MOLECULAR AND METABOLIC REGULATION OF SKELETAL MUSCLE GROWTH IN CHCIKEN

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ABSTRACT

Broiler chicken has been bred for meat production and is characterised with a very fast skeletal muscle growth rate, whilst the layer type have been bred for the production of eggs. This study aimed to understand how intense breeding programmes have developed commercial meat type chickens that have resulted in a fast muscle growth phenotype by a comparative analysis of gene expression between fast-growing broiler and slow-growing layer type chicken. Two chicken trials were carried out. In Trial 1 from fast-growing broiler Ross 308 genotype (R) fast-growing breast *Pectoralis major* (RPM) and slow-growing leg *Peroneus tertius* (RPT) muscles were collected at day 14, 36 and 42 post-hatch. In Trial 2, from fast-growing broiler Ross 308 (R) or slow growing layer Hy-Line (H) *Pectoralis major* (either RPM or HPM) and Peroneus *tertius* (either RPT or HPM) were collected at day 4, 14, 23, 35 and 42 post-hatch.

In Trial 1, there was a muscle type x age interaction in muscle weights with the RPM having the highest value at day 43 ($P<0.001$). This effect was also reflected in the protein content with RPM having more protein than RPT. However, RPT had a higher DNA content per unit tissue weight (muscle type x age interaction, $P=0.003$) with highest value seen at day 43. This suggests that muscle cells of RPM are bigger than those in RPT and that as a result they contain more protein, potentially reflecting RPM hypertrophy compare to RPT. In Trial 1, genes associated with glycolysis, GAPDH and α-enolase, were significantly higher in the faster growing RPM ($P<0.05$). Genes involved in serine biosynthesis PHGDH, PSAT and PSPH, as well as P70S6K involved in protein translation, also had a significantly higher expression in the RPM when compared to the RPT ($P<0.05$) indicating a greater rate of protein synthesis in the faster growing RPM. Expression of genes associated with smaller muscles (myostatin) or protein degradation (calpastatin, the specific endogenous inhibitor of calpain proteinases) were not different between muscles. LIM domain proteins, CSRP3 and FHL2, had a higher expression in the slower growing muscle, indicating the possibility of those two genes being negative regulators of muscle growth, or may be involved in the upregulation of muscle regenerative physiological processes, as a result of the strain on the leg muscles induced by physical activities during locomotion.

In Trial 2 there was a three-way interaction between genotype, muscle type and age in muscle growth ($P<0.001$) with the highest value seen at day 42 in the RPM. There was a 3-way interaction between genotype, muscle type and age in the expression of α-enolase ($P=0.036$) with the highest value seen at day 42 in the HPM. For β-Enolase mRNA expression, there was a separate genotype and a muscle type effect (both $P<0.001$) with HPM and HPT been higher than RPM and RPT, and PM muscles having a higher expression than PT muscles in both genotypes. When the serine synthesis pathway was examined, there was a genotype x age interaction ($P=0.046$) for PSAT and PSPH gene expression, with the highest expression at day 35 in both muscles of the Ross 308 genotype for the former, whilst the latter had the highest expression in the RPM

at day 35. For ASNS mRNA expression there were significant genotype (P=0.004) and muscle type effects (P=0.014), with the Ross 308 genotype having the higher expression and the PM being greater than PT. For P70S6K mRNA expression, PM was higher than PT (P=0.012). For P70S6K total protein there was a significant muscle type x age interaction (P=0.049), RPM and HPM being higher than RPT and HPT at days 14 and 35.

For calpain activity only at day 35 was there significant genotype x isoform interaction (P<0.001), with the Hy-Line genotype having a higher micro and milli calpain activity than the Ross 308 genotypes, irrespective of muscle. For trypsin, chymotrypsin and caspase – like activities of the proteasome, there was a significant genotype effects (P<0.001). In all three assays Ross 308 muscles had a higher activity when compared muscles from Hy-Line at both time points. For the ubiquitin ligases there was a borderline muscle type x time interaction (P=0.05) in the expression of MAFbx mRNA, with the RPT and HPT having a higher expression than the RPM and HPM respectively at day 14. For MuRF1 mRNA there was a significant genotype x age interaction (P<0.001) with the HPM and HPT having a higher expression than the RPM and RPT at day 14 and 35. For CSRP3 there was a genotype x age interaction (P<0.001), with the highest expression seen in the HPT at day 42. There was also muscle type effect within genotypes with the RPT and HPT having a higher expression than the RPM and HPM (P<0.001).

Trial 2 indicated that there is an increase in protein synthesis which leads to clear increase in protein accretion in faster growing chicken muscles, thereby supporting the wealth of literature detailing protein turnover in chicken skeletal muscles. There also appears to be an interaction between protein synthesis and degradation, however in most cases protein synthesis seems to be more dynamic and these changes seem to appear around day 35. The novel findings of this study were the observed increase in the expression genes that could limit the synthetic capacity of non-essential amino acid (non-EEA) in fast growing muscles.

CONFERENCE PAPERS PRESENTED

C. OLOMU, T. Parr, J. Brameld and J. Wiseman (2014): Differential gene expression of LIM domain proteins in two diverget growing muscles of broiler chicken (Ross 308 Genotype). Oral presentation at the proceedings of the British Socety of Animal Science (BSAS) in association with AVTRW, CFER and EBLEX. Annual Conference, held in Nottingham, UK.

C. OLOMU, T. Parr, J. Brameld and J. Wiseman (2015): Differential gene expression of glycolytic metabolic proteins in two different growing muscles of broiler chicken. Oral presentation at the proceeding of the Nutrition Society. Leicester England.

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4E-BP1	4E-binding protein 1
AAR	Amino Acid Response
ADP	Aenosine diphosphate
AEBSF	2-(4-aminoethyl)-benzenesulphonyl fluoride
α–KG	Ketoglutarate
AKT/PKB	Protein kinase B
AMP	Adenosine monophosphate
ANOVA	Analysis of Variance
ASNS	Asparagine synthetase
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BW	Body weight
C/EBP	Ccaat-enhancer binding protein
CALP	Calpastatin
cAMP	Cyclic adenosine monophosphate
CARE	C/EBP-ATF response elements
cDNA	Complimentary deoxyribonucleic acid
CO2	Carbon dioxide
CP	Crossing point
CSRP3	Cysteine and glycine-rich protein 3
DEFRA	Department for Environment Food and Rural Affairs
DEFRA	Department for Environment Food and Rural Affairs
DNA	Deoxyrionucleiic acid
Dnase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EEA	Essential amino acid
EIF2a	Eukaryotic Initiation Factor 2 α
ER	Enoplasmic reticulum
FGF2R	fibroblast growth factor receptor
FHL2	Four and a half LIM domains protein 2
FKHR	Fox ead proteins
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN2	General control non-depressible 2
GLDC	glycine dehydrogenase
GLUT	Glucose transporter protein
GSK	Glycogen synthase kinase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPM	Hy-Line Pectoralis major
HPT	Hy-Line Peroneus tertius
HRP	Horse raddish peroxidase

IGF1	insulin-like growth factor
IGF1R	insulin-like growth factor 1 receptor
IGF1R	insulin-like growth factor receptor
IR	insulin receptor
ISR	Integrated stres response
Kda	Kilodalton
Kna	Potasium tartate
KO	Knock out
MAFbx	muscle atrophy F-box
mRNA	Messanger Ribonucleic Acid
mTOR	mammalian target of rapamycin
MTSN	Myostatin
MuRF1	Muscle RING finger protein 1
MUSTN1	musculoskeletal embryonic nuclear protein 1
MW	Muscle weight
MyHC	myosin heavy chain
MYOG	myogenin
NAD	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NCBI	National Centre for Biotechnology Information
NRC	National Research Council
NSRE	nutrient sensing response elements
P70S6K	Ribosomal protein S6 kinase beta-1
PCA	pechloric acid
PCR	Polymerase chain reaction
PEPCK	Phosphophenol carboxykinase
PGC-1	Peroxisome proliferator-activated receptor
PHGDH	phosphoglycerate dehydrogenase
PIP	Phosphatidylinositol 4-phosphate
PKA	Protein kinase A
PKM	Pyruvate kinase muscle type
PM	Pectoralis maor
PPP	Pentose phosphate pathway
PSAT	Phosphoserine aminotransferase
PSPH	Phosphoserine Phosphatase
PT	Peroneus tertius
Q-RT-RCR	Quantitative reverse transcriptase polymerase chain reaction
RJF	Red jungle fowl
RPM	Ross 308 Pectoralis major
rps6	Ribosomal protein S6
RPT	Ross 308 Peroneus tertius
SDS	Sodium dodecyl sulphate
SH2	Src homology 2

SHM	serine hydroxy methyltransferase
SMTH	serine hydroxy methyltransferase
SNP	Single nucleotid polymorphism
SR	Saroplasmic reticulmu
SSC	Saline sodium citrate
Suc-LLVY	Succinyl-leucine-leucine-valine-tyrosine
TBST	Tris buffer saline Tween
TCA	Tricarboxylic acid cycle
THF	tetrahydrofolate
tRNA	Transfer Ribonucleic acid
uORF	upstream open reading frames
UPS	Ubiquitin proteasome system
Z-nLPnLD	Z-norleucine-proline-norleucine-aspartate

CHAPTER 1. (GENERAL INTRODUCTION)

1.0 INTRODUCTION

The disparity in growth between broiler and layer type chickens are evident in the former having a more rapid growth rate phenotype and a lower feed to muscle conversion ratio. This has resulted from many generations of selective breeding programs aimed at achieving these traits. As an unintended consequence these selection programmes have led to the modification of metabolic systems and expression of some genes that modulate skeletal muscle protein turnover. Selective breeding has also led to a broiler chicken phenotype characterised by a higher proportion of breast muscle relative to body weight, at the expense of the leg muscle (Al-Musawi *et* al., 2011). Furthermore, due to this long term intensive breeding programs, broiler chickens appear to have almost reached their maximum growth potential by genetic selection. Consequently, there is probably little that could be done to improve their growth efficiency through classical and conventional breeding programmes (Author and Albers 2003). Broiler chicken can therefore be used a model to elucidate and develop a better understanding of how skeletal muscle growth is regulated in humans and non-human animals. According to Zheng *et al*., (2009), the broiler (chicken for meat production) and layer (chicken for egg production) chickens are ideal model systems to study the controlling mechanisms of myogenesis rate and muscle cell size. During the past 80 years, genetic selection has been concentrated on high growth velocity and large muscle mass for broilers; in contrast, layers have been selected for egg production. Therefore, even under optimal growth conditions, the body size of layers is still much smaller than that of broilers due to their intrinsic genetic differences. Recent studies have reported an over 400% increase in broiler growth rate from 1957-2005 with a concomitant increase in *Pectoralis major* yield of 79% in males and 85% in females as a result of intense selective breeding (Zuidhof *et al*., 2014). These factors also promote a more rapid rate of muscle growth and protein turnover in broilers when compared to layers, which, in contrast, have been genetically selected for high reproductive capacity in terms of numbers of eggs laid with no attention paid to muscle accretion resulting in a much slower comparative skeletal muscle growth rate. Such unique biological feature of broilers and layers allows us to investigate many interesting questions related to muscle growth control. For example, what makes chicken skeletal muscle fibres grow two or three times faster in broilers than in layers? What is the molecular and metabolic basis for such growth difference?

One way to accomplish this is by comparatively analysing the expression of growth promoting genes within these genetically close and broiler and layer chicken, with the aim of identifying genes that can potentially

enhance growth rate and protein turnover. Although breeding programmes in chickens have been able to accomplish a phenotype of meat animals with a very fast muscle growth rate, the underlying molecular mechanisms by which this phenotype has been accomplished is not well understood. Furthermore, an appreciation of the interaction of genes associated with muscle growth points to the right direction in elucidating the mechanism and mode of action of these genes and how they induce some physiological and metabolic processes that promote growth responses in skeletal muscle. This also has the potential of developing molecular targets for manipulation. The aim of this research work was to further elucidate metabolic and molecular mechanisms modulating of muscle growth in chicken. This, in turn, could lead to the identification of molecular targets for manipulation in order to improve animal production and also develop therapies to combat muscle wasting conditions in both humans and animals.

One of the mechanisms by which skeletal muscle growth occurs is as a result of protein turnover which is the balance between protein synthesis and degradation. More synthesis than breakdown indicates an anabolic state that builds lean tissues, more breakdown than synthesis indicates a catabolic state that degrades lean tissues. Therefore, skeletal muscle growth occurs as a result of a positive overall net accumulation of muscle proteins. Having a better appreciation of the mechanisms regulating skeletal muscle growth through skeletal muscle protein turnover is therefore essential. Proteolytic systems are conventionally associated with muscle atrophy. However, in this study our observations seem somewhat contrary to the norm suggesting an alternative mechanistic role for proteolytic systems in chicken muscles which involves the redirection of muscle degraded small peptides and amino acids to protein synthesis systems for the facilitation of muscle growth. However, in atrophy conditions, an abnormally high activation of the calpain proteolytic systems has been established (Tidball and Spencer 2002; Spencer and Mellgren 2002; Williams *et al*., 1999; Fischer *et al*., 2001), as well as the increased expression of Ubiquitin proteasome system (UPS) constituents, including components of the 26S proteasome itself (Baily *et al*., 1996; Price *et al*., 1996; Tawa Jr *et al*.,1997). This gives credence to the established notion that there is a dynamic adaptation of skeletal muscle to changing requirements. In this study the animals were healthy and were not undergoing any form of muscle wasting or atrophy but had variable genetic and metabolic requirement for muscle protein accretion under normal growth conditions. Thus, our observation gives an insight into how proteolytic systems modulate skeletal muscle growth under normal physiological conditions.

It is also important to understand metabolic mechanisms that specifically modulate protein synthesis. These are made up of a group of systems that interact with each other to regulate skeletal muscle protein turnover and muscle growth. This is where the measurement of the expression of genes catalysing the synthesis of glycolytic intermediates such as Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase as well as *de novo* biosynthesis of glycolytic metabolites comes into play. Among these metabolites include non-essential amino acids serine and glycine as well as TCA derived synthesis of asparagine. Cell growth and

proliferation requires the production of building blocks for the synthesis of cell constituents which include protein, nucleic acids and lipids. Serine and glycine are biosynthetically linked and together provide the precursors for the synthesis of these biomolecules (Cooke *et al*.,2011; Amelio *et al*., 2014; Ye *et* al.,2012). Serine biosynthesis has also been reported to be upregulated during hyperplastic growth as seen in cancer cells (Possemato *et al*., 2011; Dann and Abraham 2011). However, the interaction between serine/glycine biosynthesis and cell proliferation is not fully understood. There have been no previous reports on the relationship between serine/glycine biosynthesis and muscle hypertrophy in chicken, therefore one of the objectives of this research was to determine if one existed. We therefore measured the expression the three enzymatic proteins that catalyse the three step *de novo* biosynthesis of serine namely phosphoglycerate dehydrogenase (PHGDH), phosphoserine amino transferase (PSAT) and phosphoserine phosphatase (PSPH). Another post glycolytic/TCA cycle metabolite of interest is the non-essential amino acid asparagine, which is synthesised from the transamination of glutamine to asparagine, by the amino transferase enzyme asparagine synthetase (ASNS). Evidence has also shown that asparagine plays an important role in many physiological and biological processes. Also, the gene and protein expression of ASNS is induced following amino acid deprivation (Siu *et al*., 2002). While in cancer cells, it is thought to regulate cellular amino acid homeostasis as ASNS expression is upregulated in response to amino acid shortfalls Zhang *et al*., (2014). One of the objectives in this study was to measure the differential expression of ASNS in divergent growing muscles.

Muscle protein synthesis also occurs as a result of muscle hypertrophy which is an increase in muscle fibre diameter due to the addition of more myofibrils, composed of contracting actin and myosin protein filaments to individual fibre bundles resulting in larger muscle motor unit sizes. In avian species, myofibre/myoblast number is established post embryogenesis and it is generally thought that the hypertrophy of muscle takes place by the accretion of protein and nuclei originating from the proliferation and fusion of satellite cells (Scheuermann *et al*., 2004). It has been reported that broilers compared to layers had a larger diameter type I and II myofibers and greater apparent myofiber number; the proportion of type II red myofibers decreased and that of type II fast twitch white myofibers increased during growth in the *sartorius* muscle when compared to layers (Aberle and Stewart 1983). It has also been reported that quantitative analysis of fibre type composition demonstrates that, in both broilers and layers, the *Pectoralis major* (breast muscle) is composed of more than 99% of glycolytic type IIB fibres while the *Bicep femoris* (leg) had a more heterogenous fibre type distribution with a larger population of oxidative type I fibres, with the minimum fibre diameter being larger in both muscle types of broilers when compared to layers (Cooke *et al*., 2003). Type II fibres have an upregulated glycolytic metabolic capacity when compared to type I slow oxidative fibres, due to the former's ability to generate ATP anaerobically. A comparative measure of the expression of some glycolytic genes to indicate the glycolytic capacity of the fast and slow growing muscle types between and within chicken genotypes, is therefore

essential to elucidate not only the metabolic properties in these muscles but to have an idea of how these metabolic properties may be associated with fibre type.

Skeletal muscle growth is a complicated and complex process involving several mechanisms interacting with each other physiologically and metabolically. The hypothesis in this thesis is that molecular and metabolic mechanisms known to upregulate muscle growth are modulated in such a way that protein synthesis is upregulated resulting in positive protein turnover in faster growing chicken muscles. And these biological systems are driven by the expression of some key genes that accentuate muscle growth in these faster growing chicken muscles**.** The aim of the current study was to elucidate the mechanisms that accentuates skeletal muscle growth at the molecular level using chickens as a model by investigating various biological processes that have been identified as regulators of muscle and cellular growth. This was done by investigating metabolic systems that modulate protein turnover, signalling pathways that modulate muscle protein synthesis, and myogenic genes that modulate myogenesis and interact with myogenic factors. The study also sought to investigate how changes in skeletal muscle growth pattern in differential growing genotypes and muscle types of chickens interact with proteolytic, synthesis and metabolic systems as well as the expression of some key genes involved in the modulation of these processes to regulate skeletal muscle growth.

CHAPTER 2. (LITERATURE REVIEW)

2.1 DIVERGENT SKELETAL MUSCLE GROWTH RATES IN DIFFERENT CHICKEN GENOTYPES AS A RESULT OF SELECTIVE BREEDING FOR DOMESTICATION.

There is a dynamic between chicken breeding and chicken genetics with the later providing the biological foundation for the former. Chickens have been selectively bred for various reasons ranging from religious, ceremonial, sports and food (Siegel 2006). In 2004, a draft genome sequence of the chicken was established by sequencing a single female red jungle fowl, the wild ancestor of chickens, and this genome was compared with that of a broiler, bred for meat production bred for a fast growth rate, a layer bred for egg production characterised with a slow growth rate and silkie genotype (Wong *et al*., 2004, Figure 2.1).

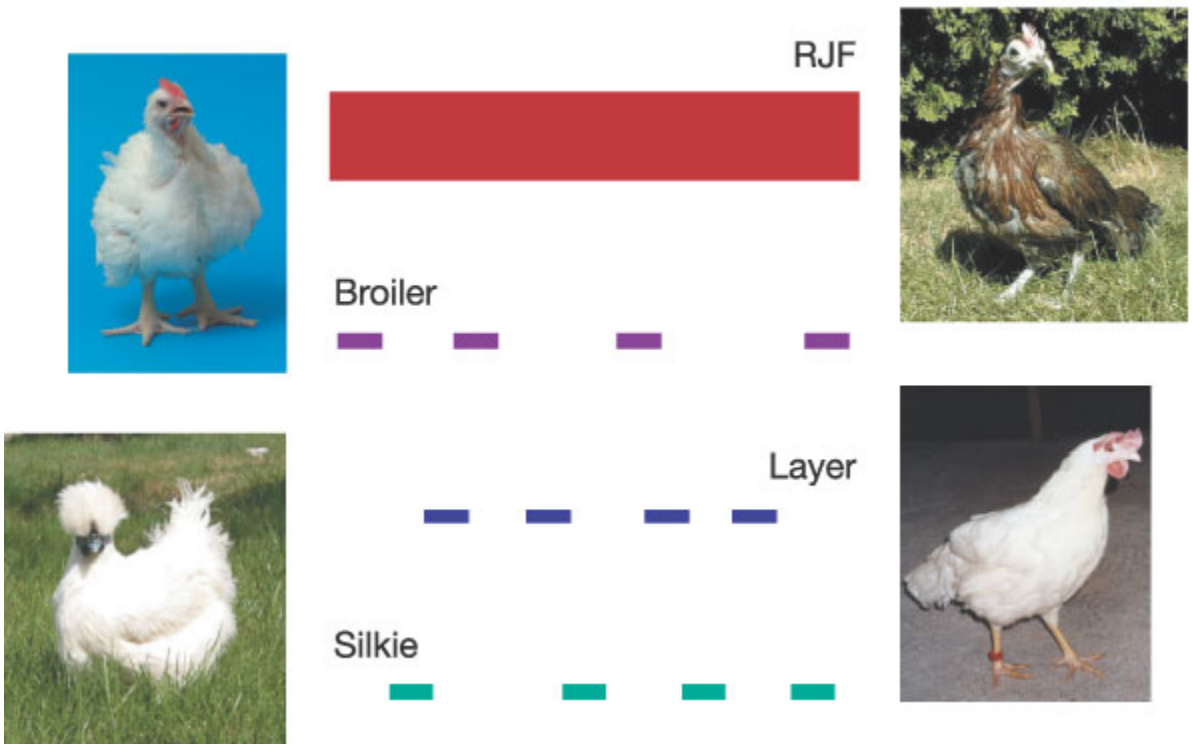


Figure 2.1: Four different type of chicken genome comparatively sequenced to identify single nucleotide polymorphisms (SNPs) which include a red jungle fowl (RJF top right), Broiler (top left), Layer (bottom left) and Silkie (bottom right) chicken photographs are provided by B. Payne (red jungle fowl), P. M. Hocking (broiler), L. Andersson (layer) and N. Yang (silkie). Source: International Chicken Polymorphism Map Consortium *Nature* 432, 717-722(9 December 2004).

