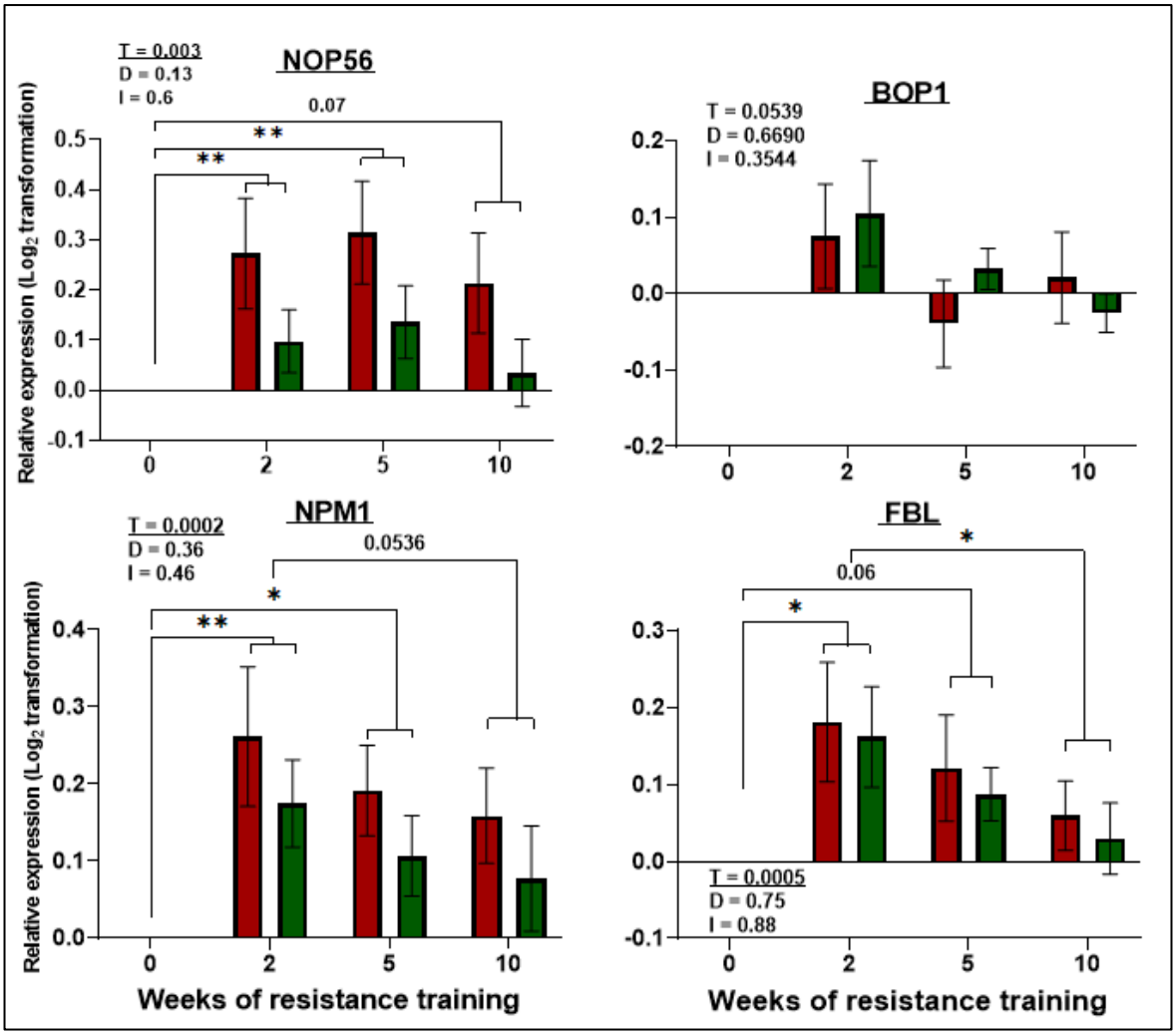


**Figure 4-12.** This figure displays the temporal response of NOP56, BOP1, NPM1 and FBL during 10 weeks of resistance training. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect. *, $P < 0.05$. **, $P < 0.01$.