This study opened the door to address important biological questions that were left unanswered which include the underlying molecular mechanisms resulting in divergent growth rates in the various muscles of different

chicken genotypes as a result of their induced evolution via selective breeding. Which is the focus of this current study.

Selective breeding of chicken has been going on for decades, but the practice was intensified post World War 2, due to a need to increase the intensity of food production. Prior to that, chickens were eaten as by-products after completing their egg production cycle. Flock sizes grew from a cockerel and few hens to some flocks with 10,000 or more chickens, but it wasn't until the 1950s and 60s when vertical integration of the broiler industry occurred and chicken factories with hundreds of thousands of birds appeared (Wiehoff 2013).

Various studies have reported data and evidence of divergent muscle growth resulting in chicken selective breeding for muscle/meat production. In a study by Zuidhof *et al*., (2014) which sought out to determine the effect of commercial selection on the growth, efficiency, and yield of broilers using two meat control strains, unselected since 1957 and 1978, and a commercial Ross 308 strain (Figure 2.2). They reported that from 1957 to 2005, broiler growth increased by over 400%, with a concurrent 50% reduction in feed conversion ratio, corresponding to a compound annual rate of increase in 42-day live body weight of 3.30% and over the same period the feed conversion ratio decreased by 2.55% each year.

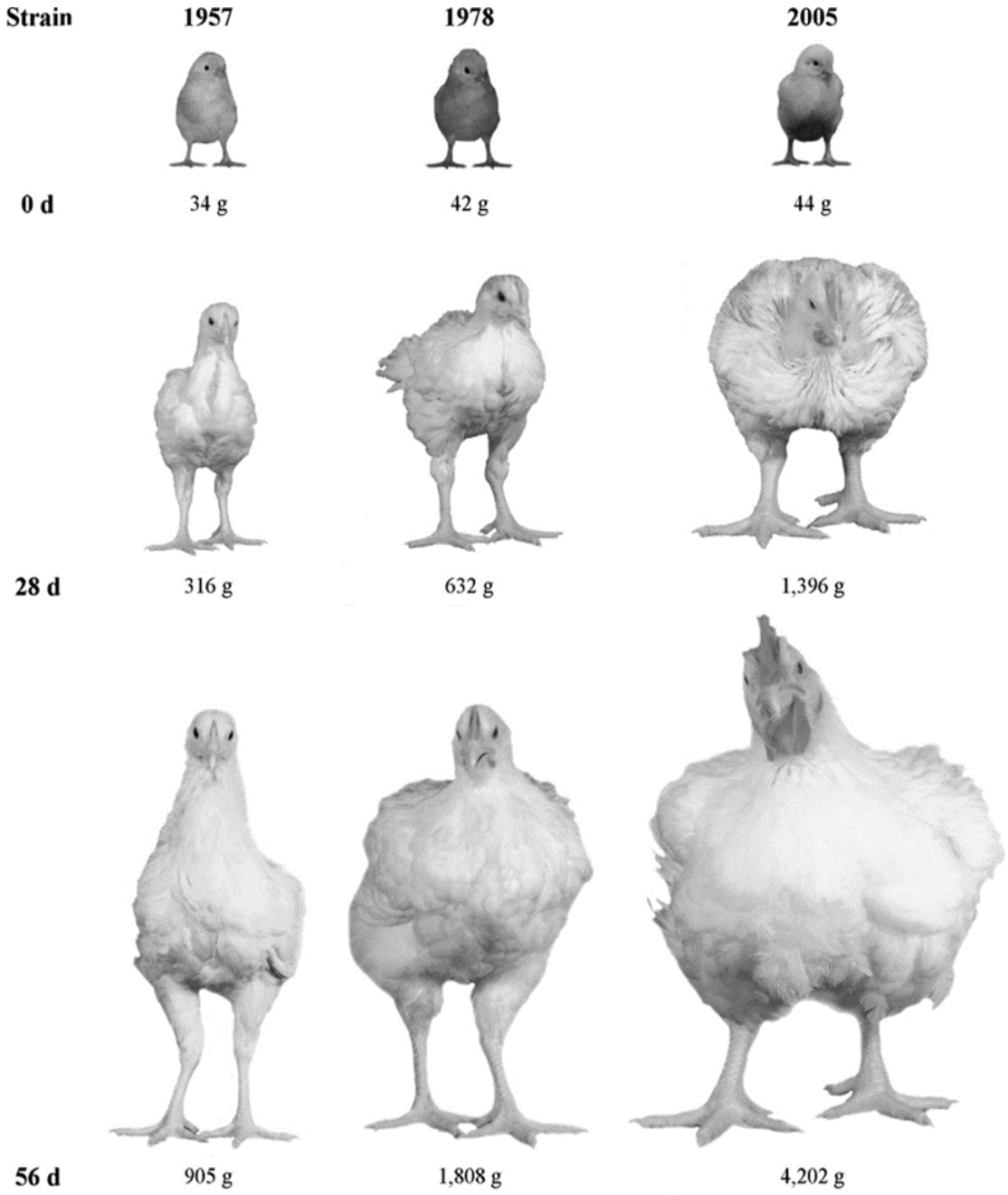


Figure 2.2: Age-related changes in size (mixed-sex body weight (BW) and front view photos) of University of Alberta Meat Control strains unselected since 1957 and 1978, and Ross 308 broilers (2005). Within each strain, images are of the same bird at 0, 28, and 56 d of age. The larger birds shown on the right side are ROSS 308 2005 strains. The smaller chickens on the left show ROSS 308 1957 strains. While the middle birds of intermediate growth in the image are 1978 ROSS 308 strains (Taken from Zuidoff *et al*., 2014).

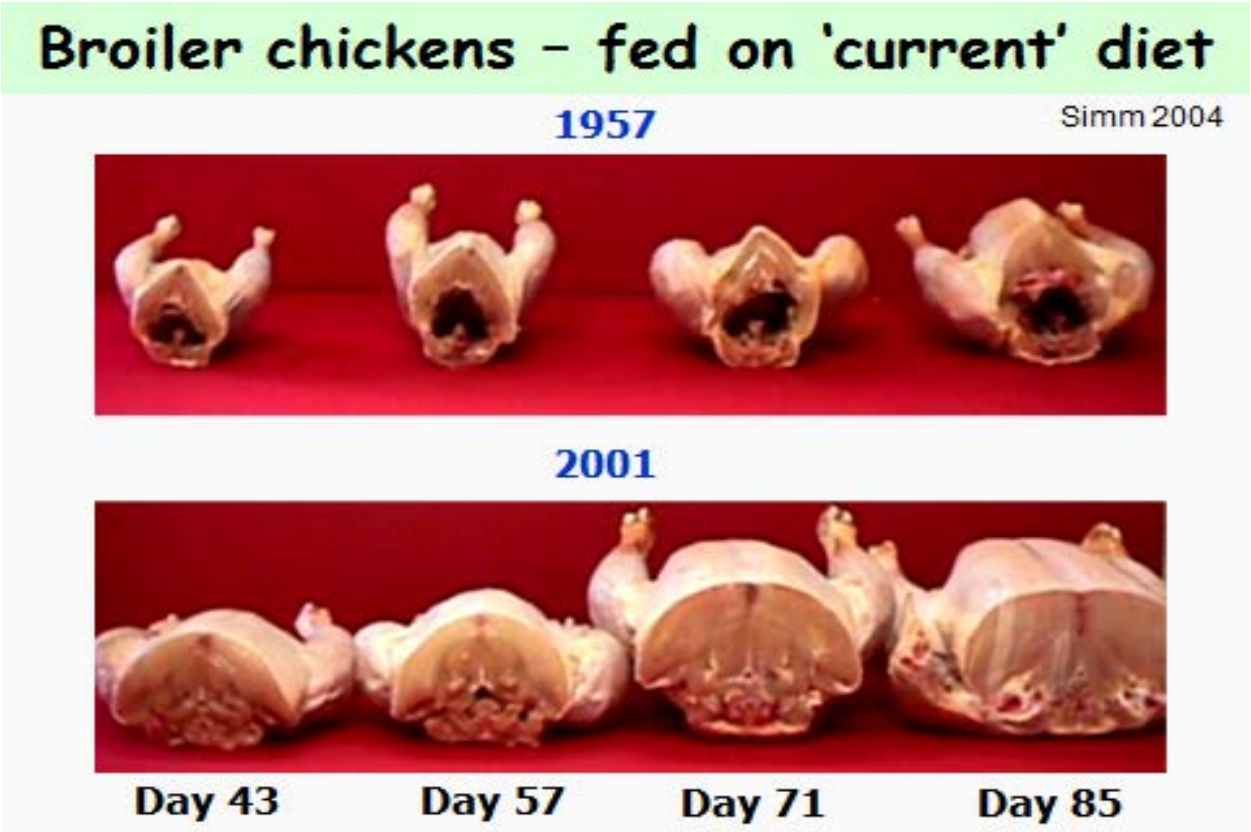


Figure 2.3: Effect of selective breeding on different broiler muscles (Simm 2004)

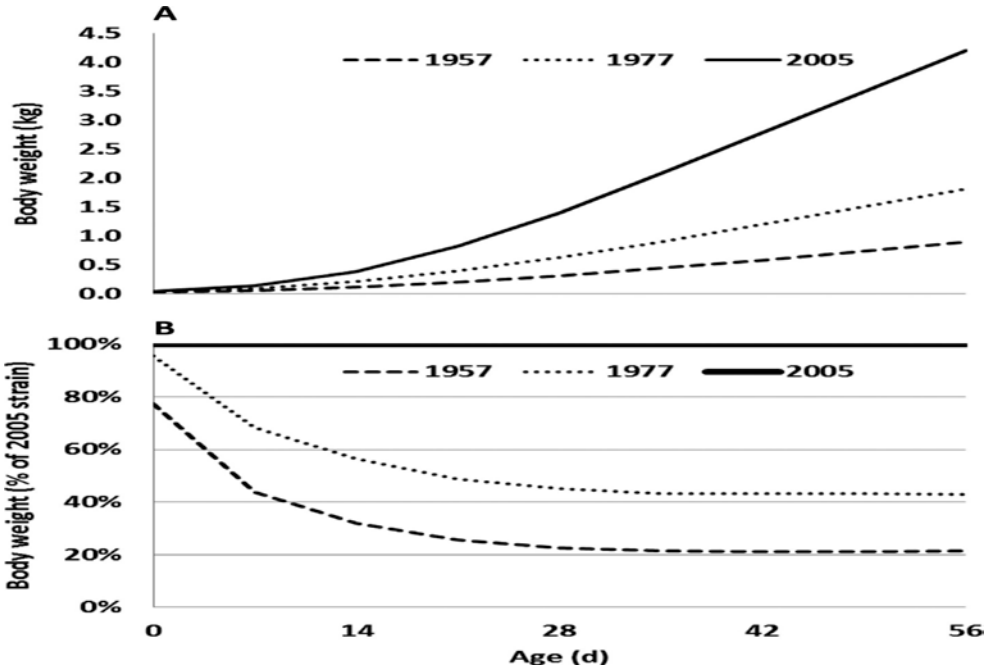


Figure 2.4: Absolute (panel A) and relative (panel B) BW of mixed sex University of Alberta Meat Control unselected since 1957 and 1978, and Ross 308 broilers 2005. (Taken from Zuidoff *et al*., 2014).

Pectoralis major growth potential increased, whereas abdominal fat decreased due to genetic selection pressure over the same time period. From 1957 to 2005, *pectoralis minor* yield at 42 d of age was 30% higher

in males and 37% higher in females; *pectoralis major* yield increased by 79% in males and 85% in females. A similar report has also been documented by Simm (2004) (see Figure 2.3). This work carried out clearly demonstrates the divergence of total body and muscle type growth rate in different types of chickens.

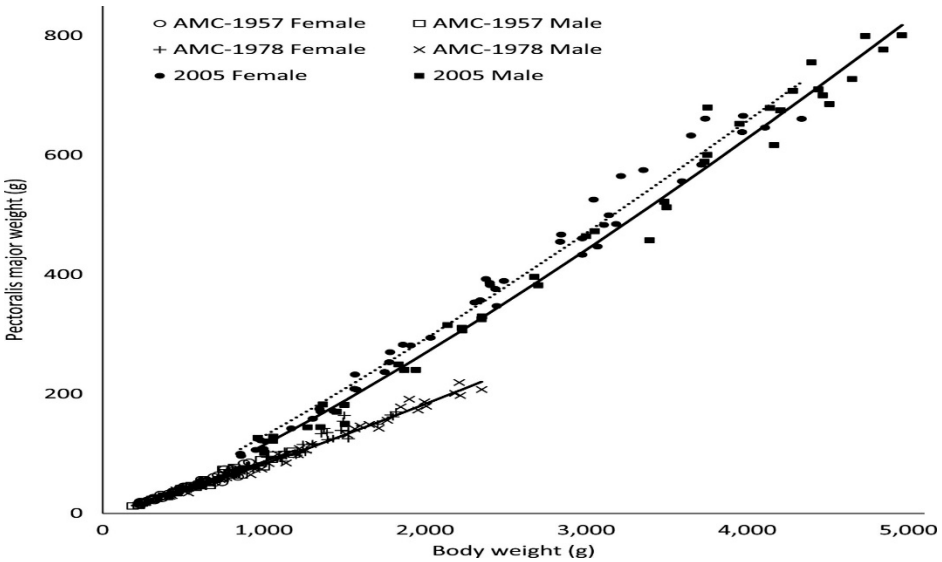


Figure 2.5: Allometric yield curves for pectoralis major from University of Alberta Meat Control (AMC) unselected since 1957 and 1978, and Ross 308 (2005) broiler males (–) and females (···). (Taken from Zuidoff *et al*., 2014).

2.2 PHYSIOLOGY OF MUSCLE GROWTH

Skeletal muscle is a collection of multinucleated muscle cells or fibres called myocytes formed by a process called myogenesis (Bentzinger *et al.*, 2012). The process involves progenitor muscle cells that undergo several rounds of proliferation to form myoblasts. They then undergo a process whereby they fuse together to form multinucleated myotubes, which then mature to form myocytes or muscle fibres. These processes are modulated by myogenic regulatory factors (Figure 2.6). This process is also activated during the repair of damaged muscle tissues where satellite cells are activated, proliferate and then fuse to existing muscle fibres.

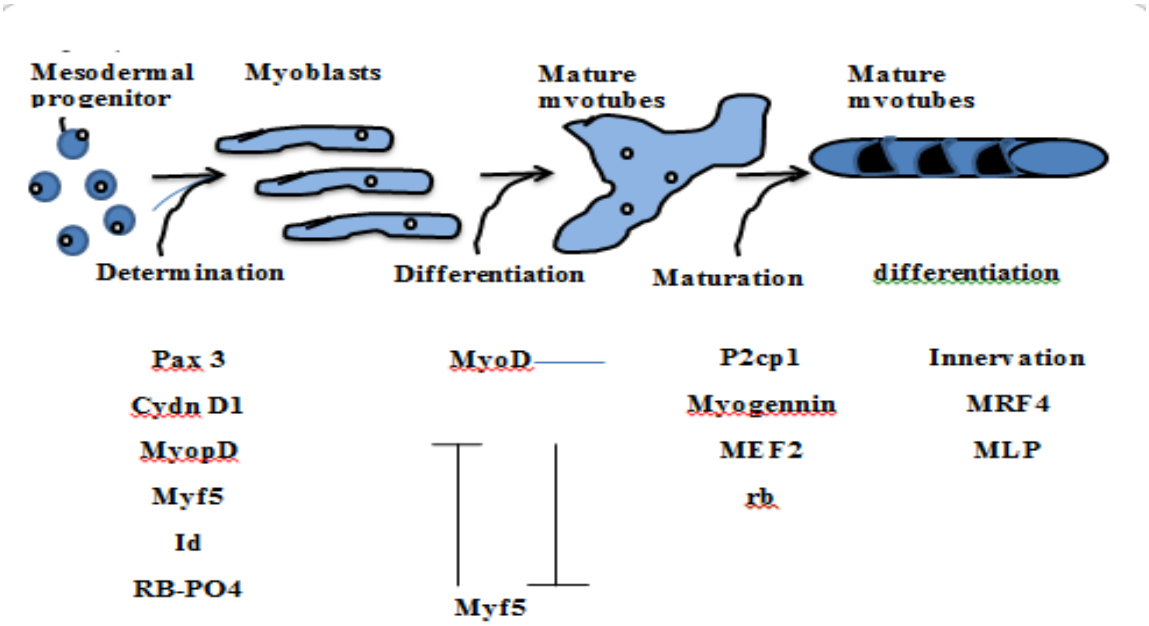


Figure 2.6: A schematic detailing the process of myogenesis, redrawn from Beaudry (2016) Progenitor cells undergo differentiation in a series of steps to from matured myofibres regulated by several myogenic factors. On induction of differentiation, the myoblasts exit the cell cycle and fuse to form multinuclear myotubes and ultimately mature into myofibres.

2.2.1 Structure of the skeletal muscle

A detailed description of skeletal muscle can be found in the review by Schiaffino and Reggiani (2011). Skeletal muscles are composed of muscle fibres which run through the entire length of the entire muscle. Each muscle fibre in turn contains individual contractile subunits known as myofibrils extending from one end of the fibre to the other. Each cylindrical myofibril is surrounded by a sarcoplasmic reticulum which is a complex of membranes forming a network composed of interconnected hollow tubes. The myofibrils contain subunits known as sacromeres made up of precise arrangements of actin and myosin filaments. Sacromeres are attached end to end throughout the length of the myofibrils and their junction points are called Z-lines. Attached to the z-lines are strands of actin and two accessory proteins that form the thin filaments. Suspended between the thin filaments are thick filament called myosin (Figure 2.7). Myosins are long cylindrical proteins that are part of the makeup of myofibrillar proteins. Other proteins in myofibrils include actin and titin. Myosins also comprise a family of ATP dependent contractile proteins and are known for a wide range of motility processes in eukaryotes.

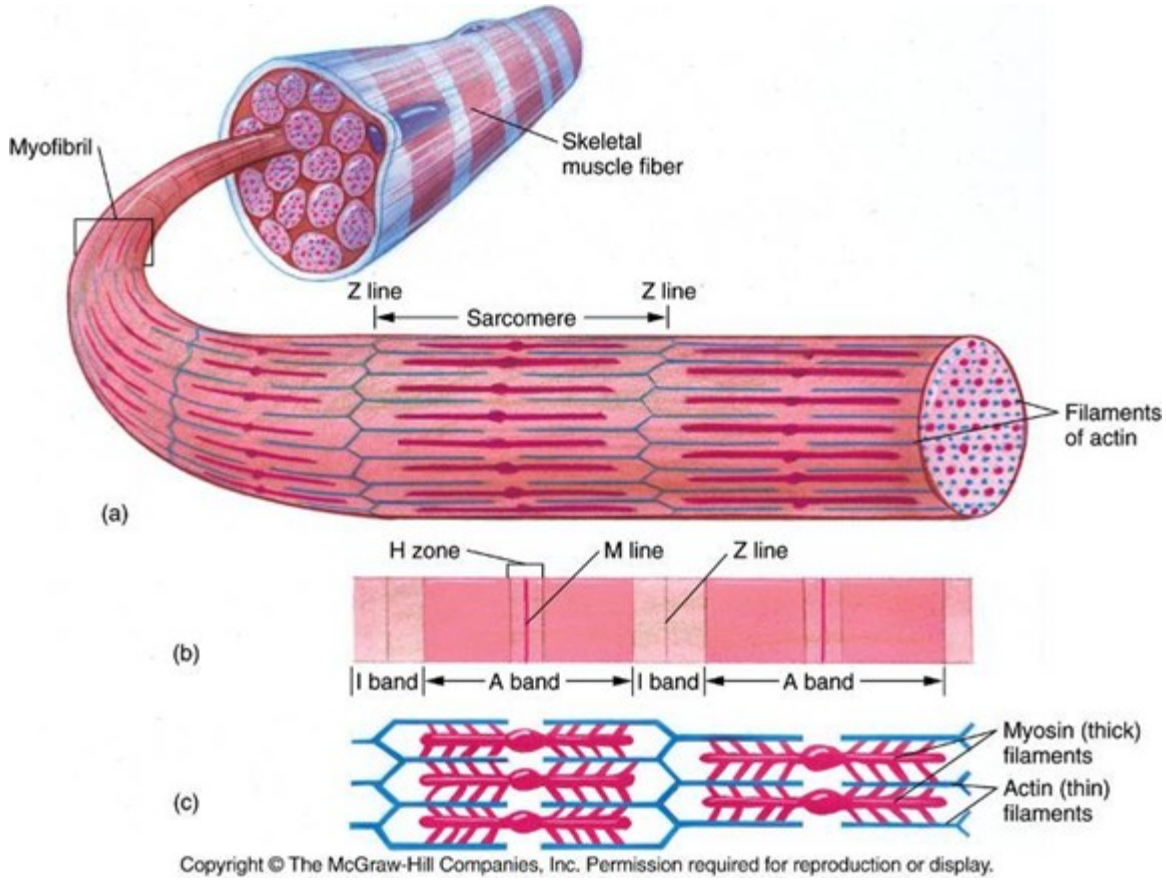


Figure 2.7: Structure of the skeletal muscle showing individual muscle fibres, myofibrils as well as thick and thin filaments. (Source: Powers and Howley 2007)

2.2.2 Myosin heavy chains and muscle fibre types

Skeletal muscle is composed of a heterogeneous population of muscle fibres, with varying physiological and metabolic characteristics. Based on fibre size and capacity to undergo muscle hypertrophy, muscle fibres can be broadly categorized into slow twitch or Type I and fast twitch or Type IIA and IIB muscle fibres (Burke *et al*., 1971). Thus, fibre types were commonly categorized as slow-twitch oxidative (type I), fast-twitch oxidative-glycolytic (type IIA) and fast-twitch glycolytic (type IIB) in human and rodents. However, the introduction of antibodies raised against components that constitute the contractile machinery of the muscle fibre, revealed there might be more than three muscle fibre types. Specifically, antibodies raised against myosin heavy chain (MyHC) isoforms highlighted four distinct fibre types. expressing type I, IIA, IIX or IIB MyHC isoforms (Schiaffino, 2010). Myosin heavy chains (MyHC) is the molecular motor of the muscle sarcomere and closely correlates with the contractile and metabolic characteristics of the muscle fibre, plus it is differentially expressed in physiologically diverse muscle fibre types, thus making it an excellent marker of muscle fibre type (Schiaffino and Reggiani 2011; Gundersen 2011). Furthermore, global expression of MyHC

isoforms in muscle tissue homogenate gives an average indication of phenotype across the sample. As it stands, measuring MyHC isoform expression as an indicator of muscle fibre type is likely a gross over-simplification, as there are many components of the muscle sarcomere that display fibre type specific expression, but it is currently considered the best and most convenient marker of muscle fibre composition (Schiaffino and Reggiani 2011). However, MyHC is a key component of the muscle sarcomere and is actively (not just passively) involved in directly influencing the physiology of the muscle fibre. Thus, it is not only a useful "biomarker" of muscle fibre type but of physiological interest to the scientific community as an influential component of the muscle fibre phenotype. Type I fibres structurally have a high mitochondrial, a high myoglobin content and low glycogen content as well as few of the enzymes involved in glycolysis but contain many of the enzymes involved in the oxidative pathway While fast twitch Type II fibres structurally have a large motor neuron and fibre diameter, a high mitochondrial density, a medium capillary density, and a medium myoglobin content and are high in glycogen content (Karp 2008). The most effective way of classifying muscle fibre types present in skeletal muscle is by characterising the myosin heavy chain profiles. Methods used in the characterisation process include myofibrillar adenosine triphosphate histochemistry, immunohistochemistry using specific antibodies to myosin heavy chain isoforms and electrophoretic analysis of myosin heavy chains in micro dissected single fibres (Pette an Staron 2000). Myosin heavy chains in muscle fibres have been well characterised in human and mouse models (Figure 2.8) (Pette and Staron 2000; Schiaffino and Reggianni 2011).

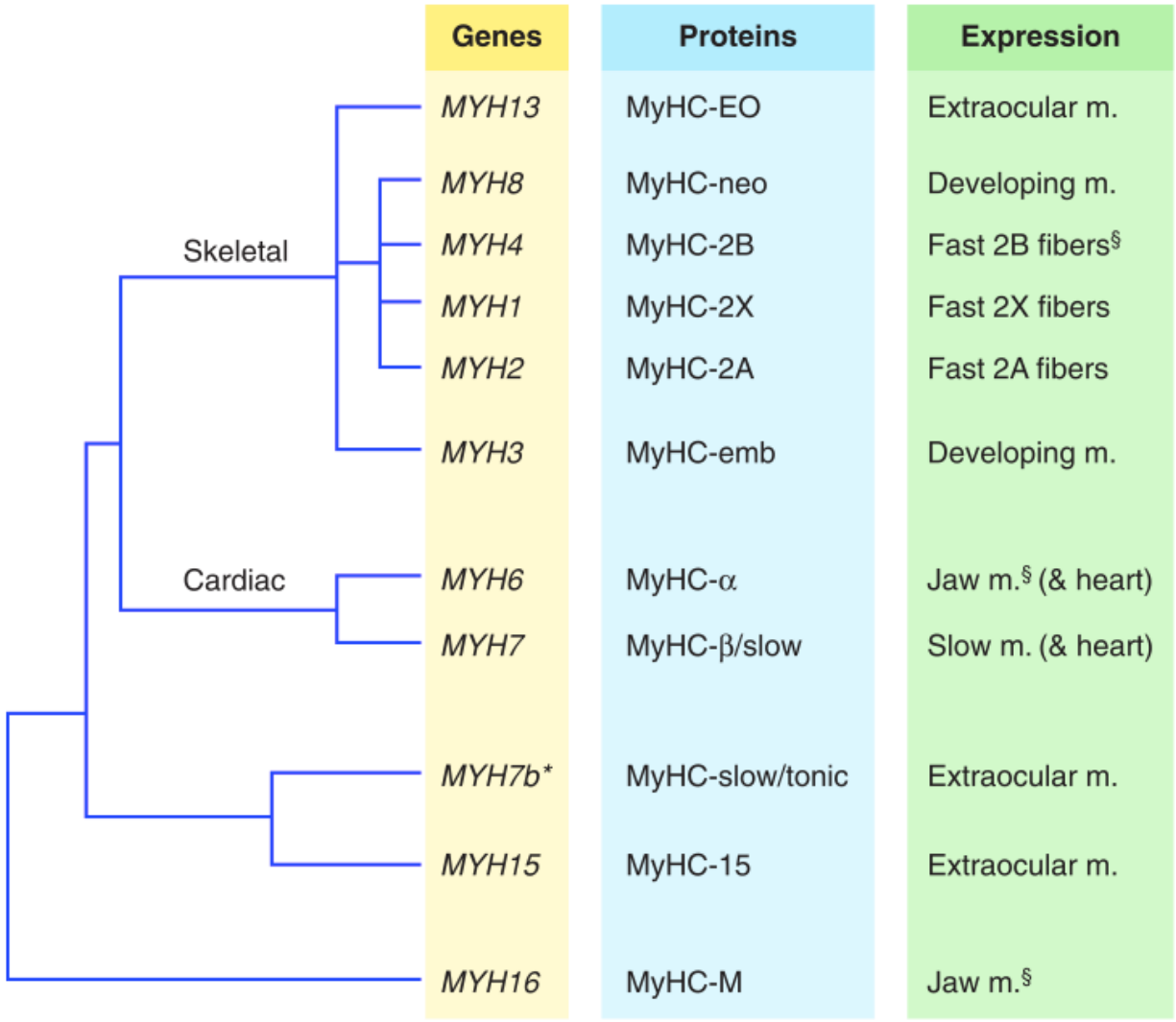


Figure 2.8: Myosin heavy chain gene family in mouse and human with their respective protein names and distribution expression (Schiaffino and Reggianni 2011).

Table 2.1 Metabolic and physiological characteristics of muscle fibres in humans and rodents

Metabolic and physiological properties	Fibre types			
	I	IIA	IIX	IIB
Contraction speed	+	+++	++++	+++++
Myofibrillar ATPase	+	+++	++++	+++++
Oxidative metabolism	+++++	++++,+++++	+,++	+
Glycolytic metabolism	+	++++	++++	+++++
Hexokinase	+++++	+++	+	+
GLUT-4	+++++	+++	+	+
Phosphocreatine	+	+++++	+++++	+++++
Glycogen	+	+++++	++++	+++++
Triglycerides	+++++	++	+	+
Vascularization	+++++	+++	+,++	+
Myoglobin	+++++	++++	++	+
Buffer capacity	+	++++	+++++	+++++
Diameter	++		++++	+++++
Fatigue resistance	+++++	++++	++	+

Source: Parr and Brameld (unpublished). +, Very low; ++, low; +++, medium;++++, high; +++++, very high

2.3 MUSCLE HYPERTROPHY AND FIBRE TYPES IN CHICKEN

Muscle hypertrophy is an increase in the size of a muscle through an increase in the size of its component cells. It differs from muscle hyperplasia, which is the formation of new muscle cells. Depending on the type of training, the hypertrophy can occur through increased sarcoplasmic volume or increased contractile proteins (Baechle and Earle 2008). As a result of many generations of selection pressure, broilers exhibit approximately 50% more muscle mass and more muscle fibres per muscle mass and larger diameter muscle fibres than laying type chickens (Mizuno and Hikami, 1971). Work done by Aberle and Stewart (1983) suggested that the selection for growth and muscularity favours factors that promote selective radial hypertrophy of type II myofibers, as seen in broiler-type chickens. The expression levels different of slow type muscle protein encoding genes in the pectoralis have been reported to be significantly higher in layers than in broilers at 2 weeks, 4 weeks, 6 weeks and 8 weeks of age. (Zheng *et al*., 2009). Broilers chickens have larger diameter type I and II myofibers and greater apparent myofiber number This attribute is more prominent in the *Pectoralis major* of the breast muscle which is composed of majorly glycolytic type 2B fibres. The leg muscles have a more heterogeneous fibre type distribution, with a larger population of oxidative type 1 fibres (Cooke *et al*., 2003; Locasale *et al*., 2011).The extensive blood vessels and myoglobin in slow fibres give these fibres a reddish colour, thus muscles dominated by slow fibres are known as red muscles. Chickens walk frequently, and the movements are performed by the slow fibres in the dark meat of their legs. As a result of repeated,

exhaustive stimulation, slow muscle fibres develop more mitochondria, a higher concentration of glycolytic enzymes, and larger glycogen reserves. (Martini, 2001).

Genetic selection has been concentrated on high growth velocity and large muscle mass for broilers; in contrast, layers have been selected for egg production while neglecting muscle growth. This kind of selective breeding makes these two chicken strains an ideal model system to study the controlling mechanisms of myogenesis rate and muscle cell size allowing us to investigate many interesting questions related to muscle growth control.

2.4 GLYCOLYTIC METABOLISM IN CHICKENS

The principal storage form of carbohydrates in muscle tissues is glycogen which is broken down into glucose and then used in metabolism. Glucose circulating in the blood is either of dietary origin, largely arising from polysaccharides that have been hydrolysed in the gut and transported across the intestinal wall, or endogenously released from its tissues, mainly the liver and kidney (Stevens 2004). Carbohydrate metabolism is linked to muscle growth in the sense that the latter is highly associated with muscle fibre type composition which in turn is characterised by the type of glycolytic metabolism predominantly occurring in the muscle which are type II fast glycolytic fibre type metabolism or type I slow oxidative fibre type metabolism. Several studies have suggested that breast muscle glycogen content is related to growth and body composition: it decreases with growth rate and breast meat yield (Le Bihan-Duval *et al.*, 2001; Sibut *et al.*,2008).

2.4.1 Intracellular transport of glucose in chickens

Chickens have a higher blood glucose concentration compared to mammals of approximately twice the value (Belo *et al.*, 1976), even in the fasting state, and are markedly resistant to the injection of high concentrations of insulin. (Akiba *et al.*, 1999 cited by Kono *et al.*, 2008). Glucose, the essential energy currency for all cells in animals, and is transported by facilitated diffusion in all cells across the plasma membrane. This process is mediated by glucose transporter proteins (GLUT) (Seki *et al.*, 2003). in mammals, GLUT 4 isoform is the transporter protein identified to be present in the muscle the activation and mobilisation of GLUT 4 in mammals is regulated by the activation of the insulin signalling cascade in mammals (Bryant *et al.*, 2002 Peyron-Caso *et al.*, 2002). The GLUT isoform in chicken skeletal muscle is yet to be identified and properly characterised as GLUT 4 has been reported to be absent in the skeletal muscle (Kono *et al.*, 2008). However, insulin injection administered in chickens has led to a 2-fold uptake of glucose in the skeletal muscle suggesting an insulin cascade dependent uptake of glucose in the muscle which may be transported by a GLUT isoform (Tokushima *et al.*, 2005).

2.4.2 Homeostatic regulation of blood glucose with insulin and glucagon

The two hormones that play perhaps the largest role in controlling the blood sugar (Glucose) level are insulin and glucagon. The combined action of glucagon and insulin is responsible for maintaining whole-body glucose homeostasis (Unger 1978 cited by Qureshi *et al*., 2004). These two hormones work in antagonistic fashions to each other. The increase in blood glucose concentration is closely followed in time by an increase in plasma insulin concentration. As insulin carries out its function and starts to bring blood glucose concentrations back down to normal, then this removes the stimulus that tells the beta cells in the pancreas to secrete the insulin in the first place. As a result, the beta cells become less and less stimulated and so the rate of secretion of insulin declines in parallel to the rate of decline in blood glucose concentration.

In direct contrast to insulin, a decrease in blood glucose concentration stimulates glucagon secretion. Therefore, circulating levels of glucagon tend to be highest during periods of starvation (fasting) or prolonged exercise (e.g. running a marathon). During these times, blood glucose concentrations are at their lowest. The glucagon brings about changes in the body's metabolism that raise blood glucose concentration back to normal. Glucagon increases glucose production by promoting glycogenolysis and gluconeogenesis in the liver and attenuation of the ability of insulin to inhibit these processes (Consoli, 1992 Cited by Qureshi *et al*., 2004).

2.4.3 Molecular mechanism for glucagon-mediated glucose regulation

Glucagon signals through its receptor on the cell surface. The binding of glucagon to the extracellular loops of the glucagon receptor results in conformational changes of the latter, leading to subsequent activation of the coupled G proteins. At least two classes of G proteins are known to be associated with and involved in the signal transduction of the glucagon receptor, namely Gsα and Gq. The activation of Gsα leads to activation of adenylate cyclase, increase in intracellular cAMP levels, and subsequent activation of protein kinase A (PKA). The activation of Gq leads to the activation of phospholipase C, production of inositol 1,4,5-triphosphate, and subsequent release of intracellular calcium (Christophe, 1995; Brucellin *et al*., 1996; Jiang and Zhang 2003) (Figure 2.9).

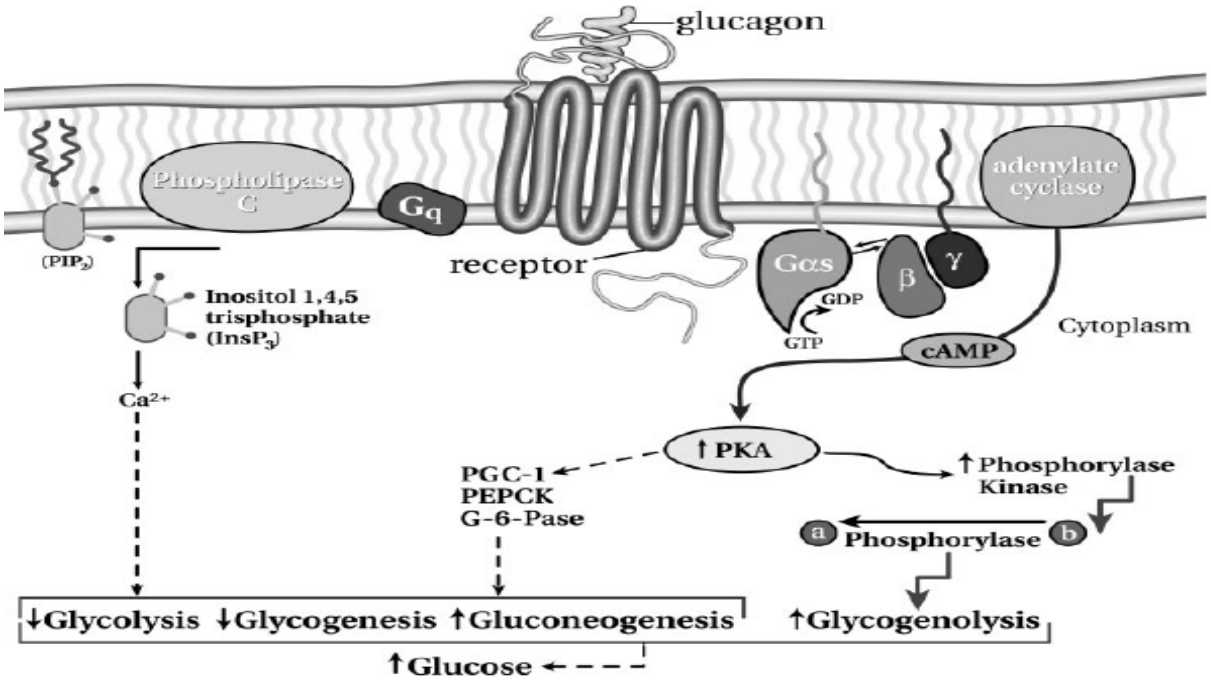


Figure 2.9: Glucagon signalling pathway (source: Jiang and Zhang, 2003). PIP= phosphatidylinositol 4-phosphate, PEPCK= Phosphoenolpyruvate carboxykinase, PGC-1= Phosphoenolpyruvate carboxykinase. Camp= cyclic adenosine monophosphate, PKA=Protein kinase A.

2.4.4 Molecular mechanism for insulin-mediated glucose regulation in the body's metabolism that raise blood

The binding of insulin to the subunit of insulin receptor (IR) not only concentrates insulin at its site of action, but also induces conformational changes in the receptor, which in turn stimulates the tyrosine kinase activity intrinsic to the β subunit of the IR and triggers the signalling cascades (Figure 2.10). Insulin receptors trans phosphorylate several immediate substrates (on Tyr residues) including IRS1, Shc, Gab 1, Cbl, APS, and P60dok. Each of these provides specific docking sites for other signalling proteins containing Src homology 2 (SH2) domains. These events lead to the activation of downstream signalling molecules including PI-3 kinase.

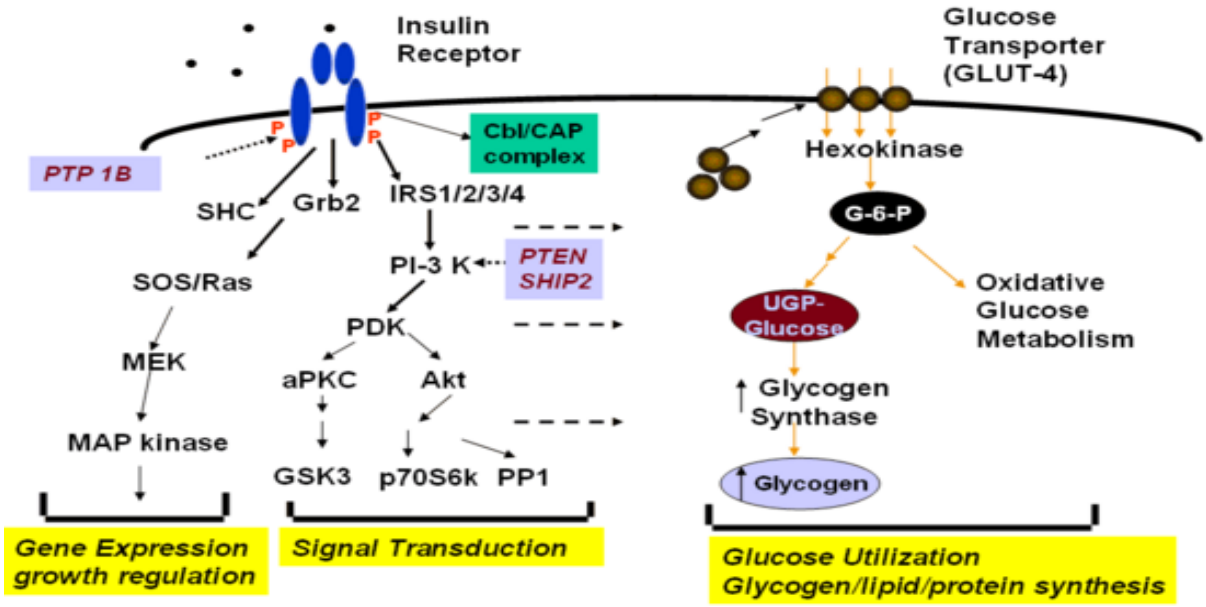


Figure 2.10: Insulin signalling pathway Zhang 2007 (www.endotext.org). IRS=insulin receptor substrate, Grb-2= Growth factor receptor-bound protein 2, SOS = Son of sevenless homolog 1, MEK= mitogen-activated, extracellular-signal-regulated protein kinase kinase.MAPK= Mitogen-activated protein kinase kinase, PI-3K= Phosphatidylinositol-4,5-bisphosphate 3-kinase, PDK= Pyruvate dehydrogenase kinase, aPKC= protein kinase C, GSK3= Glycogen synthase kinase 3.