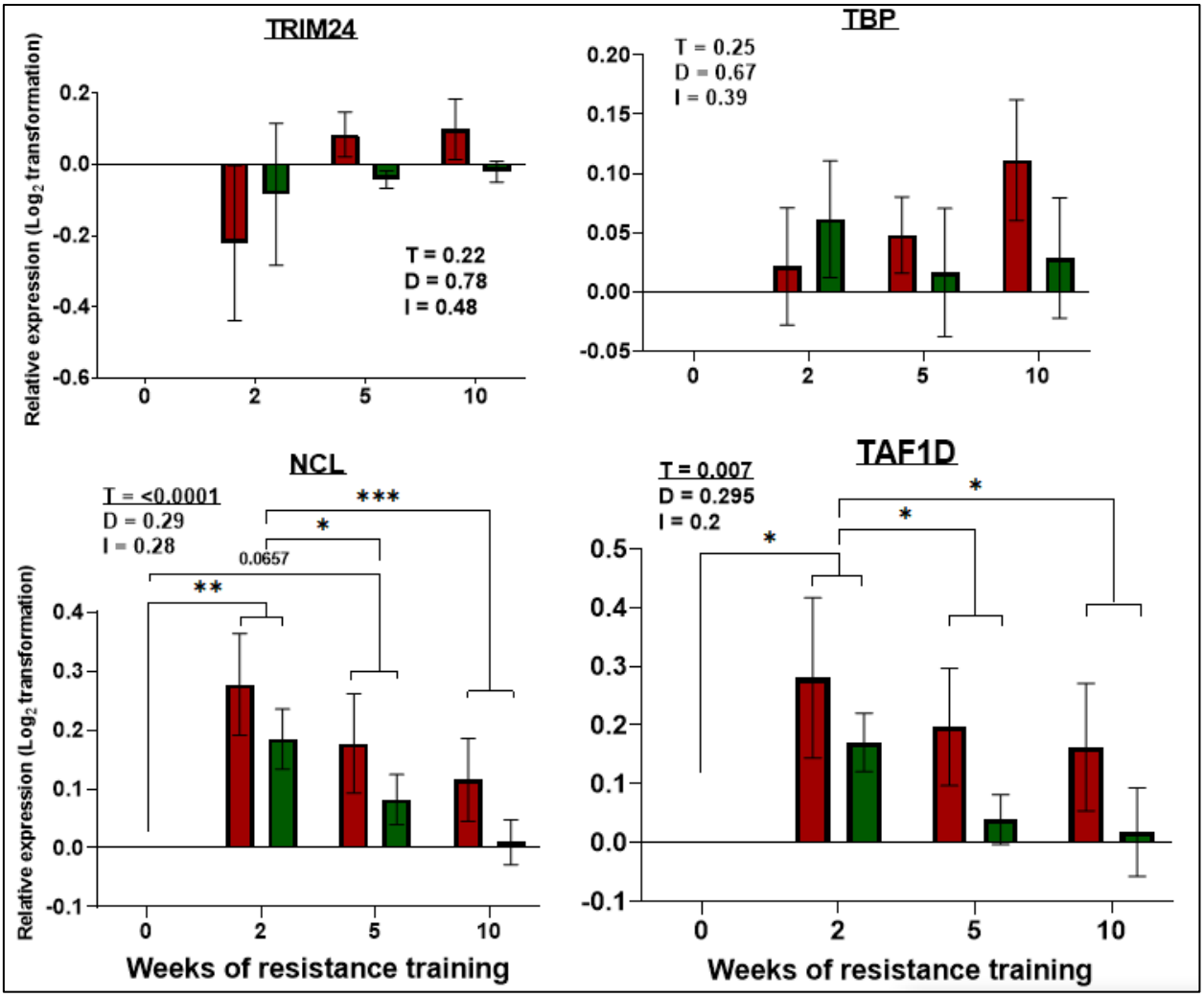


**Figure 4-13.** This figure displays the temporal response of TRIM24, TBP, NCL and TAF1D during 10 weeks of resistance training. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$

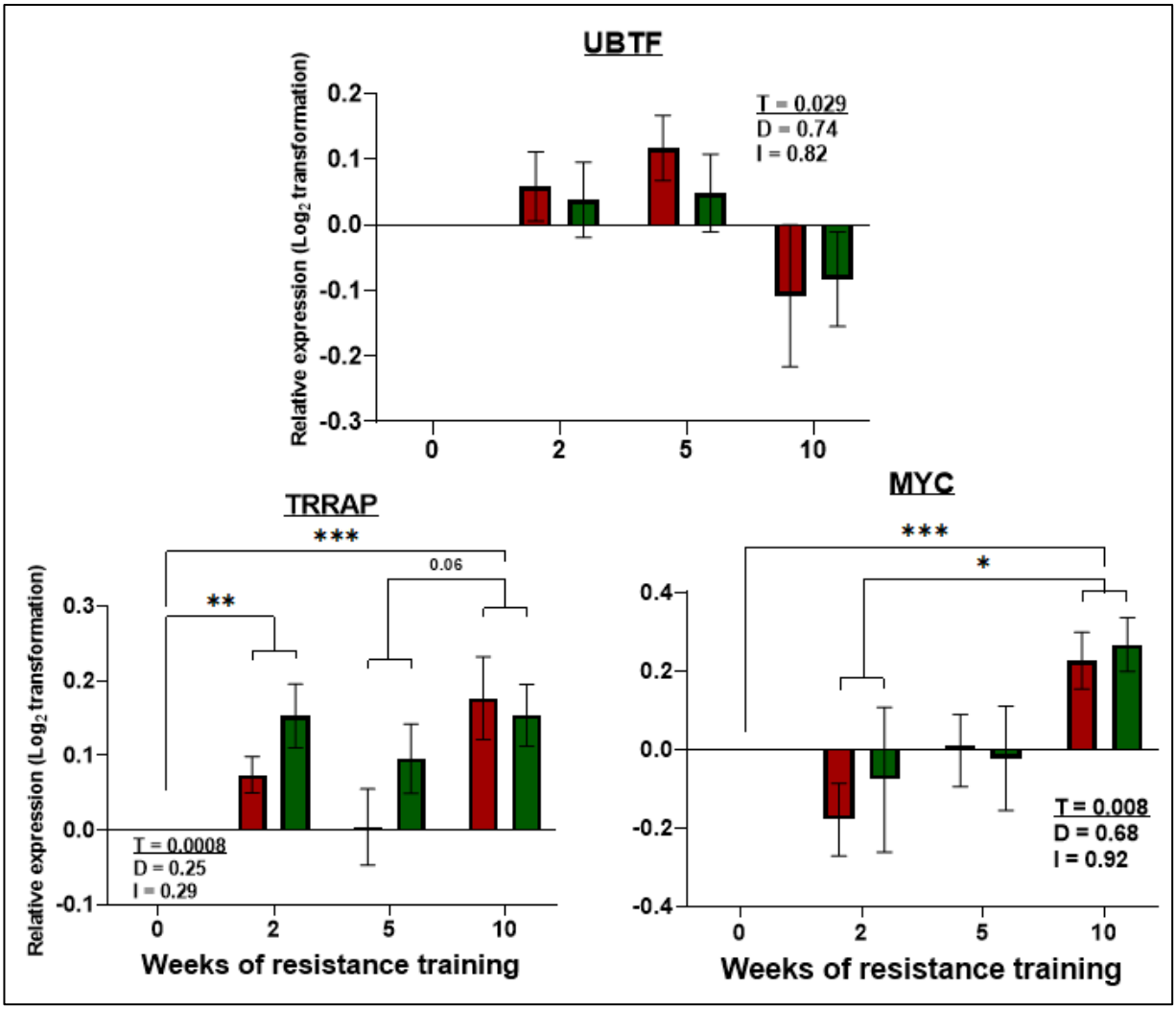


**Figure 4-14.** This figure displays the temporal response of UBTF, TRRAP and MYC during 10 weeks of resistance training. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$

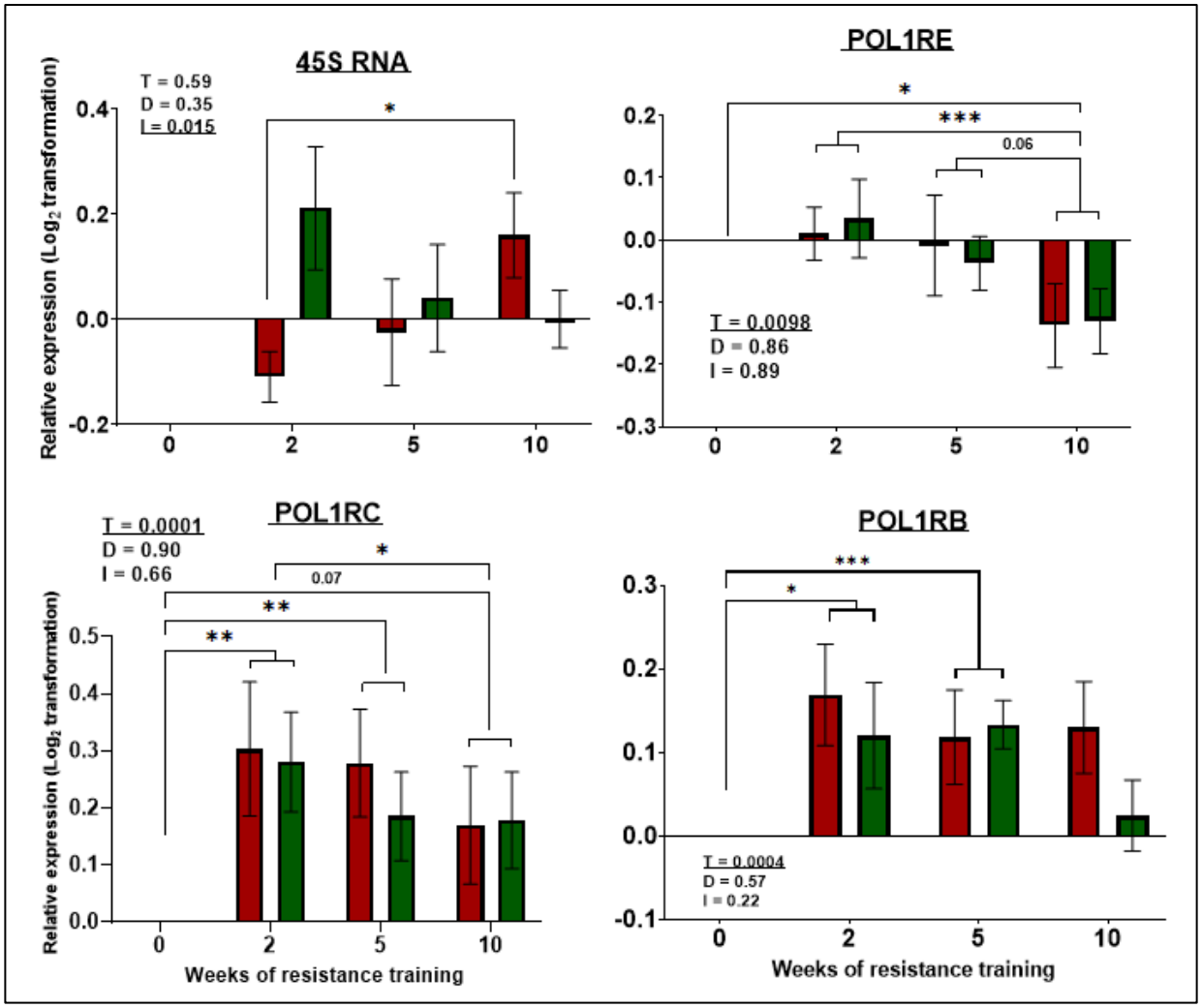


**Figure 4-15.** This figure displays the temporal response of 45SRNA, POL1RE, POL1RC and POL1RB during 10 weeks of resistance training. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$