2.4.5 Transcriptional factors involved in Insulin regulated Glycogen Synthesis and Gluconeogenesis

There are several important enzymatic checkpoints that act to control glycolysis and glycogen synthesis (glucokinase, glycogen synthase kinase-3), glycogenolysis (phosphorylase), gluconeogenesis (phosphoenolpyruvate carboxykinase, fructose 1,6 bisphosphatase), or steps that are common to the pathways (glucose-6-phosphatase). Some of them are directly controlled by insulin via phosphorylation and dephosphorylation (Zhang, 2007).

Glycogen synthase kinase 3 (GSK-3) is a cytoplasmic serine/threonine kinase that plays key roles in insulin signal transduction and metabolic regulation. It was originally identified for its ability to phosphorylate and inhibit glycogen synthase (Ding *et al*., 2000). In the insulin signalling pathway, GSK-3 is active in the absence of insulin and it phosphorylates (and thereby inhibits) glycogen synthase and several other substrates. Insulin binding to the receptor activates a phosphorylation cascade, leading to inhibitory phosphorylation of GSK-3 by Akt. Thus, insulin activates glycogen synthase by promoting its dephosphorylation through the inhibition of GSK-3 (Zhang, 2007). In addition to regulating GSK-3 via Akt, insulin also stimulates compartmentalized activation of protein phosphatase 1 (PP1). The phosphatase PP1 catalyses the metabolic effects of insulin mediated by dephosphorylation, such as the insulin dependent dephosphorylation of metabolic enzymes such as glycogen synthase (Brady and Saltiel, 2001). Schmall *et al*. (2000) reported that signalling by protein kinase

B to Forkhead proteins (FKHR) can account for the ability of insulin to regulate glucose-6-phosphatase promoter activity via the insulin response unit (IRU). Other mechanisms that are independent on the IRU, such as protein kinase B and FKHR, also are important in mediating effects of in insulin on glucose-6-phosphatase gene expression. Under basal conditions, FKHR resides in the nucleus. Upon insulin stimulation and phosphorylation by Akt kinase, FKHR is excluded from the nucleus to the cytoplasm, thereby providing a powerful mechanism by which insulin could down-regulate a number of genes including IGF-binding protein-1, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (Zhang 2007).

2.5 SERINE BIOSYNTHESIS AND MUSCLE GROWTH

Cell growth and proliferation requires the production of building blocks for the synthesis of cell constituents which include protein, nucleic acids and lipids. Serine and glycine are biosynthetically linked and together provide the precursors for the synthesis of these biomolecules (Locasale *et al*., 2011; Amelio *et al*., 2014; Ye *et al*., 2012). Serine biosynthesis has also been reported to be upregulated during hyperplastic growth as seen in cancer cells (Possemato *et al.*, 2011; Dann and Abraham 2011) as well as hypertrophic growth (Parr *et al*., 2015; Brameld *et al*., 2015). In avian species, myofibre/myoblast number is established post embryogenesis and it is generally thought that the hypertrophy of muscle takes place by the accretion of protein and nuclei originating from the proliferation and fusion of satellite cells (Scheuermann *et al*., 2004). There have been no previous reports on the relationship between serine/glycine biosynthesis and muscle hypertrophy in chicken. We therefore measured the gene expression the three enzymatic proteins that catalyse the three step de novo biosynthesis of serine namely Phosphoglycerate dehydrogenase (PHGDH), phosphoserine amino transferase (PSAT) and phosphoserine phosphatase (PSPH). As well as the protein expression of PHGDH at a time point where broiler growth has been reported to be at a rapid stage (citation needed).

Serine can be biosynthesised *de novo* from the glycolytic pathway intermediates. Glycolysis is primarily required for ATP and energy production however it also serves as a pathway that supports anabolism in cells through the *de-novo* synthesis of other biomolecules. Serine biosynthesis is a component of these glycolysis-diverting pathways. The glycolytic intermediate 3-phosphoglycerate is converted to serine following a three-step enzymatic reaction catalysed by a gate keeping enzyme known as phosphoglycerate dehydrogenase (PHGDH) where NAD oxidises ~10% of the 3-phosphoglycerate generated from glycolysis into the serine precursor 3-phosphohydroxypyruvate (DeBerardinis 2011). Subsequent enzymes in the pathway convert 3-phosphohydroxypyruvate into serine via transamination Phosphoserine aminotransferase (PSAT1) and phosphate ester hydrolysis Phosphoserine phosphatase (PSPH) reactions. Serine is a central metabolite for

biosynthetic reactions and a precursor to other metabolites. It is highly dependent on PHGDH production, as suppression of PHGDH has been shown to down regulate the synthesis of serine, even in cells cultured in media containing exogenous serine. This suggests that, besides the control of the intracellular serine level, PHGDH also controls biosynthesis of metabolites which require serine serves as a precursor (Possemato, 2011). For instance, PSAT1 uses the PHGDH product 3-phosphohydroxypyruvate to convert glutamate to α-ketoglutarate (Figure 2.11). Alpha-Ketoglutarate can potentially serve as a precursor to other biomolecules utilised in the TCA cycle. The conversion of glutamine to glutamate and then α-ketoglutarate, and other TCA intermediates, was significantly reduced in cells in which PHGDH or PSAT-1 was suppressed. Through these pathways the serine synthesis pathway is a major contributor of TCA intermediates; it is responsible for approximately half of the anaplerotic flux to the TCA cycle (Amelio *et al.*, 2014).

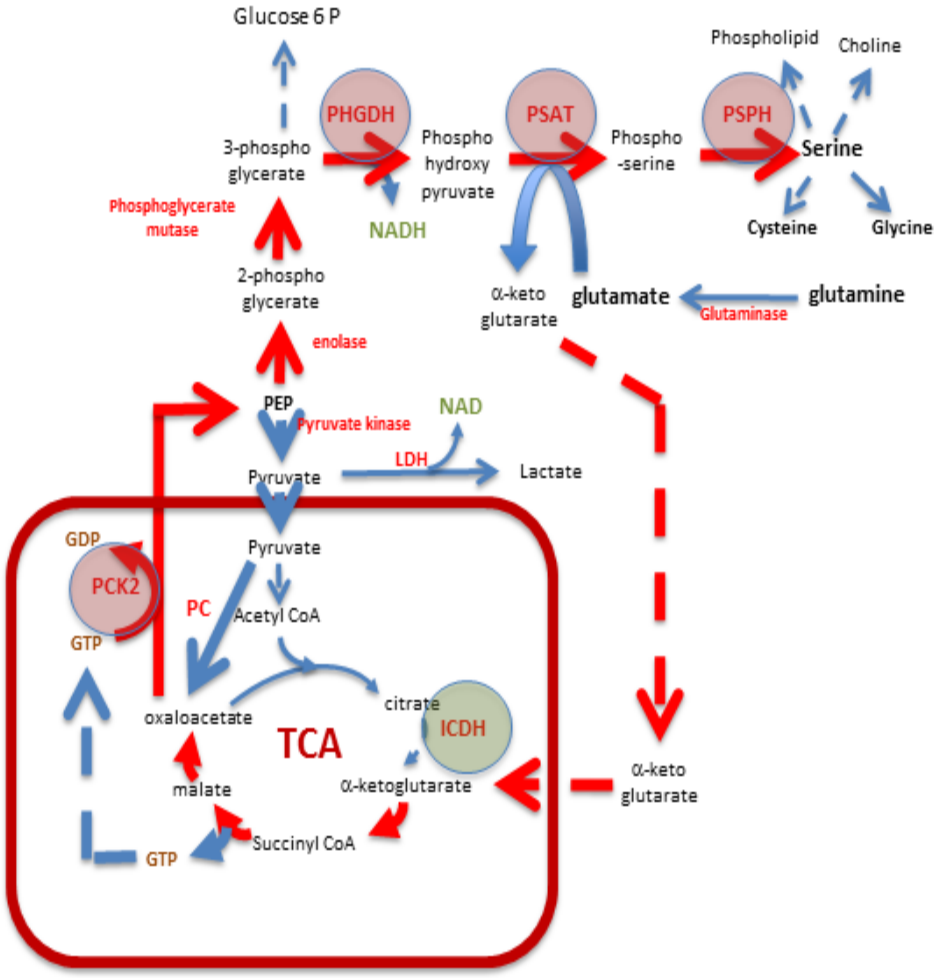


Figure 2.11: The potential links between the serine synthesis pathway and the TCA cycle. Image showing the conversion of phospho hydroxy pyruvate to phosphoserine in the serine biosynthetic pathway and the associated conversion of glutamate to α-ketoglutarate which then can enter the TCA cycle. The red arrows illustrate the potential flux of metabolites to serine. (Source: Parr *et al.*, unpublished)

De novo synthesis of serine provides precursors for a variety of biosynthetic pathways, and accordingly, a pivotal aspect of the serine *de novo* synthesis is the conversion of serine to glycine by serine hydroxy methyltransferase (SHMT). As indicated above, glycine is a major source of methyl groups for the one-carbon pools required for the biosynthesis of glutathione (GSH), protein, purines and DNA/histone methylation. There have been several reports where upregulated expression of mRNA encoding these enzymes that catalyse serine biosynthesis has been described as being associated with muscle growth. (Parr *et al*., 2015; AL Doski *et al*., 2015; Brameld *et al*., 2015) The mechanism of action of serine biosynthesis and its association with muscle hypertrophy is not well understood because there is limited information on these processes. However there have been some work done in an attempt to elucidate the relationship of serine biosynthesis and hyperplastic growth in tumor cells (Reviewed by Amelio *et al*., 2014). This may give an indication of how serine biosynthesis is regulated during skeletal muscle growth.

2.5.1 Serine and the p53 family

p53 is a key player in the cellular response to stress in the form of numerous challenges, including DNA damage, hypoxia, and oncogene activation (Vousden and Prives, 2009). The complexity and diversity of these cellular processes indicate roles for p53 in normal and cancer cell homeostasis, in addition to its traditional role as a tumour suppressor (Vousden and Ryan, 2009). The ability of p53 to respond to nutrient deficiencies lies in the established function of p53 as a mediator of the cellular stress response. The factor p53 has been associated with the capacity of cancer cells to deal with serine starvation and oxidative stress, and indeed, p53 helps cancer cells to overcome serine starvation, preserving the cellular antioxidant capacity. Cells lacking p53 fail to respond to serine starvation due to oxidative stress, which leads to reduced viability and severely impaired proliferation. During serine starvation, activation of the p53–p21 axis leads to cell cycle arrest, which promotes cell survival by efficiently channelling depleted serine stores to glutathione synthesis (Maddocks, 2013).

2.5.2 Serine and PKM2

The glycolysis enzyme PKM2 is a splice variant of the PKM gene which catalyses the last step of the glycolytic pathway converting phosphoenolpyruvate (PEP) to pyruvate and produces one molecule of ATP (Figure 2.11). The PKM2 is predominantly expressed in proliferating tissues (Mazurek, 2011) as well as tumor tissues (Reinacher and Eigenbrodt, 1981; Amelio, 2014). PKM2 has also been reported to have an association with muscle hypertrophic growth. Its expression was upregulated in growth promoter induced muscle growth in sheep with a simultaneous upregulation in the expression of genes in the serine biosynthetic pathway (Al Doski

et al., 2015). Lower PEP/pyruvate conversion favours accumulation of glycolytic intermediates, refuelling diverging anabolic pathways such as the pentose phosphate pathway (PPP) and serine biosynthesis. When serine is abundant PKM2 is fully activated allowing the consumption of glucose through aerobic glycolysis. Upon serine deprivation, PKM2 activity is reduced, and pyruvate is diverted to a fuel-efficient mode in the mitochondria, and glycolysis metabolites are diverted to serine biosynthetic pathways to sustain cell proliferation (Amelio *et al*., 2014). Through this mechanism, serine supports aerobic glycolysis and lactate production; events that are critical for cancer cell growth and survival.

2.5.3 Serine fuels glycine biosynthesis

Serine can be synthesized to glycine by the in a reaction catalysed by the enzyme serine hydroxy methyltransferase (SMTH). This reaction catalysed by SHMT represents a major source of methyl groups for the one-carbon pools that are required for *de novo* nucleotide biosynthesis and DNA methylation. Therefore, SMTH is relevant for the synthesis of glycine and *de novo* nucleotide biosynthesis (Amelio *et al*., 2014). Mammalian SHMTs catalyse the reversible transfer of the serine C_β to tetrahydrofolate (THF) or its polyglutamylated derivatives, yielding glycine (Garrow 1993).

2.5.4 One carbon metabolism

The interaction between the folate cycle to the methionine cycle constitutes a bicyclic pathway that circulates carbon units and is collectively referred to as one-carbon metabolism. The folate cycle is charged by the conversion of serine in glycine (SHMT activity) and by the glycine cleavage system; glycine dehydrogenase (GLDC) produces ammonia, carbon dioxide, and a carbon unit for the methylation of THF. The folate cycle is then coupled to the methionine cycle, with methyl-THF (mTHF) donating a carbon to homocysteine, methylating and converting it to methionine (Amelio *et al*., 2014).

A way to conceptualize the role of one-carbon metabolism in cellular physiology is that it functions as a metabolic integrator of nutrient status. Inputs in the form of glucose and amino acids enter the pathway, are processed through chemical reactions, and are then output for diverse biological functions. This analogy has been used extensively to describe growth control by the mammalian target of rapamycin (mTOR) signal transduction pathway, but has been used less for metabolic pathways per. In the case of mTOR signalling, inputs in the form of amino acids and growth factors are integrated to generate outputs such as protein translation, autophagy inhibition, and anabolic metabolism. For one-carbon metabolism, the integration is carried out through the donation of carbon units from specific amino acids (Locasale *et al*., 2013).

2.6 ASPARAGINE SYNTHESIS

Another post glycolytic/TCA cycle metabolite of interest is the non-essential amino acid asparagine which is synthesised from the transamination of glutamine on to aspartic acid to form asparagine by the amino transferase enzyme asparagine synthetase (ASNS). Asparagine is generally considered as a nutritionally non-essential amino acid in mammals (Wu et al., 2007), and chicken, (NRC 1994). Increasing evidence has also shown that asparagine plays an important role in many physiological and biological processes. Also, the gene and protein expression of ASNS is induced following amino acid deprivation (Siu et al., 2002). In cancer cells, work done by Zhang et al., (2014) reported that while intracellular glutamine is a suppressor of apoptosis in many human tumours, knockdown of ASNS, which suppress de novo synthesis of asparagine from aspartate and glutamine, leads to cell death even in the presence of glutamine, which can be reversed by addition of exogenous asparagine. The authors also reported that asparagine plays a critical role in the regulation of the cellular adaptation and promotes cellular adaptation to depletion of glutamine and other nonessential amino acids. However, although asparagine may be implicated in supporting protein synthesis in nutrient deprived cells, except aspartate, no other reported direct metabolic pathway of asparagine undergoing deamination for de novo biosynthesis of other non-essential amino acids has been reported. However, asparagine may play a role in cellular amino acid homeostasis in response to amino acid deprivation. The amino acid response (AAR) pathway has been described as being triggered by shortage of any essential amino acid, and results in an increase in activating transcription factor ATF4, which in turn affect many process (Kilberg et al., 2005). This occurs through a feedback control mechanism mediated by activating transcription factor 4 (ATF4). This transcription factor can be activated by a number but asparagine depletion has been reported to activate the serine threonine kinase general control non-depressible 2 (GCN2) (Dong et al., 2000; Hao et al.,2005) which phosphorylates the translation initiation factor eIF2□, resulting in increased translation of the transcription factor ATF4 (Harding et al., 2000) which can increase expression of a range of genes including upregulation of ASNS gene expression (Balasubramanian et al., 2013). Any subsequent asparagine accumulation then suppresses GCN2 and reduces ATF4 expression (Park et al., 2017).

2.7 PROTEIN METABOLISM IN SKELETAL MUSCLE

The dietary requirements of proteins, amino acids and nitrogen are determined by the nature of the metabolic demand that must be satisfied. Nitrogen is needed for both protein tissue synthesis and production of several nitrogenous compounds involved in a range of functions. The achievement of nitrogen homeostasis involves a complex series of changes in rates of whole body protein turnover (Tome and Bos, 2000).

The amino acid pool in the cell is composed of a mixture of amino acids degraded from dietary and endogenous proteins. The uptake of these amino acids for protein synthesis determines the rate of protein turnover because proteins are constantly being degraded and resynthesized in the body thus giving rise to the concept of positive and negative nitrogen balance depending on the net protein retention in the (Ophardt 2003)

2.7.1 Protein metabolism and turnover in broiler and layer chickens

Broiler chickens have larger diameter muscle fibres and larger muscles when compared to layers. This situation is also as a result of a greater rate of a positive protein turnover resulting from the utilisationn of amino acids from the amino acid pool to synthesize muscle protein fibres thus increasing fibre size Jones *et al*. (1986) reported that the ratio of fractional muscle protein synthesis to breakdown indicated that 16% of the layer pectoral muscle was retained, as compared to 45% observed in broilers. Confirmation of this can be found in studies conducted by Tesseraud *et al*. (2000) that reported that the total amount of protein synthesized, gained and degraded were significantly higher ($p<0.001$) in the *pectoralis* muscle of fast growing line broiler chickens than slow growing line layer chickens (Figure 2.11).

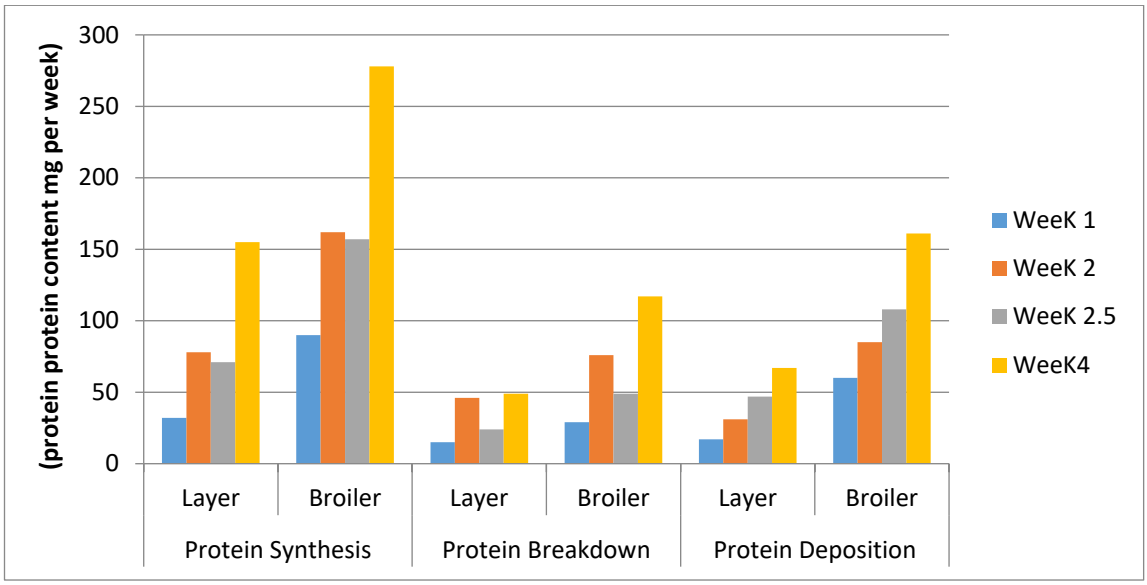


Figure 2.12: Protein turnover in broiler chicken showing total protein synthesis, breakdown and net protein deposition. Data derived from Tesseraud *et al*., (2000).

2.8 PROTEIN SYNTHESIS AND SKELETAL MUSCLE GROWTH

Mammalian cells have two recognized pathways for monitoring and responding to amino acid availability. These two pathways change the rate of protein synthesis in opposite directions, but whether they are linked in a more direct manner is not known (Kilberg *et al*., 2005). The mammalian target of rapamycin (mTOR) pathway functions to confirm a sufficient level of amino acids to support protein synthesis and cell growth. Through an unknown amino acid detection mechanism, amino acid sufficiency activates the mTOR kinase cascade that ultimately results in phosphorylation of the ribosome-associated S6 kinase. The second known amino acid responsive pathway is designed to detect amino acid deficiency. Limiting the availability for any single amino acid initiates this signalling cascade, which is referred to here as the amino acid response (AAR) pathway (Kilberg *et al*., 2005).

2.8.1 AKT/mTOR signalling pathway

The mTOR pathway is thought to regulate protein synthesis through translational capacity and efficiency which leads to an upregulated mRNA translation resulting in increased fibre size (Zanchi and Lancha, 2011). The mammalian target of rapamycin **(**mTOR) exists in 2 protein isoforms namely the mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The protein mTORC1 can be stimulated by insulin, growth factors, energy signals (ATP level), and amino acids (Deng *et al*., 2013). One of the mechanisms by which mTOR controls this process is by the modulation of the activation of the downstream intermediate P70S6 kinase which in turn phosphorylates S6 ribosomal protein (Nader *et al*., 2005; Brown *et al*., 1995). Phosphorylation of S6 ribosomal protein correlates with an increase in translation of mRNA transcripts that contain an oligopyrimidine tract in their 5' untranslated regions (Deng *et al*., 2014) reported an upregulated phosphorylation of mTOR, P70S6K and 4E-BP1 in chick pectoral muscles influenced by an increased dietary leucine. These same observations have also been reported in rats (Kimball and Jefferson 2014). Leucine and other branched chain amino acids have been reported to upregulate skeletal muscle protein synthesis by initiating and modulating the activation of mTOR and its downstream intermediates P70S6K and 4EBP1 (Anthony *et al*., 2001).