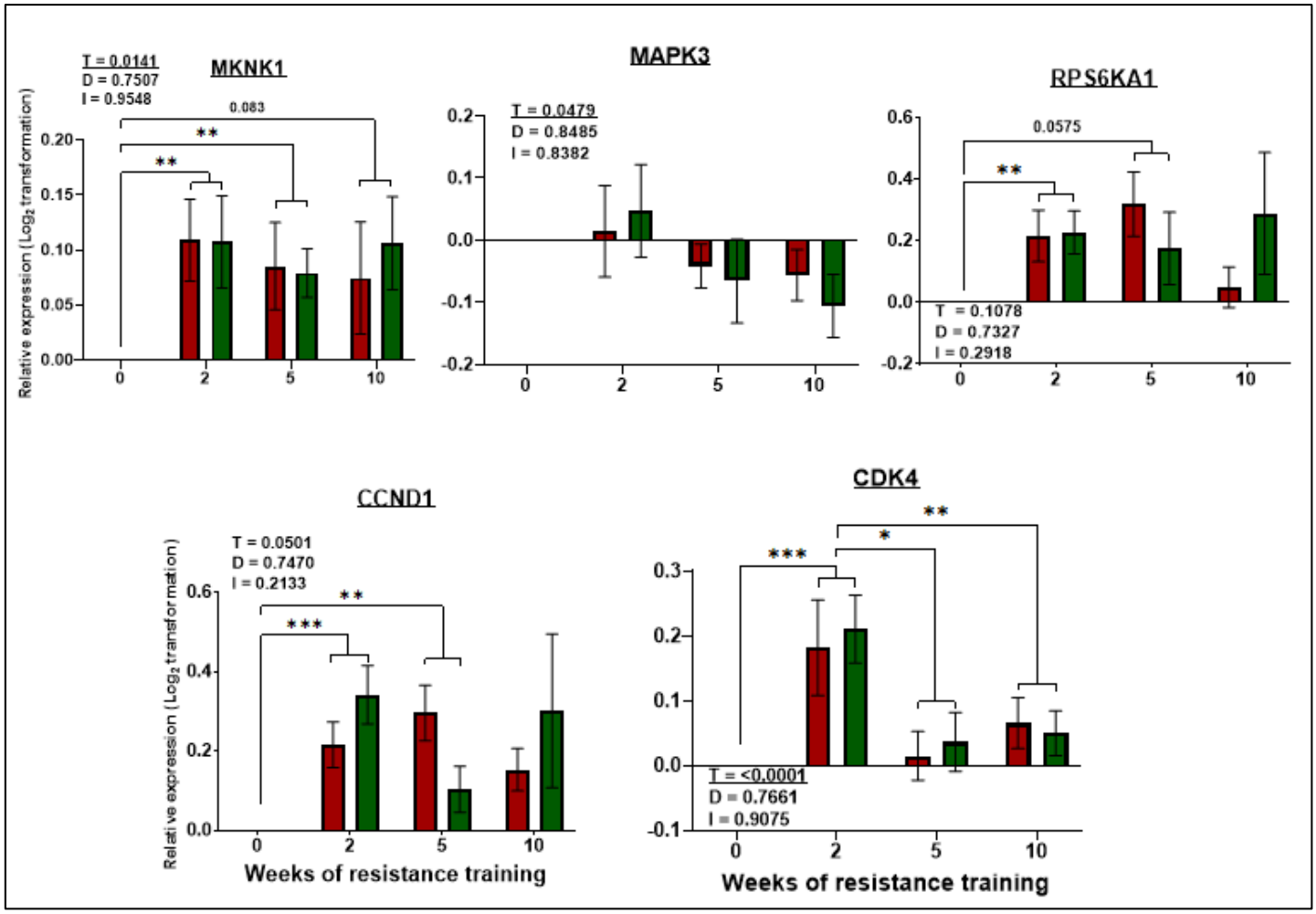


**Figure 4-16.** This figure displays the temporal response of MKNK1, MAPK3, RPS6KA1, CCND1 and CDK4 during 10 weeks of resistance training. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$