Phosphorylation of S6 ribosomal protein correlates with an increase in translation of mRNA transcripts that contain an oligopyrimidine tract in their 5' untranslated regions. Deng *et al*. (2015) reported an upregulated phosphorylation of mTOR, P70S6K and 4E-BP1 in chick pectoral muscles influenced by an increased dietary

leucine. These same observations have also been reported in rats (Kimball and Jefferson 2004). Leucine and other branched chain amino acids have been reported to upregulate skeletal muscle protein synthesis by initiating and modulating the activation of mTOR and its downstream intermediates P70S6K and 4EBP1 (Anthony *et al*., 2001).

The mechanism by which mTOR signalling controls protein synthesis has been well characterised. This is via mTORC1 directly phosphorylating the translational regulators eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1), which, in turn, promote protein synthesis via elevating translation (Ma and Blenis, 2009). The phosphorylation of 4E-BP1 prevents its binding to the cap-binding protein eIF4E, enabling the latter to participate in the formation of the eIF4F complex which is required for the initiation of cap-dependent translation. The activation of S6K1 leads, through a variety of effectors, to an increase in mRNA biogenesis, as well as translational initiation and elongation (Laplante and Sabatini 2012). The overall rate of protein synthesis is reduced when there is an inhibition of the activation of mTOR. (Thoreen *et al*., 2009; Yu *et al*., 2009).

Growth factors, such as insulin and insulin-like growth factor 1 (IGF1), stimulate the intermediate PI3K upstream the mTOR pathways. The effector kinases of these pathways—protein kinase B (Akt/PKB), extracellular-signal-regulated kinase 1/2 (ERK1/2), and ribosomal S6 kinase (RSK1)-- directly phosphorylate the TSC1/TSC2 complex to inactivate it and thus activate mTORC1 (Inoki *et al*., 2002; Ma *et al*., 2005; Manning *et al*., 2002; Potter *et al*., 2002; Roux *et al*., 2004). Akt also signals to mTORC1 in a TSC1/2-independent fashion by phosphorylating and causing the dissociation from raptor of PRAS40, an mTORC1 inhibitor (Sancak *et al*., 2007; Thedieck *et al*., 2007; Vander Haar *et al*., 2007; Wang *et al*., 2007). Pro-inflammatory cytokines, like tumor necrosis factor-α (TNFα), activate mTORC1 through a conceptually similar mechanism as growth factors: IκB kinase β (IKKβ) phosphorylates TSC1, causing TSC1/2 inhibition (Lee *et al*., 2007). Lastly, the canonical Wnt pathway, a major regulator of cell growth, proliferation, polarity, differentiation, and development, also activates mTORC1 through TSC1/2. In this case, Wnt signalling inhibits glycogen synthase kinase 3β (GSK3-β), which normally phosphorylates and promotes TSC2 activity (Inoki *et al*., 2006).

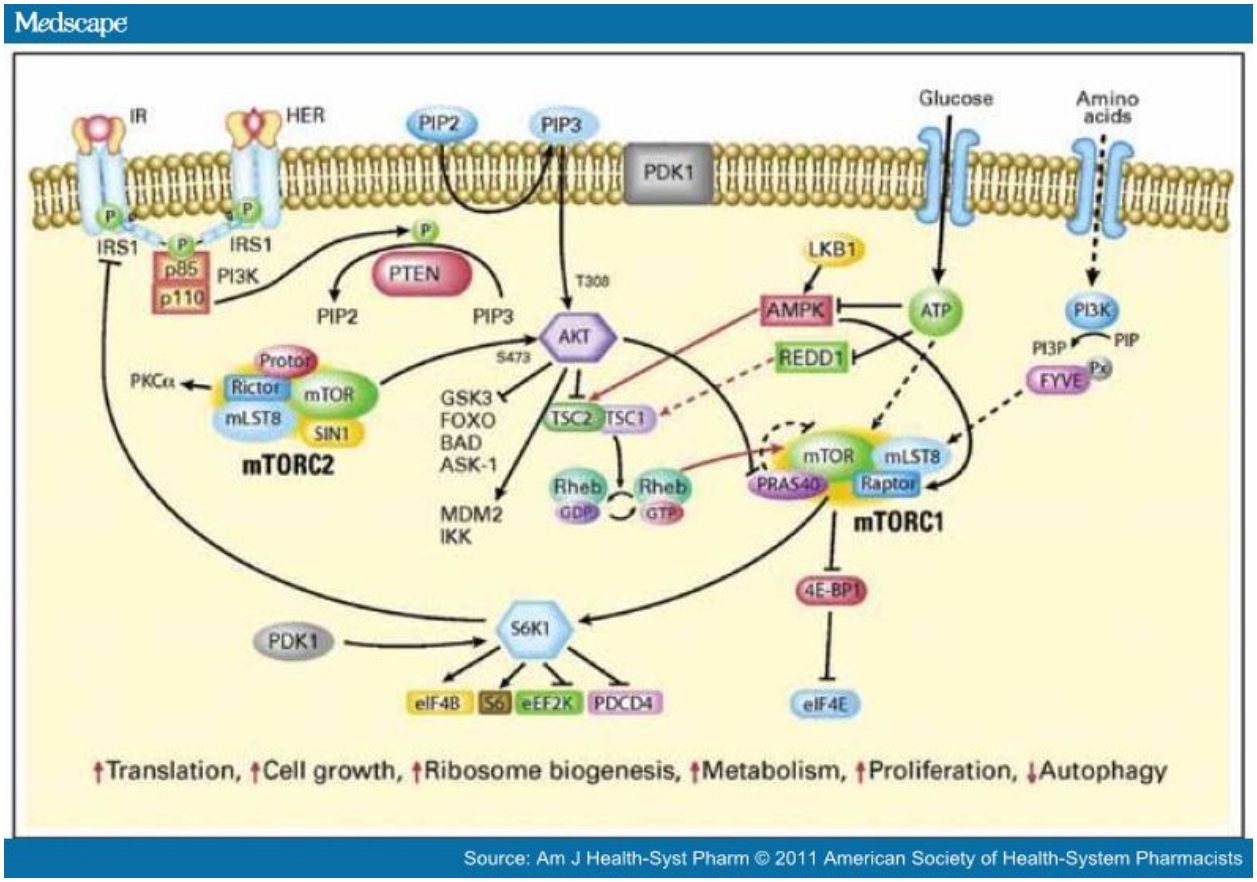


Figure 2.13: Overview of mTOR signalling upstream and downstream as it modulates protein translation as well as other cellular processes. source: Borders *et al*., 2010.

2.8.2 Amino Acid Response (AAR) Pathway

Limiting the extracellular supply of an essential amino acid or blocking the synthesis of an otherwise nonessential one, results in an increase in uncharged tRNA that binds to and activates the general control nonderepressible protein 2 (GCN2) kinase (Berlanga *et al*., 1999) which leads to phosphorylation of the translation initiation factor 2 (eIF2α) on serine-51. The kinase activity of GCN2 is activated when the protein binds any one of the uncharged tRNA molecules. Thus, depletion at the cellular level of any individual amino acid can trigger the AAR (Kilberg *et al*., 2009). Phospho-eIF2α suppresses general protein synthesis but promotes a paradoxical increase in translation of selected mRNA species. Among these are activating transcription factor 4 and 5 (ATF4 and ATF5) (Lu *et al*., 2004). ATF4 triggers increased transcription by binding to C/EBP-ATF response elements (CARE), so named because they are composed of a half-site for the C/EBP family and a half-site for the ATF family of transcription factors (Wolfang *et al*., 1997; Fawcett *et al*., 1999). The products of these CARE-containing genes modulate a wide spectrum of cellular events designed to adapt to dietary stress. If adaptation is unsuccessful, ATF4-induced apoptosis can

also occur. Microarray analysis of ATF4-deficient fibroblasts (Harding *et al*., 2003) and hepatoma cells (Lee *et al*., 2008) have documented that ATF4 regulates a wide array of genes involved in amino acid transport, metabolism, oxidation status, and energy management. However, it is also clear that individual tissues, particularly developing bone, use ATF4 signalling to regulate tissue-specific genes as well (Yang and Karsety 2004). As an illustration of inter-tissue homeostasis, feeding GCN2 knockout mice a leucine-deficient diet resulted in muscle loss, but a concurrent sparing of liver mass, whereas in wild-type mice, the leucine deficiency caused muscle and hepatic weight loss (Anthony *et al*., 2004).

The ATF4 mRNA contains two upstream open reading frames, uORF1 and uORF2, that are located 5′ to the ATF4 coding sequence, and both are translated in the non-stressed condition to the exclusion of ATF4 itself the uORF2 overlaps with that for ATF4 but is out of frame. During amino acid limitation, as a consequence of phospho-eIF2α inhibition of eIF2B and the corresponding decrease in functional eIF2 complex, ribosome scanning bypasses the uORF2 and translation re-initiation occurs at the ATF4 coding region. Thus, synthesis of ATF4 protein is selectively elevated in response to amino acid deficiency (Kilberg *et al*., 2009).

ASNS transcription has also been reported to be induced during AAR through a bipartite regulatory unit in the promoter that contains nutrient sensing response elements I and II (NSRE-I and II). Together these two elements function as an enhancer and mediate activation of the ASNS gene in response to ATF4 (Chen *et al*., 2004). Also, expression of enzymes in the serine biosynthetic pathway (PHGDH, PSAT and PSPH) have also been reported to be upregulated in response to increased expression of ATF4 (Adams 2007).

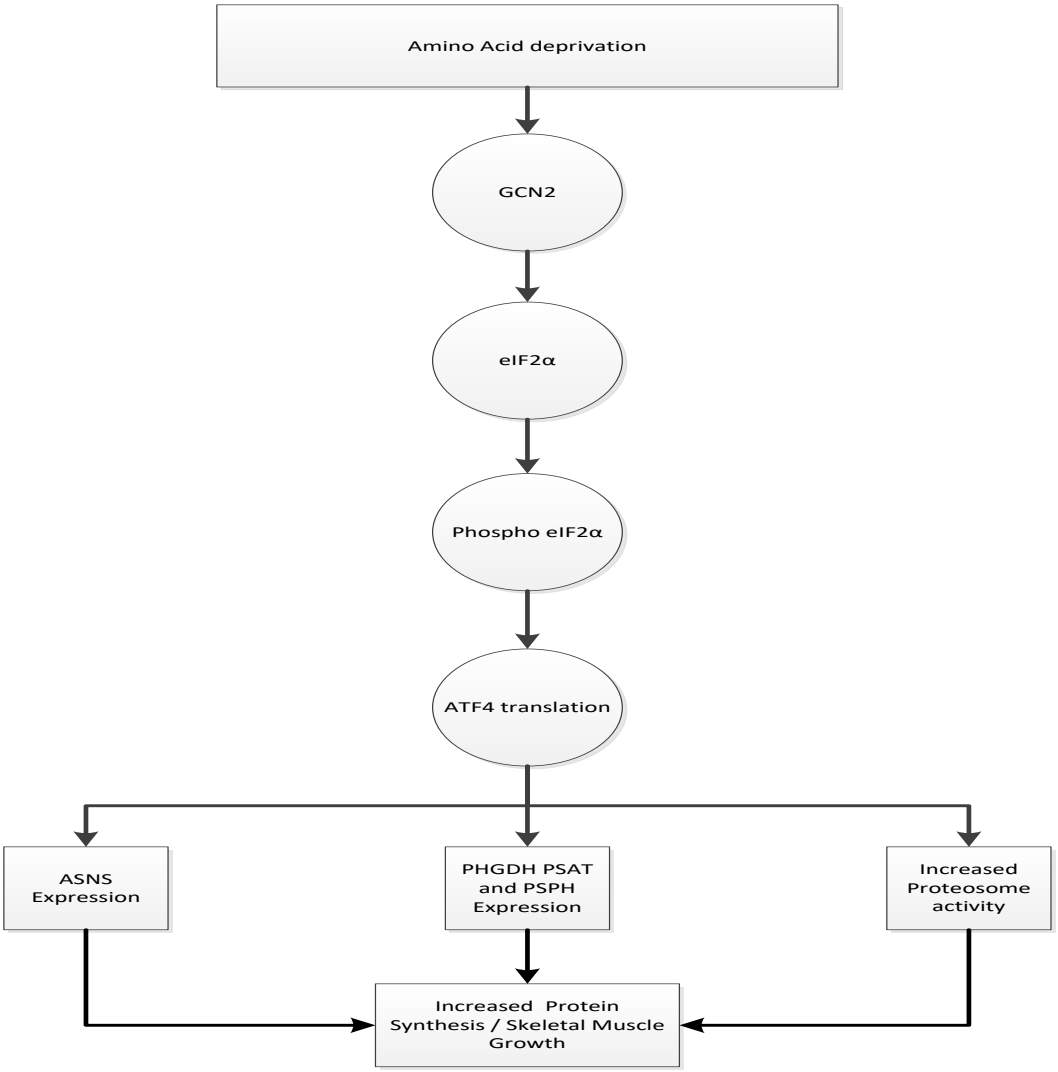


Figure 2.14: A schematic detailing the Amino acid response pathway as it activates the expression of ATF4 leading to the upregulated expression of genes in the serine biosynthetic pathway (PHGDH, PSAT and PSPSH) as well as asparagine synthesis catalysing enzyme ASNS

2.9 PROTEOLYTIC SYSTEM AND SKELETAL MUSCLE GROWTH

Skeletal muscle contains four proteolytic systems that could be involved in protein turnover the lysosomes, the calpains, the caspase system, and the ubiquitin proteasome system (UPS) (Murton *et al*., 2008). The contribution of lysosomal proteases to metabolic turnover of myofibrillar proteins is probably small. Due to their low pH optima as cathepsins are not active at the pH of cell cytoplasm. Furthermore, inhibitors of lysosomal proteinases were reported to fail to diminish the release of N-methylhistidine which is an amino acid only found in actin and myosin on its release during proteolysis (Bradford *et al*., 1986). It is also unlikely that the caspase system displays significant activity in normal-functioning muscle cells, since the caspases are activated by events that initiate apoptosis (Goll *et al*., 2008). Therefore, the calpain proteolytic system and the

UPS are likely to be the main proteolytic pathways involved in metabolic turnover of myofibrillar proteins, in such a way that calpains initiate the process by disassembling and releasing myofilaments, which would be later degraded to small peptides or amino acids by the UPS (Huang and Forsberg 1998). Thus, in this study proteolytic systems were limited to the calpain and the ubiquitin proteolytic systems.

Proteolytic systems are conventionally associated with muscle atrophy. However, in atrophy conditions, an abnormally high activation of the calpain proteolytic systems have been established (Tidball and Spencer 2002; Spencer and Mellgren 2002; Williams *et al*., 1999; Fischer *et al*., 2001). As well as the increased expression of UPS constituents, including components of the 26S proteasome itself (Baily *et al*., 1996). This gives credence to established notion that there is a dynamic adaptation of skeletal muscle to changing requirements. A mechanism by which muscle growth has been demonstrated to be enhanced is by a decrease of protein degradation relative to synthesis, as is seen in beta-adrenergic stimulated growth (Parr *et al*., 2016). But in increased muscle growth can theoretically achieved by increasing protein degradation as long as protein synthesis is sufficient to lead to net protein accretion. These changes in degradation and synthesis are potential most likely to occur in rapid growth where selective degradation is required form remodelling tissues.

2.9.1 Calpain proteolytic system

The calpain proteolytic system serves as the gateway system for skeletal muscle degradation because the calpain acts on larger muscle myofibrils releasing myofibrillar proteins, which serve as substrates for other muscle protein systems such as the ubiquitin proteasome system to act on and further breakdown (Goll *et al*., 2008). Calpains are a class of proteins that belong to the Ca^{2+}-dependent, non-lysosomal cysteine proteases (Koohmaraie and Geesink 2006). There are three major types of calpains which show proteolytic activities expressed in skeletal muscle, namely, micro-calpain (μ-calpain), milli-calpain (m-calpain), and calpain 3 (p94). Micro-calpain and m-calpain are calcium-activated proteases and require micro- and milli-molar concentrations of Ca^{2+}, respectively, for their activation (Goll *et al*., 2003). They are expressed ubiquitously in the skeletal muscle. Calpain 3 requires very little or no Ca^{2+} for its activation and is characterized by a rapid and complete autolysis (Sorimachi *et al.*, 1993). It was originally proposed that calpains are responsible for initiating metabolic turnover of myofibrillar proteins and therefore they affect the rate of muscle protein degradation (Dayton et al., 1975). Calpastatin is an endogenous inhibitor of μ- and m-calpain. It has been reported to greatly reduce calpain activity in muscle, resulting in increased muscle fibre number, and leads to a reduced muscle fibre wasting in transgenic mice (Tidball and Spencer 2002).

Recent investigations have shown that the calpain activity is mandatory for myoblast fusion and for cell proliferation (Barnoy *et al.,* 1996). Calpains also affect the number of skeletal muscle cells in domestic animals by changing the rate of myoblast proliferation and myoblast fusion modulation. In addition to calpains, their specific inhibitor, calpastatin, is also found in the skeletal muscle, which is a highly polymorphic protein and calpain-specific endogenous inhibitor. It is found to be associated with calpain proteolytic enzyme family (Wendt *et al.,* 2004). Several experiments have indicated that calcium ions and calpastatin are the main regulators of the calpain proteolytic system (Koohmaraie 1988; Shackelford *et al.,* 1994). It has been established that one calpastatin molecule can inhibit up to four μ- or m- calpain molecules (Goll *et al.,* 1990) with the requirement of calcium ions which are lower than that required for calpain activation (Kapprell and Goll 1989). Calpastatin is an unstructured protein that adopts a structure that allows inhibition to take place when bound to calpains (Kemp *et al*., 2010).

There is ample evidence that the calpains have main roles, both in normal and postnatal skeletal muscle growth, muscle wasting, and loss of muscle mass. For example, the administration of β-adrenergic agonists to an animal results in a 10-30% elevation in the rate of accumulation of muscle mass (Yang and McElligot 1989). But it has also been reported that the administration of β-adrenergic agonist affects the activity of calpain system (Forsberg *et al.,* 1989) and elevates the muscle calpastatin activity (Higgins *et al.,* 1988) ranging from 52% to 430% (Kretchmar *et al.,*1989). Muscle μ-calpain activity is either reduced or unchanged, while m-calpain activity seems to be elevated (Koohmaraie and Shackelford 1991). Cockett *et al*. (1994) reported that the skeletal muscle mass in the callipyge phenotype sheep was 30-40% greater than that in half-siblings not expressing the callipyge trait. Koohmaraie *et al.* (1995) also reported that calpastatin activity in the affected muscles from callipyge lambs are 68-126% greater than in the same muscles from normal lambs. This finding suggests that elevated rates of skeletal muscle growth can result from a reduction in the rate of muscle protein degradation. This reduced rate of muscle protein degradation is associated with the reduction in the activity of the calpain system.

The calpains are unique among proteolytic enzymes in that they do not rapidly degrade the major muscle proteins, actin and myosin, which also are the major components of the easily releasable filaments. Large size and ordered structure of intact myofibrils would prevent them from being taken up into lysosomes (Lowell *et al.,* 1986). Neither myofibrils nor structurally recognizable fragments of myofibrils have been observed in lysosomal structures. Calpains initiate disassembly of the myofibril by specific cleavages of Z-disk proteins at the surface of the myofibril, releasing the thin filaments from their attachments to the myofibril. The myosin thick filaments that are attached to the released thin filaments dissociate in the presence of ATP in the cell. Calpain induced cleavage of C-protein and M-protein leads to further dissociation of thick filaments to

individual myosin molecules, and these are degraded by proteasome or taken up into lysosomes and degraded by lysosomal cathepsins. Calpain-induced cleavage of tropomyosin as well as troponin T and I, together with the degradation of nebulin, favours dissociation of thin filaments to actin monomers, and these are degraded by the proteasome (Pandurangan and Hwang 2012).

Although available evidence points to the fact that calpains play a role in skeletal muscle protein turnover, it is clear that the calpains cannot degrade the myofibrillar or any other class of proteins to amino acids. Consequently, at least two or three proteolytic systems are involved in the turnover of the myofibrillar proteins.

2.9.2 Caspase proteolytic system

Caspases are cysteine-dependent aspartate specific proteases. They were first identified as the proteases interleukin 1b converting enzyme and an apoptotic receptor (CED-3.1). Their enzymatic properties are governed by a dominant specificity for substrates containing Asparagine, and by the use of a Cysteine side chain for catalysing peptide bond cleavage. (Stennicke and Salvesen 1999). Caspases are essential in cells for apoptosis, or programmed cell death, in development and most other stages of adult life, and have been termed "executioner" proteins for their roles in the cell. Apoptosis, or programmed cell death, plays a central role in the development and homeostasis of all multi-cellular organisms (Horvitz 1999; Jacobson *et al.*,. 1997 cited by Shi 2002). The caspases are responsible for degradation of proteins during apoptosis. The caspases are cysteine proteases, but they do not require Ca^{2+} for activity, as do the calpains.