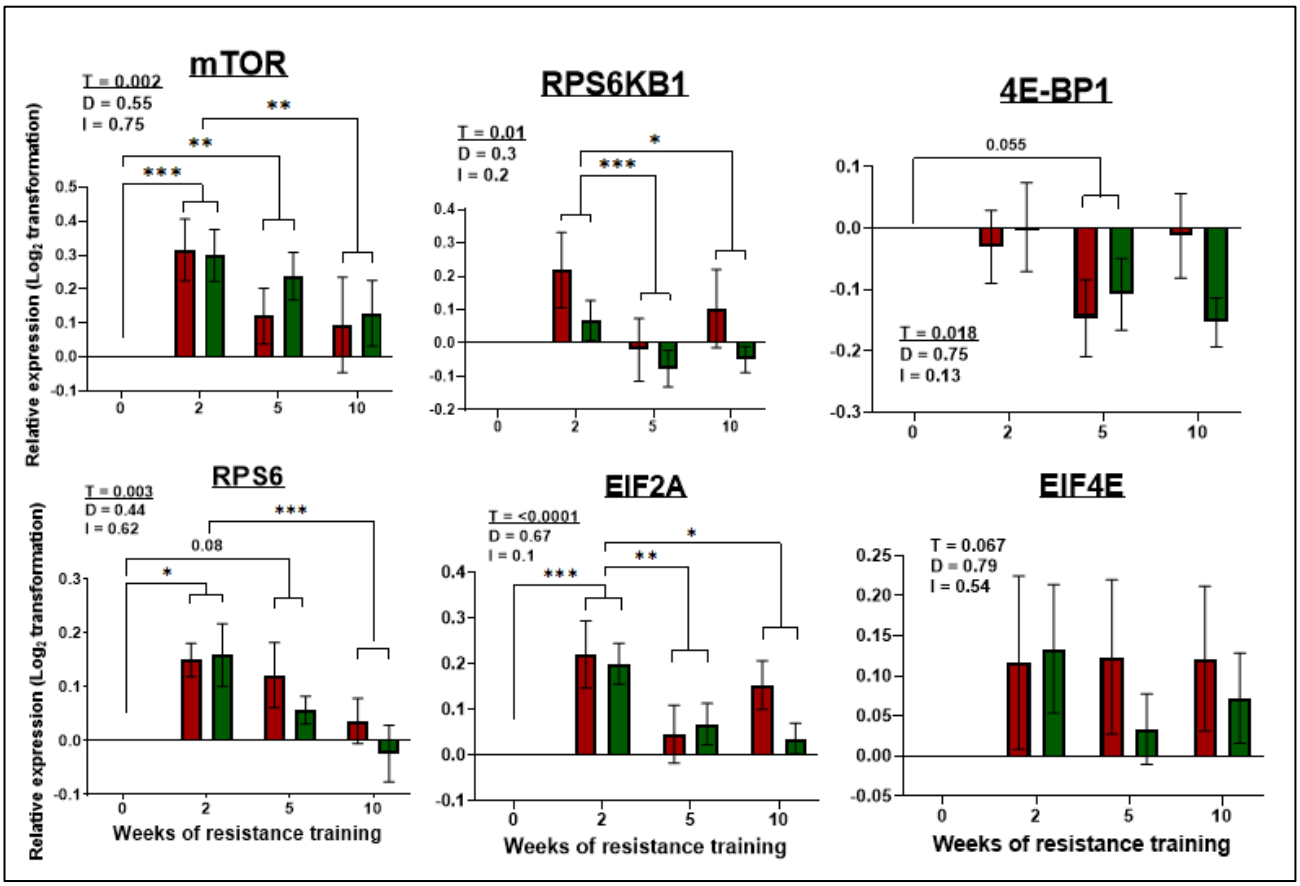


**Figure 4-17.** This figure displays the temporal response of mTOR, RPS6KB1, 4E-BP1, RPS6, EIF2A and EIF4E during 10 weeks of resistance training. Values are mean ± SEM. Red bars, OMN group. Green bars, VEG group. T, time effect, D, diet effect, I, interaction effect. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$