2.9.3 The Lysosomal System (Cathepsins)

Lysosomal proteases, most of which are cathepsins, are responsible for breaking down peptide polymers to their amino acid building blocks. In addition to their digestive functions, cathepsins are involved in proteolytic maturation of lysosomal proteins and enzymes (Hiraiwa 1999). Cathepsins are located inside lysosomal structures and have acidic pH optima ranging from 3.5 up to 6.5. Therefore, any role of cathepsins in muscle protein turnover would have to occur inside lysosomes. Because of their inactivity in the cytoplasm as a result of their low pH for activity, the presence of cystatin in muscle cells (an inhibitor of cathepsin) and the large diameter of myofibrils, which makes them too large to be engulfed in the lysosome, as well as the relatively small amount of lysosomes in muscle cell, it seems unlikely that lysosomal proteases are involved in metabolic turnover of myofibrillar proteins. (Wildenthal *et al.,* 1980; Lowell *et al.,* 1986 cited by Goll *et al.,* 2007),

2.9.4 The Proteasome system

The proteasome is ubiquitous and essential in eukaryotes. It is estimated that 80 to 90% of all proteins in a cell are ultimately degraded via the proteasome pathway, and it is not surprising therefore that numerous reports have implicated the proteasome in muscle protein turnover (Goll *et al.*, 2008). Protein substrates of this major pathway are first marked for degradation by the covalent attachment of chains of Ub molecules. Proteolysis is then catalysed by the 26S proteasome complex that degrades proteins to small peptides (Lecker *et al.*, 1999). This pathway plays a special role in the accelerated breakdown of myofibrillar proteins in fasting and disease states. The presence of a powerful proteolytic enzyme system within the cell requires the evolution of mechanisms to safeguard against the nonspecific digestion of essential proteins. The first safeguard is the requirement for Ub-conjugation prior to degradation of the protein. Second, the active sites of the proteasome are geographically isolated within the central chamber and away from cytosolic milieu. Third, only unfolded proteins can enter the narrow opening in the α rings on either end of the 20S proteasome (Lecker *et al*., 1999). The ubiquitin proteasome system is a major non-lysosomal protein degradation machinery of the cell that cleaves regulatory misfolded proteins and damaged proteins into small peptide (Struckeberg *et al*., 2010). In this process, cellular proteins for degradation are tagged by multimers of the evolutionary conserved protein ubiquitin followed by degradation of the 26S proteasome. The proteasome (26S proteasome) is a hollow cylinder–shaped multi-protein structure of 2.5 MDa which comprises a core particle (CP or 20S proteasome) covered in one or both sides by a regulatory particle (RP or 19S proteasome). RP comprises a lid and a base sub-complex and functions in ubiquitinated proteins recognition, de-ubiquitination which allows ubiquitin molecules to be recycled, unfolding of the target proteins, and delivery of the proteins to the CP (Voutsadakis 2017). Before tagged proteins are ushered into the 26S proteasome for degradation, A cascade of three types of enzymes known as E1 (or ubiquitin-activating enzyme), E2 (or ubiquitin-conjugating enzymes), and E3 (or ubiquitin ligases) attach the small protein ubiquitin to the substrate protein that is then recognized and degraded by the proteasome particle (Voutsadakis 2010). The 26S proteasome is a giant cytosolic protease composed of two regulatory cap domains (19S-subunits), and a hollow proteolytical active cylinder or domain (20S-subunit) composed of four stacked rings. Two inner β-rings that are identical in sub-unit composition and two identical α–rings (Ciechanover 1998). The proteasome possess three enzymatic activities, a post-glutamyl (caspase-like or post-acidic residues cleavage) activity, a trypsin-like (post-basic residues cleavage) activity, and a chymotrypsin-like (post-hydrophobic residues cleavage) activity that reside in sub-units PSMB6, PSMB7, and PSMB5 (β1, β2, and β5), respectively, and degrade target proteins producing fragments of four to 14 amino acids (Voutsadakis 2008). The chymotrypsin-like site cleaves peptide bonds after hydrophobic residues, and the trypsin-like site cuts after basic residues, whereas the caspase-like site preferentially cuts after acidic residues and has generally been termed postglutamyl peptide hydrolase (PGPH). But it tends to

also cleave after aspartates in fluorogenic substrates of caspases thus the name caspase-like has been suggested (Kisselev *et al*., 2003).

The release amino acids from intact proteins can be distinguished in two parts based on utilization of metabolic energy; the proximal ATP-dependent steps are followed by ATP-independent events. Cellular proteins targeted for degradation by ATP-dependent 26S proteasome are tagged with ubiquitin which is covalently bound to the target protein by an isopeptide linkage between the carboxy terminal glycine of ubiquitin molecule to form polyubiquitin chains (Ciechanover and Iwai 2004). An enzyme E1, transfers ubiquitin to a carrier enzyme E2 which in turn tags ubiquitin to the substrate to be degraded with the help of E3 enzymes/ligases such as Muscle Ring-finger protein-1 or Muscle Atrophy F-box (MAFbx or atrogen-1). Bodine *et al*., (2001) used a differential display approach to identify MuRF1 and MAFbx as genes that are similarly altered under disparate atrophy conditions, including immobilization, denervation, hindlimb unloading, dexamethasone treatment, and interleukin-1-induced cachexia. While in a report by Gomes *et al*., (2001) Affymetrix microarray was used to identify differentially expressed genes from mouse gastrocnemius muscle following 2 days of food deprivation. The most highly upregulated gene in their analysis was a novel F-box protein that they named atrogin-1, which turned out to be identical to MAFbx. Northern blot analysis of additional catabolic models showed that MAFbx/atrogin-1 and MuRF1 was significantly increased in models of diabetes, cancer cachexia, and renal failure (Lecker *et al*., 2004). The generation of mice with a null deletion of either MuRF1 or MAFbx has allowed for the examination of the physiological function and importance of these two E3 ligases in the regulation of skeletal muscle mass. The first atrophy model to be tested using the knockout mice was denervation, where it was demonstrated that deletion of each gene individually resulted in significant sparing of mass in both the tibialis anterior and gastrocnemius muscles; MAFbx-KO showed sparing at both 7 and 14 days, and the MuRF1-KO showed sparing at 14 days (Bodine *et al*., 2001).

Although the proteasome is responsible for much of the intracellular protein degradation that occurs in muscle and other cells, neither myofibrils nor thick and thin filaments can be degraded by the proteasome, because they cannot enter the central catalytic chamber of the proteasome, whether they are ubiquitinated or not. The proteasome only degrades myosin and actin after ubiquitination, after their release from the myofibril Goll *et al.,* (2008).

The negative connotation of protein degradation associated with the proteasome is particularly evident in skeletal muscle where excessive protein destruction is the principle mechanism behind muscle atrophy and weakness. However, recent studies offer an alternative function for the proteasome, for what otherwise has been largely considered to be a conveyor of muscle wasting and pathology. For example, the surge in protein breakdown following resistance and endurance exercise has been hypothesized to be adaptive, as it rids

muscles of damaged proteins and facilitates myofilament restructuring and muscle growth (Bell *et al*., 2016). A study by Cunha *et al*., (2012) reported that chronic loading of mice skeletal muscle using the functional overload model led to skeletal muscle hypertrophy that was characterized by increased protein synthesis and degradation via the ubiquitin proteasome pathway (UPP). However, in contrast another study by Baehr *et al*., (2014) reported an increased proteasome activity independent of MuRF1 and MAFbx expression.

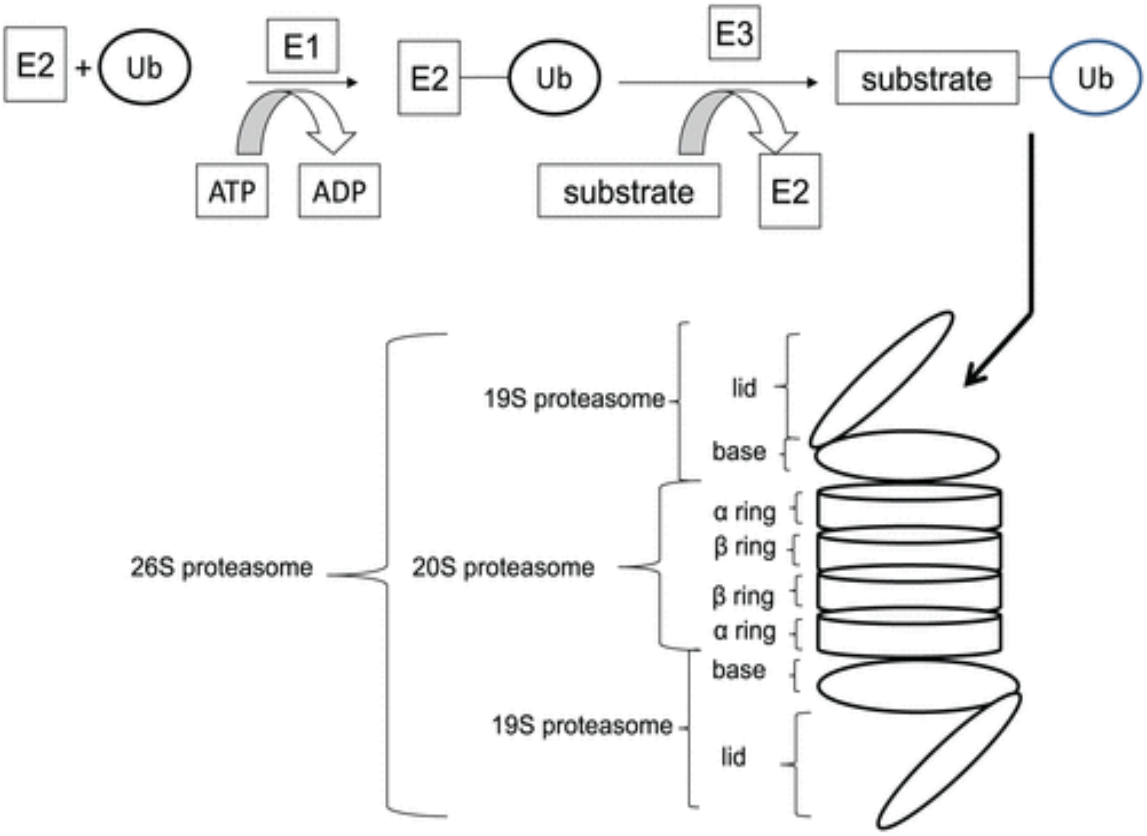


Figure 2.15: The ATP-dependent ubiquitination cascade leading to delivery of target proteins to the proteasome for degradation. The 26S proteasome with its different components is depicted in the lower part of the figure. E1: ubiquitin-activating enzyme; E2: ubiquitin-conjugating enzyme; E3: ubiquitin ligase. (Source: Voutsadakis 2017)

2.10 GROWTH FACTORS AND MUSCLE GROWTH

2.10.1 Insulin growth like factor 1 (IGF-1)

The insulin-like growth factors (IGF-I and IGF-II) have been shown to stimulate multiple growth responses, affecting rates of protein synthesis and degradation, DNA synthesis, and transport of substrates. IGF-1 is a polypeptide structurally related to insulin synthesized in the liver, brain, skeletal and cardiac muscle. IGF-1

from chickens has 88% similarity with its human counterpart. The predominant effect is on growth promoting (Stevens 2004).

2.10.2 Overview of the IGF1-Akt/PKB pathway

A simplified scheme of the IGF1-Akt pathway is shown in Figure 2.16. Binding of IGF1 to its receptor leads to activation of its intrinsic tyrosine kinase and autophosphorylation, thus generating docking sites for insulin receptor substrate (IRS), which is also phosphorylated by the IGF1 receptor. Phosphorylated IRS then acts as docking site to recruit and activate phosphatidylinositol-3-kinase (PI3K) which phosphorylates membrane phospholipids, generating phosphoinositide-3,4,5-triphosphate (PIP3) from phosphoinositide-4,5-biphosphate (PIP2). PIP3 acts in turn as a docking site for two kinases, phosphoinositide-dependent kinase 1 (PDK1) and Akt, and the subsequent phosphorylation of Akt at serine 308 by PDK1, leading to Akt activation. All these steps take place at the inner surface of the plasma membrane. Akt inhibits protein degradation by phosphorylating and thus repressing the transcription factors of the FoxO family and stimulates protein synthesis via the mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3β (GSK3β) (Manning and Cantling, 2007). FoxO factors are required for the transcriptional regulation of the ubiquitin ligases atrogin-1, also called muscle atrophy F-box (MAFbx) and muscle ring finger 1 (MuRF1), leading to the ubiquitylation of myosin and other muscle proteins (see below), and their degradation via the proteasome (Schiaffiano and Reggiani, 2011).

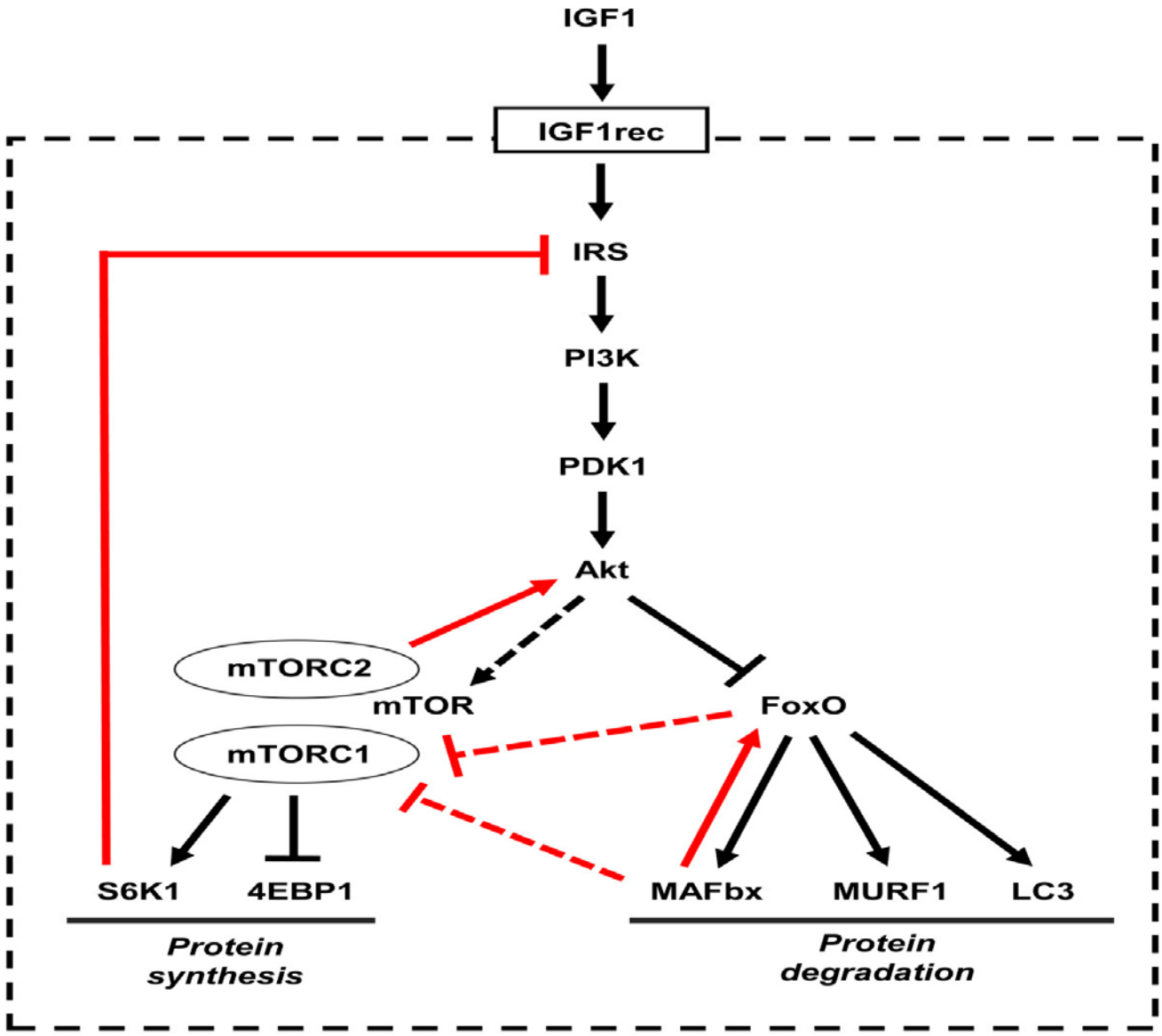


Figure 2.16: The insulin-like growth factor 1 (IGF1)-Akt pathway controls muscle growth via mammalian target of rapamycin (mTOR) and FoxO**.** The internal feedback loops that control the IGF1-Akt pathway are indicated in red. The dotted line indicates that the effect of Akt on mTOR is indirect, being mediated by the tuberous sclerosis complex (TSC) proteins 1 and 2 and by Rheb (Ras homolog enriched in brain) Source: Schiaffino and Mammucari 2011

2.10.3 The role of the IGF1-Akt pathway in adult skeletal muscle

IGF-1 regulates muscle mass during development, due to its effect on myogenic cell proliferation and differentiation. As to the role of IGF1 in adult skeletal muscle, several studies indicate that IGF1 can induce hypertrophy and block atrophy (Schiaffino and Mammucari 2011). Liu *et* al., 2012 reported that *in vivo* feeding of ducks with IGF-1 stimulated growth and especially lead to increased muscle hypertrophy. They observed that muscle fibre diameter (MFD), cross-sectional area (CSA), the number of myofibres per unit area (MFN) and frequency of satellite cell activation and mitosis at the embryo stage of 27 days (27E) and the postnatal stage of 2 days, were increased in the breast and thigh muscle tissues. And observed that growth rate was more

profound in the thigh than the breast muscle. Adams and Mccue (1998) infused either 0.9% saline or non-systemic doses of recombinant human IGF-I (rhIGF-1) directly into a non-weight-bearing muscle of rats, the tibialis anterior (TA). Saline infusion had no effect on the mass, protein content, or DNA content of TA muscles. Local IGF-I infusion had no effect on body or heart weight. The absolute weight of the infused TA muscles was significantly ~9% greater than that of the contra-lateral TA muscles. IGF-I infusion resulted in significant increases in the total protein and DNA content of TA muscles suggesting that IGF-I may be acting to directly stimulate processes such as protein synthesis and satellite cell proliferation, which result in skeletal muscle hypertrophy.

2.10.4 The effect of IGF1-Akt pathway activation on satellite cells

Muscle growth and muscle regeneration require the participation of satellite cells, and other cell types, including cells of blood vessels and, in the case of regeneration, inflammatory cells. This aspect must be considered in genetic models involving over production of IGF1, as this growth factor is known to act on different cell types, because of the presence of the IGF1 receptor in satellite cells and non-muscle cells. The effect on satellite cells is especially important for muscle hypertrophy. Satellite cells were reported to play a crucial role in the hypertrophic response induced by viral-mediated gene transfer of IGF1 in adult mouse muscles, as shown by the fact that gamma-irradiation, used to block satellite-cell proliferation, was found to reduce the hypertrophic effect of IGF1 overexpression (Schiaffino and Mammucari 2011).

2.10.5 Myostatin

Myostatin is a secreted protein that negatively regulates skeletal muscle mass determining both muscle fibre number and size. The myostatin pathway is conserved and regulates muscle mass in a number of animal species ranging from fish to humans. Myostatin regulates activation, proliferation and self-renewal of the muscle satellite cell pool. Moreover, loss of myostatin results in enhanced skeletal muscle regeneration in response to injury, whereas increased post-natal myostatin expression is associated with many skeletal muscle wasting conditions. Furthermore, myostatin has been shown to directly induce cachexia following subcutaneous injection of myostatin over-expressing cells into mice. Mice carrying a targeted deletion of the myostatin gene have a dramatic and widespread increase in skeletal muscle mass. Individual muscles of myostatin null mice weigh approximately twice as much as those of wild-type mice as a result of a combination of muscle fibre hyperplasia and hypertrophy (Lee and Mcpheron 2001).

Myostatin acts by inhibiting the growth of muscles, it prevents them from growing too large. It is also known as growth and differentiation factor 8 (GDF-8). It is a protein made up of two identical subunits. Each subunit

contains 110 amino acids. The gene encoding myostatin is termed MSTN (or GDF8) and is on chromosome 2 in band 2q32.1 in human beings. Myostatin is also a member of the transforming growth factor beta (TGF-beta) family. All of the members of this gene family regulate growth and differentiation from early embryogenesis to mature cell types and tissues. Myostatin is a transforming growth factor-β family member that acts as a negative regulator of skeletal muscle mass. *Mstn*$^{-/-}$ mice exhibit an approximate doubling of skeletal muscle mass throughout the body as a result of a combination of increased numbers of muscle fibres and increased muscle fibre sizes. The function of myostatin is highly conserved among mammals: naturally occurring mutations in the *MSTN* gene resulting in increased muscling have been identified in cattle, sheep, dogs, and humans (Lee *et al*., 2012). Figure 2.17 shows genetically modified animals engineered to knock out the MTSN gene.

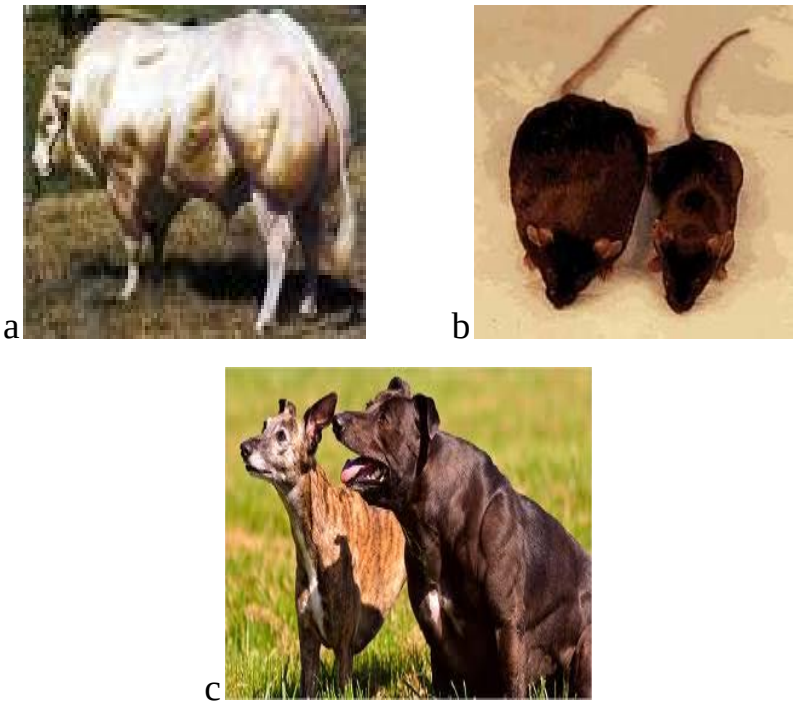


Figure 2.17: Genetically modified animals engineered to knock out the MTSN gene. The larger looking mouse in figure b and dog in figure c shown in pictures are genetically modified while the smaller ones are the conventional breeds.

Characterization of the intracellular signalling pathway for myostatin is still in its infancy. Much of what is known has been inferred by knowledge of the Transforming Growth Factor β-Like TGF-β signalling pathway. (Kollas and Dernott 2008). In a study conducted by Rebbapragada *et al*., (2003) the myostatin signal

transduction pathway was characterized. They showed that myostatin binds the type II Ser/Thr kinase receptor. ActRIIB, and then partners with a type I receptor, either activin receptor-like kinase 4 (ALK4 or ActRIB) or ALK5 (TβRI), to induce phosphorylation of Smad2/Smad3 and activate a TGF-β-like signalling pathway. (see fig 2.18). Myostatin is a member of the TGF-β family of secreted proteins but unlike TGF-β, it is predominantly expressed in skeletal muscle. In skeletal muscle, myostatin is produced as a prepromyostatin and is processed to promyostatin, consisting of a propeptide and myostatin. The propeptide binds to myostatin to produce an inactive latent complex which is activated by proteolysis yielding free myostatin which binds to ActRIIB (high-affinity type-2 activin receptor) on muscle membranes (Lee and McPheron 2001). Myostatin-ActRIIB binding results in the activation of type-1 activin receptor serine kinases, ALK4 or ALK5, which phosphorylate Smads 2/3 to exert changes in gene transcription eventually leading to muscle wasting (Han and Mitch 2011).

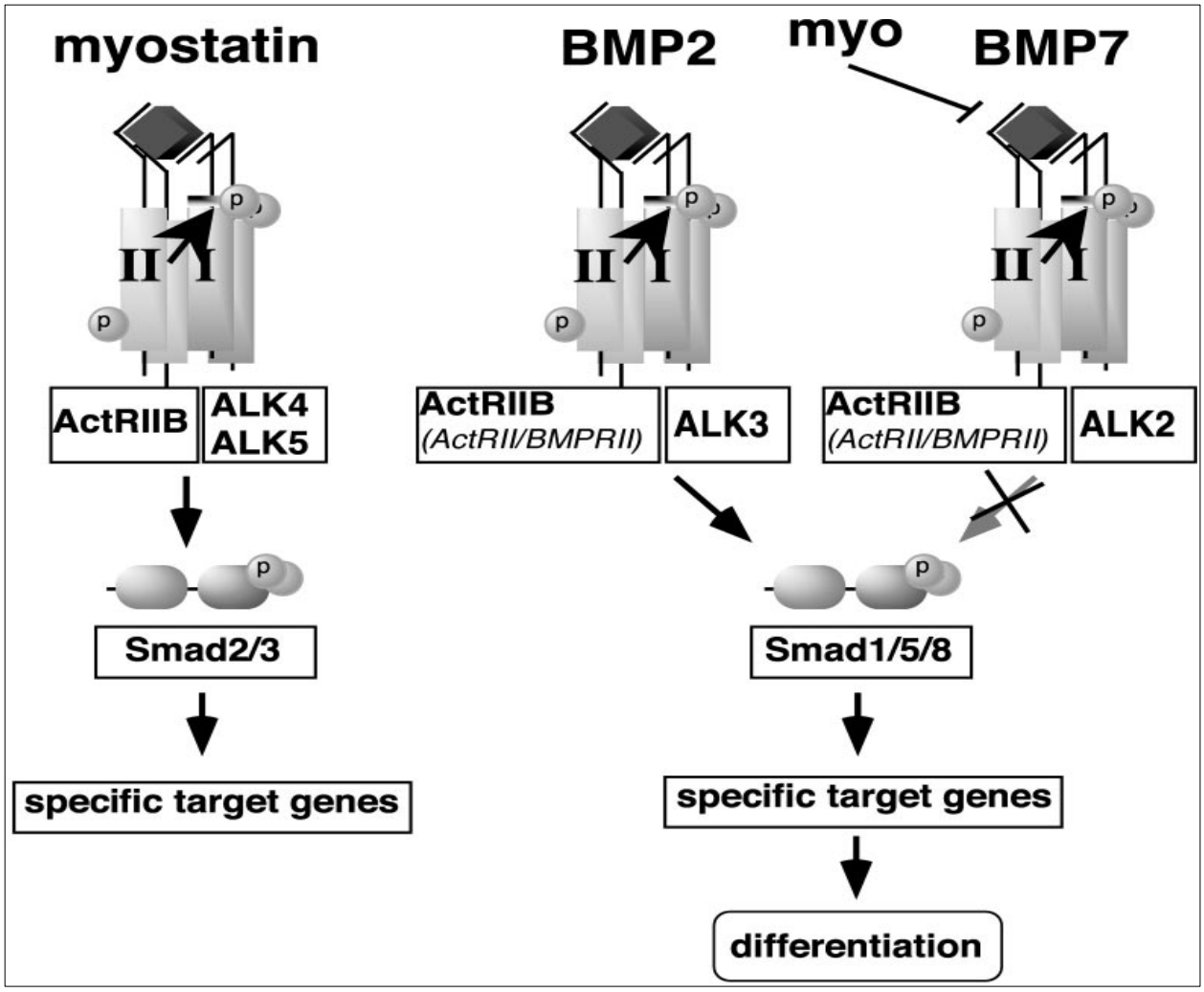


Figure 2.18: Model of extracellular and intracellular antagonism of BMP signalling by myostatin and TGF-_. Myostatin signals through ActRIIB and either ALK4 or ALK5 to activate a TGF-_-like signalling pathway. Myostatin potently antagonizes BMP7 but not BMP2 by competing for BMP7 binding to the ActRIIB type II receptor. (Source: Rebbapragada *et al*., 2003).

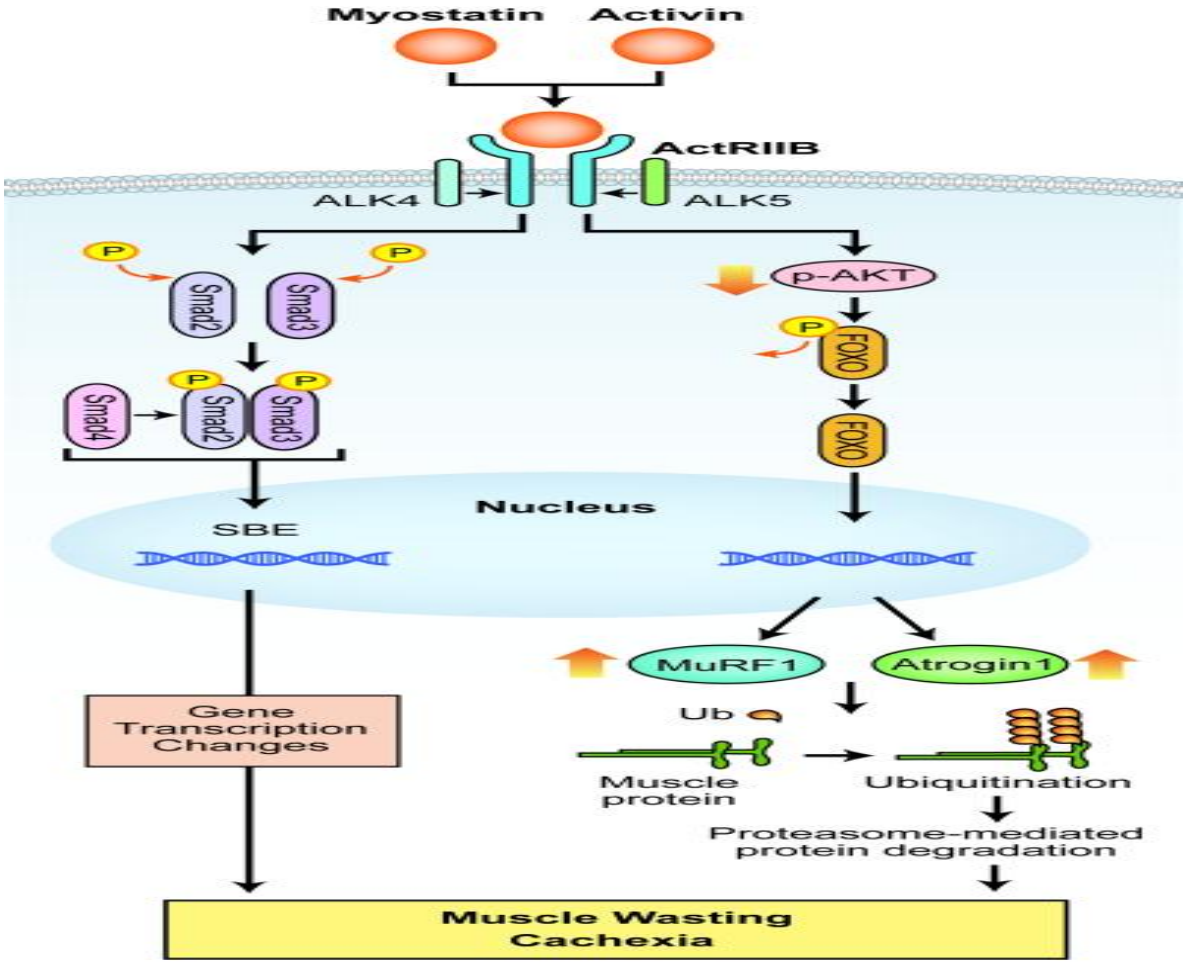


Figure 2.19: Myostatin and activin signalling in muscle. Myostatin or activin binds to type IIB activin receptor (ActRIIB) on muscle membrane to cause its dimerization, which leads to recruitment and activation of type I activin receptor transmembrane kinase ALK4 or ALK5. This in turn causes phosphorylation of Smad2 and Smad3 and the recruitment of Smad4 into a Smad complex. The Smad complex translocates into the nucleus to elicit transcription changes of downstream genes, which result in muscle wasting. Myostatin/activin binding to the receptor also reduces AKT activity and consequently diminishes FOXO phosphorylation. Dephosphorylated FOXO enters the nucleus to activate transcription of atrophy-specific E3 ligases MuRF1 and Atrogin1, which cause muscle protein ubiquitination and degradation by the proteasome. (SBE = S mad binding element; Ub = Ubiquitin). Source: Rebbapragada *et al*., 2003).

2.10.6 Fibroblast growth factor (FGF)

Fibroblast growth factor has been known to be involved in the repair and regeneration of tissues comprising of about 22 members (Yun *et al.,* 2010). They are known to be associated with regulation of skeletal muscle development, and, therefore, potential activators and mediators of satellite cells in vivo during postnatal hypertrophy (Mitchell *et al*., 1998). They have also been shown to activate dormant satellite cells (Chakkalakal *et al*.,2012).

FGFs act as signal molecules that bind and activate FGFRs. Activated FGFRs mediate signalling by recruiting specific molecules that bind to phosphorylated tyrosine at the cytosolic part of the receptor, triggering a number of signalling pathways leading to specific cellular responses. These then serve as docking sites for the recruitment of SH2 (Src homology-2) or PTB (phosphotyrosine binding) domains of adaptors docking proteins or signalling enzymes. Signalling complexes are formed and recruited to the active receptors resulting in a cascade of phosphorylation events. The best understood pathways are the RAS/MAP kinase pathway, PI3 kinase/AKT pathway, and PLCγ pathway. Figure 2.20 schematically describes the three pathways of the FGF signal, the RAS/MAP kinase pathway, PI3 kinase/AKT pathway, and PLCγ pathway (Yun *et al.*, 2010).

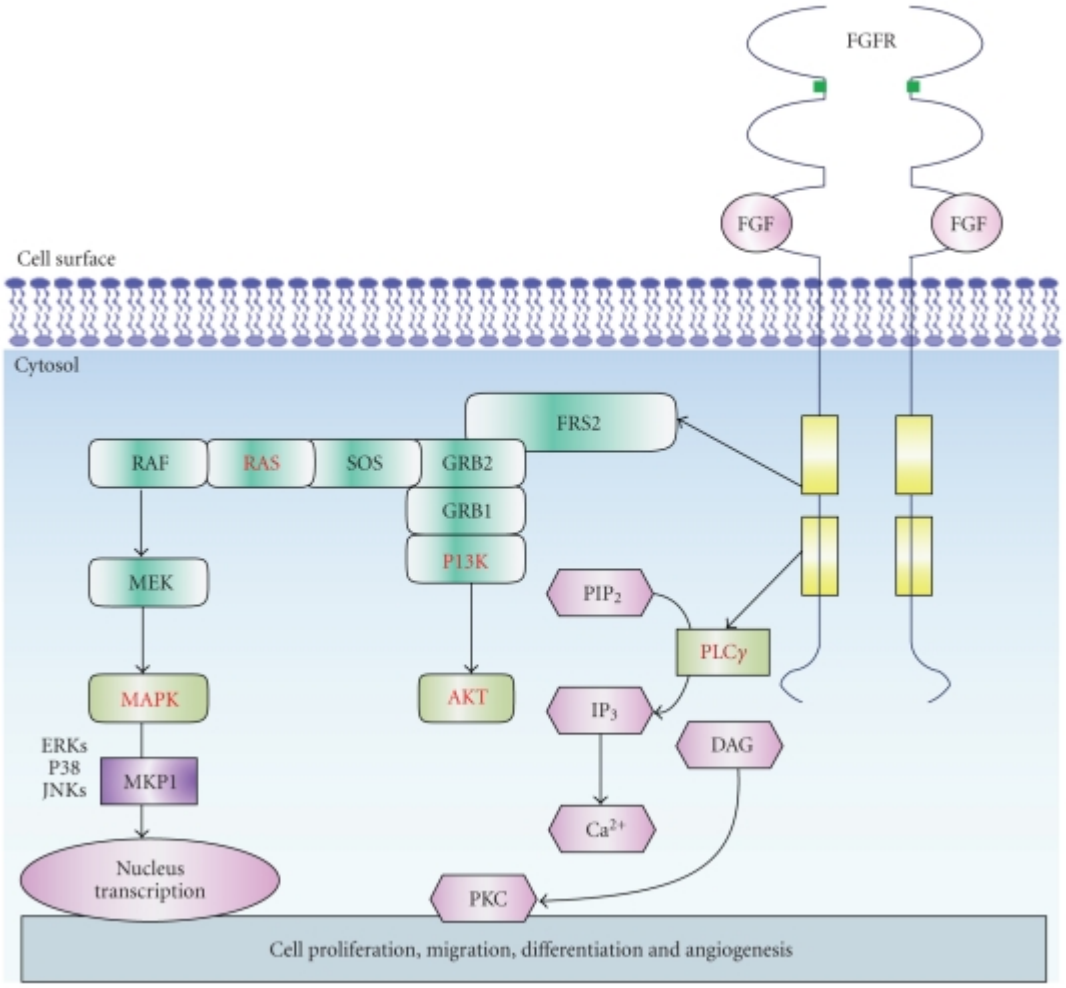


Figure 2.20: FGF signalling pathway FGF signal pathway. FGFs stimulate tyrosine phosphorylation of the docking protein FRS, followed by forming the GRB2-SHP2-GAB-1 complex resulting in activation of RAS-MAP kinase pathway and PI3 kinase/AKT pathway. In PLCγ pathway, activated PLCγ hydrolyses phosphatidylinositol, generating IP3 and DAG and results in the activation of PKC. FRS2: fibroblast growth factor receptor substrate 2, GRB: guanine nucleotide exchange factor, SOS: son of sevenless, RAS: monomeric G-protein, RAF: kinase, MEK: kinase, MKP1: MAP kinase phosphatase, PIP2: phosphatidylinositol (4,5)-bisphosphate, IP3: inositol triphosphate, DAG: diacylglycerol, PKC: protein kinase C. (Source: Yun *et al*., 2010)

2.11 LIM DOMAIN PROTEINS

LIM domain proteins perform critical roles in embryonic development and tissue differentiation as well as translational regulation MLP is a member of the cysteine and glycine rich protein (CSRP) family that is composed by CSRP1, CSRP2 and CSRP3 or muscle LIM proteins (MLP). CSPRs, in turn, belong to the larger family of LIM domain proteins. (Kadrmas and Beckerle 2004). MLP is also involved in myocyte differentiation by activating transcription factors such as MyoD and myogenin. MLP protein levels or intracellular localization are altered in skeletal myopathies, such as fascioscapulohumeral muscular dystrophy, nemaline myopathy and limb girdle muscular dystrophy type 2B (Reviewed by Rashid *et al*., 2015). FHL2 is another LIM domain protein belonging to a subfamily containing four and a half LIM domains reported to induce differentiation in mouse myoblast and also downregulated in expression during rhabdomyosarcoma (Martin *et al*., 2002). Suggesting that FHL2 plays a role in the regulation of muscle growth.

Muscular LIM domain proteins have been implicated in skeletal muscle dystrophies. An increase in the protein expression of a novel isoform of a particular MLP was reported to have a higher protein expression in patients with neuromuscular dystrophic diseases (Vafiadaki *et al*., 2014) In a comparative study between broiler and layer chickens, Zheng *et al.,* (2009) identified 543 differentially expressed genes in the *Pectoralis major* (PM) across five developmental stages after hatching. The LIM domain proteins genes with the greatest differential expression which were upregulated in the slower growing muscles were those encoding LIM domain proteins, Cysteine and Glycine Rich Protein 3 LIM protein (CSRP3) and four and a half LIM domain protein (FHL2). CSRP3 has also been reported to undergo an upregulated expression in bovine skeletal muscle in response to and during recovery from chronic and severe undernutrition (Lehnert 2006).

2.12 SUMMARY AND OBJECTIVE OF THE THESIS

Our understanding of muscle growth has increased over time and continued advances in the understanding of skeletal muscle biology will likely give more insight on the mechanisms regulating skeletal muscle growth. Chickens are a good model to study these mechanisms because due to genetic selection for fast growth rate in the breast muscle broiler chickens, we can use chickens as a model to study molecular basis for diversity in muscle growth rate. This can be achieved by differentiating the way biological systems work to accentuate or attenuate muscle growth in genetically close yet phenotypically diverse growing chickens having different muscle growth rate. Thus, elucidatng the molecular mechanisms that accentuate muscle growth in fast growing muscles. This thesis aimed to develop on already understood mechanisms that upregulate muscle growth and identify which mechanism is more pronounced in genetically selected chickens that exhibit a fast muscle growth rate phenotype.

The starting point of comprehending skeletal muscle growth is an understanding of the mechanisms modulating skeletal muscle protein turnover and muscle fibre size. Muscle fibre size increment occurs as a result of the continued synthesis and deposition of skeletal muscle proteins. In order for muscle growth to occur, there has to be a positive skeletal muscle protein turnover where protein synthesis and muscle fibre protein deposition supersedes muscle protein degradation. Thus, the questions we sought to answer was what molecular mechanisms upregulating muscle growth in fast growing chickens are more activated and how do these mechanisms interact? It has been established that broiler chickens have a greater muscle protein turnover rate than layers Teseraud *et al*., 2000 Jones *et al*., 1986). But the molecular mechanisms regulating this process has not been fully understood. Apart from protein turnover, there are other molecular mechanisms that modulate muscle growth such as growth factors, fibre type composition, signal transduction, metabolic factors modulating protein synthesis and degradation as well as protein degrading modulating genes. This study aimed to investigate these mechanisms on an individual basis and find out the most prominent mechanism that can be attributed to this phenotype in broiler chickens that has occured as a result of selective breeding. The intention was to build on knowledge already gained on processes that modulate muscle growth which include metabolic systems, protein synthesis, endogenous proteolytic systems, growth factors and myogenic regulatory factors.