**Table 4-4.** This table outlines the mRNA expression results for genes not described in the full text.

| | Gene | Week 0 | Week 2 | | Week 5 | | Week 10 | | Time | Diet | Interaction |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | OMN | VEG | OMN | VEG | OMN | VEG | | | |
| **Ribosomal proteins** | RPL3 | 0 | 0.29±0.07 | | 0.14±0.06 | | 0.21±0.06 | | **0.0003** | 0.75 | 0.75 |
| | RPL12 | 0 | 0.196±0.03*** | | 0.015±0.03$$$$ | | 0.049±0.036* | | **<0.0001** | 0.54 | 0.09 |
| | RPS16 | 0 | 0.05±0.07 | -0.01±0.1 | -0.12±0.05 | -0.01±0.1 | -0.01±0.04 | -0.01 ±0.04 | 0.23 | 0.92 | 0.23 |
| | RPS19 | 0 | 0.05±0.04 | | -0.1±0.02**$$ | | -0.17±0.03****$$$$ | | **<0.0001** | 0.67 | 0.35 |
| | RPS4X | 0 | -0.1±0.05 | 0.02±0.05 | -0.08±0.04 | -0.1±0.03* | 0.01±0.05 | -0.08 ±0.03 | 0.101 | 0.99 | **0.03** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **rDNA transcription** | POl1RB | 0 | 0.14* | 0.13*** | 0.07 | **0.0004** | 0.5 | 0.2 |
| | POL1RC | 0 | 0.29** | 0.229** | 0.17$ | **0.0001** | 0.9 | 0.66 |
| | POl1RE | 0 | 0.023 | -0.024 | -0.13*$$$ | **0.0098** | 0.86 | 0.89 |
| | UBTF | 0 | 0.047 | 0.08 | -0.09 | **0.029** | 0.74 | 0.82 |
| | TAF1D | 0 | 0.22±0.07* | 0.11±0.05$ | 0.086±0.07$ | **0.007** | 0.296 | 0.22 |
| | TBP | 0 | 0.02±0.04 0.06±0.05 | 0.05±0.034 0.016±0.05 | 0.11±0.04 0.029±0.05 | 0.25 | 0.67 | 0.39 |
| | MYC | 0 | -0.13±0.1 | -0.014±0.08 | 0.25±0.05***$ | **0.0083** | 0.68 | 0.92 |
| | TRRAP | 0 | 0.12±0.03** | 0.05 ± 0.03 | 0.16 ± 0.03*** | **0.0008** | 0.25 | 0.29 |
| | TRIM24 | 0 | -0.22±0.23 -0.08 ±0.19 | 0.08±0.06 -0.04±0.02 | 0.099±0.086 -0.02±0.03 | 0.23 | 0.78 | 0.48 |
| | NCL | 0 | 0.23±0.046** | 0.13±0.04$ | 0.059±0.04$$$ | **<0.0001** | 0.29 | 0.28 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **rRNA processing** | NPM1 | 0 | 0.22±0.05** | 0.15±0.04* | 0.12±0.05 | **0.0002** | 0.36 | 0.46 |
| | BOP1 | 0 | 0.09±0.05 | -0.002±0.028 | -0.004±0.03 | 0.0539 | 0.67 | 0.35 |
| | NOP56 | 0 | 0.18±0.04** | 0.22±0.05** | 0.12±0.04 | **0.0033** | 0.13 | 0.6 |
| | FBL | 0 | 0.17±0.04* | 0.10±0.04 | 0.04±0.03$^{\$}$ | **0.0005** | 0.75 | 0.88 |
| **ERK 1/2 pathway** | MAPK3 | 0 | 0.03±0.05 | -0.05±0.04 | -0.08±0.03 | **0.048** | 0.85 | 0.83 |
| | MKNK1 | 0 | 0.11±0.03** | 0.08±0.02 ** | 0.09±0.03 | **0.01** | 0.75 | 0.95 |
| | CDK4 | 0 | 0.198±0.04*** | 0.03±0.03$^{\$\$}$ | 0.058±0.03$^{\$}$ | **<0.0001** | 0.77 | 0.9 |
| | CCND1 | 0 | 0.28±0.05*** | 0.19±0.05** | 0.23±0.1 | 0.0501 | 0.75 | 0.21 |
| | RPS6KA1 | 0 | 0.22±0.05** | 0.24±0.08 | 0.18±0.11 | 0.11 | 0.7 | 0.29 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **mTOR pathway** | MTOR | 0 | 0.3±0.05*** | | 0.18±0.05** | | 0.11±0.08$^{\$\$}$ | **0.0023** | 0.55 | 0.75 |
| | RPS6KB1 | 0 | 0.14±0.06 | | -0.05±0.05$^{\$\$\$}$ | | 0.02±0.06$^{\$}$ | **0.01** | 0.34 | 0.22 |
| | RPS6 | 0 | 0.15±0.03** | | 0.088±0.03 | | 0.005±0.03$^{\$\$\$}$ | **0.0034** | 0.44 | 0.61 |
| | 4E-BP1 | 0 | -0.01±0.05 | | -0.13±0.04 | | -0.088±0.04 | **0.0178** | 0.75 | 0.13 |
| | EIF2A | 0 | 0.21±0.04*** | | 0.06±0.04$^{\$\$}$ | | 0.09±0.03$^{\$}$ | **<0.0001** | 0.67 | 0.14 |
| | EIF4E | 0 | 0.11±0.1 | 0.13±0.08 | 0.12±0.08 | 0.03±0.04 | 0.12±0.09 | 0.07±0.056 | 0.067 | 0.79 | 0.54 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Growth factors** | IRS-1 | 0 | 0.039±0.05 | | -0.19±0.06*$$ | | -0.15±0.05 | | **0.0006** | 0.99 | 0.08 |
| | IGF-1 | 0 | 0.18±0.05* | | 0.06±0.05 | | 0.08±0.07 | | **0.0299** | 0.95 | 0.43 |
| | MSTN | 0 | -0.007±0.08 | | -0.22±0.07*$$ | | -0.11±0.06 | | **0.006** | 0.53 | 0.17 |
| **Protein degradation** | TRIM63 | 0 | 0.037±0.07 | 0.07±0.06 | -0.09±0.03 | -0.29±0.18 | -0.057±0.07 | -0.19±0.06 | 0.0599 | 0.29 | 0.45 |
| | TRIM32 | 0 | 0.05±0.05 | | 0.19±0.06* | | 0.098±0.06 | | **0.0052** | 0.56 | 0.3 |
| | FBXO32 | 0 | -0.12±0.1 | -0.09±0.1 | -0.05±0.04 | -0.1±0.05 | 0.045±0.06 | -0.19±0.08 | 0.26 | 0.21 | 0.06 |
| | NFKB1 | 0 | 0.12±0.07 | -0.01±0.08 | -0.03±0.04 | 0.03±0.07 | -0.04±0.06 | -0.06±0.06 | 0.23 | 0.74 | 0.29 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Amino acid transporters** | SLC7A5 | 0 | 0.07±0.06 | 0.2±0.05 | -0.04 ±0.04* | 0.058±0.06 | 0.078±0.08 | -0.04 ±0.01$^{\$\$}$ | **0.007** | 0.4997 | **0.02** |
| | SLC38A2 | 0 | 0.10±0.1 | 0.02±0.06 | 0.03±0.04 | -0.01±0.08 | 0.07±0.09 | -0.07±0.05 | 0.45 | 0.42 | 0.38 |
| | SLC7A1 | 0 | -0.03±0.06 | -0.01±0.2 | 0.06±0.05 | -0.03±0.04 | 0.18±0.086 | 0.17±0.15 | 0.12 | 0.8 | 0.9 |
| | SLC36A1 | 0 | 0.13±0.04* | | 0.11±0.05 | | 0.06±0.05 | | <u>0.097</u> | 0.56 | 0.96 |
| | ATF4 | 0 | 0.07±0.04 | | -0.07±0.04 | | -0.05±0.04 | | **0.0187** | 0.4 | 0.4 |
| | LARS | 0 | 0.02±0.05 | | -0.038±0.046 | | -0.12±0.06$^{\$\#}$ | | <u>0.0955</u> | 0.60 | 0.30 |

Data is presented as mean ± SEM and units are relative expression ($log_2$ transformed). If a time effect was found, the mean ± SEM presented when VEG and OMN are collated together. Numbers in bold represent a significant effect. Numbers underlined represent a trend for an effect or a forced post hoc test. *, significant difference from week 0. $^{\$}$ = significant difference from week 3. $^{\#}$, significant difference from week 6. * or $^{\$}$ or $^{\#}$, $P < 0.05$. ** or $^{\$\$}$ or $^{\#\#}$, $P < 0.01$. *** or $^{\$\$\$}$ or $^{\#\#\#}$, $P < 0.001$. **** or $^{\$\$\$\$}$ or $^{\#\#\#\#}$, $P < 0.0001$

## CHAPTER 5

# GENERAL DISCUSSION

## A summary of the thesis aims and primary findings

The aims of the present thesis were to firstly provide a temporal transcriptional regulation of many different areas of ribosome biogenesis after 3 days and several weeks of resistance training with high protein consumption. Second, investigate whether the type of protein consumed (animal or non-animal derived) differentially impact the transcriptional regulation of ribosome biogenesis. Prior to this, there were no studies investigating the temporal molecular regulation of ribosome biogenesis during a prolonged resistance training program. Furthermore, no other study has investigated every area of ribosome biogenesis from transcriptional related signalling to mature rRNA transcripts. Additionally, it was unclear whether the type of protein consumed (animal or non-animal derived) would impact ribosome biogenesis.

As a result, the first study aimed to characterise the acute regulation of ribosome biogenesis after 3 days of high protein animal or non-animal derived consumption. It was found that 3 days of resistance training and protein consumption produced a robust stimulation of every stage of ribosome biogenesis including ribosomal proteins, rRNA transcripts and rDNA transcription. However, this did not lead to appreciable increases indirect measures of translational capacity. Lastly, the consumption of a primarily animal or non-animal derived diet did not have a significant impact on the transcriptional regulation of ribosome biogenesis.

To understand how daily regulation of translational capacity translates to weekly regulation, the second study aimed to characterise the weekly transcriptional regulation and concentration of the ribosome, through temporal muscle biopsies, during a 10 week resistance training program. Additionally, the impact of consuming

a primarily animal or non-animal derived diet was investigated. It was found that, again, there was robust stimulation of every stage of ribosome biogenesis. However, through temporal measurements, it was found that the greatest 'activity' of ribosome biogenesis occurred at and around 2 weeks of resistance training. This was likely to accommodate the large degree of skeletal muscle hypertrophy reported. After this time point, the majority of ribosomal related signalling began to return to baseline values. Furthermore, the robust stimulation of every stage of ribosome biogenesis led to significant increases indirect markers of translational capacity and this response was maintained throughout the 10-week training period. Lastly, the consumption of a primarily animal or non-animal derived for 10 weeks did not have any significant impact on the transcriptional regulation and indirect abundance of translational capacity.

## Areas of future research

**Directly measuring the abundance of the 40S and 60S subunit**

Study 1 and 2 found a diverse transcriptional regulation of the 60S and 40S subunits. Specifically, in both experimental chapters, the 60S subunit was upregulated but the 40S subunit was unaltered or decreased over time. This is certainly an interesting finding. No previous study has reported this, which may be due to the lack of detailed investigation of both rRNA transcripts and ribosomal protein coded genes. Additionally, this finding was supported in two different experimental conditions which took muscle in the 'rested and 'active' state. However, to further strengthen these findings is the measurement of more ribosomal protein coded genes and also 5.8S RNA and 5S RNA, which forms part of the 60S subunit. Additionally, measuring ribosomal proteins on the protein level would further strengthen the findings of the

present thesis as this effect reported in the present thesis may only occur on the transcriptional level.

Historically, the direct measurements of ribosome subunits has been achieved via sucrose gradients in cell lines. However, until recently there has been a development in this method to measure ribosome subunits in tissue using a more efficient method when compared to sucrose gradients (Yoshikawa *et al.*, 2018, 2021). But, no work has been completed in human skeletal muscle tissue. If this mechanism of diverse regulation of the ribosome subunits exists, then certainly measuring the 60S and 40S subunit directly in human muscle tissues is required.

**Ribosome location**

It has been recently established that the location of mTOR (a primary dictator of muscle protein synthesis) provides a new perspective on its role in dictating protein synthesis (Song *et al.*, 2017; Abou Sawan *et al.*, 2018). This work should be expanded to the ribosome, to answer some fundamental questions. Where the ribosome is located within the muscle cell? How does it interact with other proteins in the mTOR pathway in response to nutrition and exercise? Does the ribosome stay in closer proximity to signalling molecules in the trained compared to the untrained state? Currently, there has only been one study investigating the location of the ribosome, but this was conducted in rat tissue and provided no context on how the ribosome interacts with other proteins (Horne and Hesketh, 1990). Additionally, the ribosome is not a single protein but made up many different proteins, therefore there is currently no staining method to directly detect the whole ribosome, as a result antibodies of the specific ribosomal proteins have to be utilized but this only provides an indirect marker

of ribosome location. In the present thesis, there was no measurement of ribosome location, therefore it is difficult to answer the abovementioned questions. As a result, future studies should aim to understand characterise the subcellular location of the ribosome and its possible role in interacting with other proteins involved in protein synthesis.

**Ribophagy**

Ribophagy is the process of ribosome degradation via autophagy. Little is known about this area and only one receptor, nuclear fragile X mental retardation-interacting protein 1 (NUFIP1) has been established as a mediator of ribophagy (Wyant *et al.*, 2018). Recently, a detailed study by Kim *et al.* (2020) showed a reduction in muscle mass and ribosomal capacity in a model of ovarian cancer. This was attributed to an increase in ribophagy via NUFIP1 and also a decrease in ribosome synthesis. Additionally, Figueiredo *et al.* (2020) showed that RNA degradation is increased in response to hind limb suspension (an animal model to induce muscle loss), however NUFIP1 expression was not measured. Presumably in a muscle hypertrophy model, there would be a decrease in ribophagy, although this has not been investigated previously and the present thesis did not measure markers of ribophagy. Muscle protein balance, just like ribosome concentration, is dictated through the balance between synthesis and breakdown. In the trained and rested state, muscle protein breakdown is elevated (Kim, Staron and Phillips, 2005), to possibly accommodate the high protein turnover. Therefore, it is possible that ribophagy may be elevated compared to basal levels in a trained state to accommodate the high degree of ribosome synthesis (Sieljacks *et al.*, 2019). However, since indirect markers of ribosome concentration are increased in the trained state (established in Chapter 4), ribophagy would not be increased to the same

extent as ribosome synthesis. Currently, ribophagy has not been investigated in a training study and requires further investigation utilizing stable isotope methodology, to measure RNA degradation rates, and measuring protein expression of NUFIP1.

**Genetic and epigenetic regulation of ribosome biogenesis**

A recent study by Figueiredo *et al*. (2021) aimed to investigate the regulation of rDNA transcription. The present study did in fact highlight the regulation of rDNA transcription but this study took it one step further and looked at the influence of rDNA gene dosage and methylation of rDNA on rDNA transcription. One key finding from the study is that the association of MYC to areas of rDNA is highly influential in rDNA transcription and further highlights the importance of MYC in ribosome biogenesis. Another interesting finding was that rDNA gene dosage was correlated with the extent of 45S rRNA expression at 24 hours post exercise. This firstly shows that 24 hours post resistance exercise displays a concentrated period of ribosome biogenesis. Secondly, the findings of rDNA gene dosage are fascinating because it may show that rDNA dosage is a genetic factor in skeletal muscle hypertrophy. However, due to the recent development of this method, the present thesis did not utilize this measurement. Therefore, it is unclear currently, if rDNA gene dosage could influence the extent of skeletal muscle hypertrophy. Due to the possibility of predicting the degree of skeletal muscle hypertrophy through rDNA gene dosage alone and the ramifications in the athletic world, this will most likely be an area of further interest.

**RNA synthesis**

RNA concentration is dictated through RNA synthesis (ribosome biogenesis) and RNA breakdown (ribophagy). The present thesis measured RNA concentration and

measured the transcriptional regulation of ribosome biogenesis, however it did not directly measure ribosome biogenesis. The introduction of a deuterium oxide tracer has allowed the measurement of total RNA synthesis, which as mentioned previously, is ~80% comprised of ribosomal RNA. Therefore, this tracer allows the option to characterise daily, weekly or monthly rates of ribosome synthesis and degradation. Since the introduction of this method by Brook *et al.* (2017), many different training studies have utilised this measure (Sieljacks *et al.*, 2019). Additionally, it has been shown repeatedly that ribosome biogenesis is elevated during a resistance exercise training program (Sieljacks *et al.*, 2019; Hammarström *et al.*, 2020), which supports the transcriptional findings of the present thesis. However, the measurement of ribosome biogenesis and the transcriptional regulation of ribosome biogenesis have not been utilized in the same study. Therefore, a similar approach to the present study with the addition of stable isotope methodology to measure ribosome biogenesis should be carried out in future studies to fully understand and appreciate the process of ribosome biogenesis.

**Protein expression**

The ability to measure the expression of many different genes through a reproducible method, such as the microfluidic cards, provides the opportunity to understand many different areas of ribosome biogenesis. This therefore prevents the need to piece different studies together to understand the 'full picture' of ribosome biogenesis, which can be difficult as studies differ in many different factors, such as instruments and population used. The work in the present thesis provides the groundwork to understand what occurs on the protein level. Therefore, protein expression data is the next step.  However, the standard measurement for protein expression, western

blotting would be difficult to measure up to 48 proteins (in order to replicate the findings of the present thesis). However, the use of an omics approach, such as proteomics, would combat this limitation. A recent study by Lin *et al*. (2021) applied a proteomic and phosphoproteomic approach in a muscle atrophy animal model. Although the condition and population cannot be compared to the present thesis it provides a novel method which can be applied to skeletal muscle hypertrophy models and should be a future direction in understanding ribosome biogenesis on the protein level.

**Different populations**

The present thesis concluded the findings in a young population, however it is unclear how the regulation of ribosomal biogenesis is impacted by other populations. A study by Brook *et al*. (2016) found that in older humans, a blunted hypertrophic effect of resistance exercise was attributed to decreases in cumulative muscle protein synthesis but most importantly ribosome biogenesis. However, it is unclear what specific area of ribosome biogenesis impacted as a detailed insight into the regulation of ribosome biogenesis was lacking. Therefore, a similar investigation from the present thesis should be applied to an older population to pin down what area of ribosome biogenesis is likely driving the age related deficits in skeletal muscle hypertrophy.

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