The expression of growth factor genes such as IGF-1, myostatin and FGFR2 were comparatively measured to determine if these genes have been altered as a result of selective breeding. Metabolic systems were also investigated. Since the muscle fibre type in chicken have been fully characterised and fast muscle growing chickens have been shown to have a fast fibre type profile **(**Aberle and Stewart 1983; Cooke *et al*., 2003**).** It was our intention to investigate glycolytic metabolic properties of the different muscle types in the divergent

growing muscles of chickens. This was done by measuring the expression of glycolytic enzymes to ascertain the glycolytic capacity in these muscles and relate it to the their known fibre type composition. In addition to that, literature points to the fact that glycolytic metabolites are affiliated with cell growth as seen in growth promoter treated animals specifically pigs and sheep **(**Al-Doski *et al*., 2015, Brameld *et al*., 2015, Parr *et al*., 2015**)** and hyperplastic cancer cells **(**Posemato *et al*., 2011; Dann and Abraham 2011**).** Thus, an investigation of the *de novo* biosynthesis of glycolytic metabolites was carried out. Notably among these were the expression of genes in the serine biosynthesis pathway (PHGDH, PSAT and PSPH) as well as asparagine biosynthesis (ASNS). The objective was to comparatively measure the expression of these genes in divergent growing muscles and relate it with the rate at which those muscle grow. The metabolites identified were mainly non-essential Amino acids (EEAs). Our understanding of the expression of the non-EEAs amino acid synthesising catalysing enzymes may help us understand the amino acid metabolic demand from muscle cells for muscle growth. This knowledge may be particularly important as it may give information on animal nutrition to help improve the nutrition regimen and diet formulation of meat animals. It may also be important because most of the amino acids are precursors to secondary metabolites for example, serine is a precursor to the biosynthesis of other metabolites such as choline and nucleic acids. Thus, our understanding of the metabolic properties of fast growing broiler chicken in relation to non-EEA synthesis may open an array of questions for further investigation such as; is there an upregulated synthesis of non-EEAs in differentiated hypertrophic fast-growing chicken skeletal muscle as seen in highly proliferative cancer cells? If so what mechanism could explain this relationship between fast growing muscles and upregulated non-EEA synthesis? Could the effect be as a result of these synthesised amino acid being used for protein synthesis? or is there a secondary effect as a result of the metabolites of these non-EEAs such as the synthesis of nucleic acids to transcribe and translate more muscle protein materials for deposition?

Furthermore, our knowledge of how protein synthesis and degradation determine protein turnover and muscle growth allowed us to investigate and gain more understanding of protein synthesis and proteolytic systems. Other workers have identified that protein synthesis is modulated by two signalling pathways which are the AKT/mTOR and the amino acid response (AAR) signalling pathways. The AKT/mTOR pathway is well characterised and Growth factors such as IGF1 and FGF are known to accentuate protein synthesis via this pathway. Apart from that, investigating the expression of intermediates of this pathway that are known to upregulate protein synthesis would give us an understanding of how this pathway is pathway in regulated in divergent growing chicken muscles. Also, the synthesis of non-EEAs has been established to be driven by the AAR pathway particularly the synthesis of serine and asparagine. Thus, investigating the synthesis of these non-EEA's in divergent growing chicken muscles could point in the right direction in elucidating how the AAR signalling pathway modulates protein synthesis and muscle growth.

Lastly investigating proteolytic systems may give us an idea on how the interaction between protein synthesis and degradation could impact on muscle protein turover. Proteolytic systems are conventionally associated with muscle atrophy. A condition where the calpain and proteasome proteolytic system have been reported to be upregulated. (Tidball and Spencer 2002; Fischer *et al*., 2001; Baily *et al*., 1996). However, in this study the animals were healthy and were not undergoing any form of muscle wasting or atrophy but had variable genetic and metabolic requirement for muscle protein accretion under normal growth conditions. Thus, our observation will give an insight into how these two proteolytic systems modulate skeletal muscle growth under normal physiological conditions and how it interacts with protein synthesis systems to regulate protein turnover.

The hypothesis was that the metabolic systems regulating the expression of glycolytic genes and synthesis of non-essential amino acids are upregulated in faster growing chicken muscles. In addition, turnover is coordinated in such a way that proteins synthesis and degradation interact to bring about a net positive turnover rate in faster growing muscles of chickens and genes positively regulating protein synthesis are upregulated in faster growing chicken muscles.

In summary, this thesis intended to conduct the following bod of work:

- Characterise and establish a relationship between metabolic parameters, gene expression of glycolytic metabolic enzyme, expression of genes associated with muscle protein degradation and muscle mass regulation.
- Characterise the expression of genes associated with serine biosynthesis and asparagine biosynthesis to gain an understanding of how products of the AAR signalling pathway are regulated in differential growing mucles.
- Characterisation of gene and protein expression of intermediates involved in the mTOR/AKT signalling cascade and to elucidate the relationship with those intermediates and skeletal muscle in muscles growing at different rates.
- To characterise the expression of genes involved in skeletal muscle cell cycle (Myogenin, MUSTN1 and FGF2R) in divergent growing muscle of chickens.
- To determine the relationship between gene expression of LIM domain proteins and muscle growth in the two-divergent growing *Pectoralis major* (PM) and *Peroneous tertius* (PT) muscles.
- Investigate the regulation of muscle growth by proteolytic systems (calpain and proteasome) in chicken.

Two trials were therefore carried out to investigate these objectives. Trial 1 was a pilot study while trial 2 was an extended study to verify the findings of trial 1 and to further our investigation.

CHAPTER 3. (MATERIALS AND METHODS)

3.1 TRIAL DESIGN AND SAMPLE COLLECTION

Two chicken growth trials were carried out where birds were raised on a standard broiler diet and given feed and water *ad libitum* from one day old to slaughter, in both trials. The birds were housed accordingly to standards set by the Department for Environment Food and Rural affairs (DEFRA). The birds were housed at 32^{o}C from day 1 and this dropped by 1^{o}C per day down to 21^{o}C. The animals were grouped housed with the floor area allowance of 250cm^2 for birds <200 (g) and 2100 cm^2 for birds that weighed >2400 (g). The light/dark cycle consisted of providing light for 12 hours daily and were thereafter housed in darkness for the remaining 12 hours. Birds were humanely euthanized by a trained animal technician by method of a Schedule 1 to the animals (Scientific Procedures) act 1986 according to the Home Office. Birds under 1.5kg were euthanized with CO_2 gas followed by cervical dislocation. Birds over 1.5kg were euthanized with pentobarbital IV injection (0.7 ml/kg body weight).

Trial 1

A total of 18 male Ross 308 broiler chickens (PD Hooks Hatcheries Limited, Oxfordshire, UK) were raised as described above. Six birds were randomly selected for euthanization and sample collection at each time point, days 14, 36 and 43 post hatch. These time points were selected in order to study the variation in parameters measured at different stages of development of the chickens. After death the birds were weighed, after which a sample of the *Pectoralis major* (PM) of the breast muscle and *Peroneus tertius* (PT) of the leg muscle were immediately dissected, snap frozen in liquid nitrogen and stored at -80^{o}C prior to analyses. The contra lateral muscles were dissected out and weighed.

Trial 2

A total of 30 intact male Ross 308 male broiler chickens (PD Hooks Hatcheries Limited, Oxfordshire, UK) and 30 intact male Hy-Line layer type cockerels (Hy-Line UK Limited, Warwickshire, UK) were raised on a standard broiler diet from one day old to slaughter. The birds were grouped housed, with 6 per group, and fed *ad libitum* from day old to slaughter. The birds were euthanized at five different time points at day 4, 14, 23, 35 or 42 days old post hatch. Six birds were randomly selected from each genotype at each time point for euthanization. After which the birds were weighed and a sample of the PM, PT were immediately dissected

and snap frozen in liquid nitrogen and stored at -80^{o}C prior to analyses, whilst the contra lateral muscles were dissected out and weighed.

3.2 PRODUCTION AND METABOLIC PARAMETERS

3.2.1 Glycogen assay

This was done with a glycogen standard curve using an assay utilising amyloglucosidase in 96 well plates. A range of glycogen dilutions for the standard curve was prepared for digestion to glucose. This was done using 80 unit per ml amyloglucosidase (extracted from *Aspergillus niger*) (Dreiling *et al*., 1987; Adamo and Graham 1998). Neutralised solution was prepared by mixing 8% (v/v) pechloric acid (PCA)**,** saturated Na_2CO_3 and 0.4M sodium acetate pH 4.8 in the ratio of 1:1.25:1.25. Glycogen was weighed into a tube and a stock solution was prepared by diluting the known weight in neutralised solution to 5mg glycogen per ml of solution. From this solution, a range of standards with concentrations from 0.05-0.75mg/ml was prepared.

Samples which had been crushed under liquid nitrogen were weighed and diluted in 8% (w/v) PCA at a concentration of 0.25g/ml. The samples were homogenized in the PCA with a homogeniser (Polytron) in a fume hood for approximately 45 seconds each. A 1ml aliquot was removed from the solution and placed in a 1.5ml tube. The tubes were centrifuged in a bench top centrifuge for 5 min at 13,000xg at room temperature after which 100µl of each of the supernatants were transferred into separate 1.5ml tube. To each supernatant sample 125 µl saturated sodium bicarbonate solution was added. The solution was then mixed, after which 125 µl of 0.4M sodium acetate buffer pH 4.8 was added and mixed. This solution, the neutralised supernatant solution, had a final volume of 350µl. The sample extract supernatants were further diluted in neutralised solution in order to fall within the range of the standard curve. The dilution factor was dependent on the concentration of glycogen in tissue samples. To the neutralised supernatant solution 6.5µl of amyloglucosidase solution (80mg/ml) was added and incubated on a heating block at 38°C for 30min. After the incubation, the mixtures were heated for 5 min at 100^{o}C to stop enzyme activity then cooled on ice.

To assess the quantity of glucose present, an enzymatic glucose assay was carried out with glucose oxidase to produce a product that can be detected by development of a colour. An aliquot of 10µl of each solution were taken (from tissue extracts and standards) and transferred into the wells of a 96 well plate in triplicate. To this was added 150µl of enzymatic glucose assay solution glucose oxidase (Thermo Electron Corporation). The plate was incubated at room temperature for 20min following this absorbance of the plate was then measured at 550nm wavelength using a micro plate reader 680XR (BioRad).

3.2.2 Lowry assay for protein quantification

This was done with a protein standard curve using Lowry assay in 96 well plates (Lowry *et al*., 1951). Protein extraction was done by weighing 200 mg of ground muscle powder (crushed under liquid nitrogen) in 5 volumes (1ml) of extraction buffer (PH 7.5) containing 150mM NaCl, 50mM HEPES, 2.5mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 200µg/ml 2-(4-aminoethyl)-benzenesulphonyl fluoride (AEBSF) and 1 tablet each of protease inhibitor (ROCHE cOmplete protease inhibitor cocktail) and phosphatase inhibitors (ROCHE PhosSTOP) per 10ml extraction buffer. For Lowry assay, 50µl of whole homogenate was removed and 950µl of 0.1M NaOH was added in labelled 1.5ml tubes.

A range of protein standards of known concentration ranging from 0.2-1.2mg/ml was prepared by diluting Bovine Serum Albumin (BSA) in extraction buffer. To 5ml of these standards were loaded in triplicates into 96 well plates to obtain a standard curve for quantifying protein concentration. To 5µl of extracted protein homogenates was also loaded on to the plates in triplicates and 195 µl of 0.1M NaOH was added to the wells containing the standards and homogenates, making it up to 200 µl. Following this, 50µl of reagent 1 containing 2% (w/v) Na_2CO_3 1% (w/v) $CuSO_4$ and 2% (w/v) KNa tartrate was thereafter added to each well. Following incubation for 5 minutes at room temperature, 50µl of reagent 2 containing 5ml 0.1M NaOH and 0.5 ml Folins and Coicalteau reagent was added, then incubated at room temperature for another 20 minutes for colour change to take effect. Absorbance of the plate was then measured at 665nm wavelength using a micro plate reader 680XR (BioRad).

3.2.3 DNA Assay

This was done with a DNA standard curve using DNA assay in 96 well plates. DNA standards were prepared by dissolving 12mg DNA from extracted from calf thymus (Rago *et* al., 1990) in 12 ml of SDS SSC solution (0.05% (w/v) sodium dodecyl sulphate (SDS), 0.15M of sodium chloride and 15mM of sodium citrate). The stock solution was then placed on a roller mixer in a cold room at about 15^{o}C for 3 days to allow the DNA to dissolve. After which the DNA strands in the solution were fragmented with a sonicater for two minutes. DNA standards were made having a highest standard concentration of 20µg/ml, by pipetting 20µl out of the stock solution and then diluting to 1ml, then making sequential dilution by 50-fold dilutions in SDS SSC buffer, to get standards with the concentration range of 0.3-20 µg/ml. A blank containing no DNA in buffer was also prepared to correct the standard curve.

DNA extraction was done by homogenizing 1g of crushed samples (crushed under liquid nitrogen) in 3ml of SDS SSC buffer for 45 seconds. The homogenates were then centrifuged at 13,000xg for 5 minutes at -20^{o}C, after which a known volume of the supernatant solution was loaded into another set of 1.5 ml centrifuge tubes and diluted by 50-fold in 0.1M NaOH (10µl supernatant solution to 490 0.1M NaOH). To quantify DNA concentration, 100ul of DNA standards and extracted muscle DNA supernatants, as well the blank, were loaded into 96 well plates in duplicates after which 100ul 1 X TNE solution (100mM Tris, 1M NaCl, 10mM EDTA, pH 7.4) containing 1mg/ml Hoechst dye.

3.3 BIOINFORMATIC STUDIES

3.3.1 Sequence retrieval and sequence alignments

Relevant RNA sequences for genes of interest were searched for using the National Centre for Biotechnology Information (NCBI) database. *Gallus* mRNA/cDNA sequences were aligned using ClustalW2 software to check for similarities between transcripts that had different isoforms. The same software was also used to check for similarities mRNA sequences of some chicken genes with similar genes of other species.

3.3.2 Specific primer design

Specific primers are single stranded oligonucleotides that are designed to prime and amplify a specific cDNA during a Q-RT-PCR reaction. Primers were designed using Primer Express software (Applied Biosystems) and NCBI website. Primers were designed to specifically amplify only the gene of interest been measured and did not detect or react with any other sequence in the synthesised cDNA. Primers were also designed to span exon-exon boundaries, thus preventing the amplification of sequences from residual genomic DNA carried over from the RNA extraction step. This is because genomic DNA contains both non-coding introns and coding exons, whilst cDNA contains only spliced exons. All working primer sequences designed and used for this study are shown in Table 3.1 below.

Table 3.1 List of chicken gene transcripts measured and the forward and reverse primer sequences

Gene transcript	Forward primer (5' to 3')	Reverse primer (5' to 3')
Cyclophilin	GGGATTTGGCTACAAGGGCT	GCCTAAAGTCACCACCCTGA
β-actin	AATCAAGATCATTTGCCCCACCT	ATCCTGAGTCAAGCGCCAAA
GAPDH	TGACCACTGTCCATGCCATC	GGGAACAGAACTGGCCTCTC
MTSN	CCTGGAACAAGCACCTAACA	TCGTCCCTCTGGACATCATA
IGF1-R	CCGGACACAGAGGAGCTTGA	GTGCTTGTCAGTGGGTTGGA
PHGDH	GGCATGAAGACCATCGGCTA	GTGTGGGAAGTGCGTTTGTC
PSAT	GTGAGGACTTGCTGGGATTT	CTGTAGCATGGTGGAGTGTTAT
PSPH	CCTCAGCTAACACCAGGAATAC	TGCTCCACAATGCTCTGAAA
Calpastatin	AGCTTCTACGTCGGGAGGAA	TGGTGTTGGCTTGACAGGTT
α-enolase	ATTCTGAAGATCCACGCCCG	GCAGCTCTGAACAGACCCTT
Myogenin	AGCCTCAACCAGCAGGAG	TGCGCCAGCTCAGTTTTGGA
P70s6k	GGAGAAGTTCAGGCTCACCC	GTTCCACCTTTCGTGCCAAC
4EBP1	CCTCTCCGTGTGGGTGTGA	CCCCACAGCCCATCATCA
MUSTN1	TGAAGGAGGAAGATCTCAAAGGA	GCCCATTTGTTCACACTGCTT
FGF2R	TGGACGGCTTCCAGTAAAGTG	TCTCCCACATTAGCACACCAAT
CSRP3	CCAAAAAGATGGTTGACGTGG	GCATGTTTTGTGCCAAGGTTT
FHL2	GTTTGCCATGCAGTGCGTC	GCATGTTTTGTGCCAAGGTTT
PCK2	ACGGGTCTCCGGAGCAA	GACCTTCGTTATCTCGGAGGAA
ASNS	CAGCACATGGTCTTGAGCTAAGA	CGCAGTTCTGCTGGTAGAGATAAA
β-enolase	GATGAGGATCGAGGAGGCTCTT	CTGCCCTTATTTGGCTTTGG
MAFBX	GGGTGCACAGGATGGAGACT	GCAGGCCTGGTGATTTGAAT
MURF1	AGCAAGCCTGTGGTGATCCT	GCCTCGGCTCTGCCAGTAT

3.3.3 BLAST Searches

BLAST searches were routinely used on the NCBI and ensembl databases, to identify similar sequences in the database, and to ensure primers designed were specific to cDNA of interest only. The cDNA sequence of the gene of interest (saved in Fasta format) was inserted into the query window within the standard nucleotide BLAST search webpage. Parameters were set for database types to search through (e. g. *Gallus, Mus musculus*, human, the number of alignments to be made and how many to be viewed). The BLAST output showed listed sequences aligned to sequence of interest in order of greatest homology, measured by a Score, E Value and % sequence identities.

3.4 GENE EXPRESSION ANALYSIS

3.4.1 Overview

To assess changes in gene expression, total RNA was extracted from the samples from the muscles isolated from the two studies. The mRNA was then reverse transcribed to form cDNA, and specific transcripts of interest were amplified by Q-RT-PCR to quantify relative gene expression (using the standard curve method, Applied Biosystems).

3.4.2 RNA extraction from skeletal muscles

Total RNA was extracted from muscle tissues using an RNA isolation kit (QIAGEN RNeasy fibrous tissue mini kit). To lyse the muscle tissue samples, 300µl of lysis-binding solution (Buffer RLT) was added directly to the 30µg of crushed muscle powder (crushed under liquid nitrogen) in 1.5ml centrifuge tube. After which the solution was homogenised on ice using a Polytron tissue homogeniser. To the tube was added 590µl of RNase-free water containing the homogenates after which 10µl of protinase K (20mg/ml) was added. The solutions were then mixed and incubated at 55^{O}C in a heating block for 10 minutes then centrifuged at 10,000xg for 3 minutes at -2^{o}C. To the supernatant was added 0.5 volumes (450µl) of ethanol and the solution was mixed. From this mixture 700µl of the solution was then transferred to RNeasy mini column and centrifuged for 15 seconds at10,000xg after which the flow through was discarded. This process was repeated until all the supernatant solution had been used. Then 350µl of wash buffer (RW1) was added to RNeasy column after which the lids were closed and tubes centrifuged for 15 seconds at 10,000xg at 2^{o}C. A solution containing 10µl of DNase stock solution mixed with 70µl of buffer RDD was added to each column and incubated for 15 minutes at 20-30^{O}C. Then 350µl of buffer RW1 was then added to each column and it was centrifuged for 15 seconds at 10,000xg at -2^{o}C, after which flow through was discarded. To each column was added 500µl of buffer RPE which was then centrifuged at 10,000xg at -2^{O}C for 15 seconds, after the lids were closed. The same process was repeated but instead the columns were centrifuged for 2 minutes. The columns were then placed in new 2ml tubes and centrifuged for 1 minute at 13,000xg at room temperature. After which the columns were placed in new 1.5ml tubes and 40µl of RNeasy-free water was added, the lids were closed and tubes centrifuged at 10,000xg for 1 minute at -2^{o}C. This elution process was repeated with the RNA eluate. RNA was stored at -80°C until used. RNA concentration was determined using a Nanodrop ND-1000 (Thermo Scientific, 45 Wilmington, USA) and concentrations were adjusted to 50ng/µl for cDNA synthesis.

3.4.3 RNA integrity check (non-denaturing agarose gel electrophoresis)

As an indicator of RNA integrity, ribosomal RNA (28S and 18S) bands were visualised by non-denaturing agarose gel electrophoresis to ensure bands were intact and not degraded. Agarose powder at the weight of 1.2g was added to 120ml of 1X TAE Buffer (0.04M Tris acetate, 0.001M EDTA, pH 8.0) for preparing 1% (w/v) gels. The mixture was then heated in microwave for 3 minutes and was left to cool before being poured into a gel casting module (Biorad). A gel comb was placed into the gel before it solidified. The solidified gel was placed in the gel tank, and 1X TAE buffer was used to fill the tanks as running buffer. In order to electrophorese DNA samples, 1µl of 5X loading dye (Promega) was mixed with 5µl DNA and the sample then loaded into a well. Five µl of 100bp DNA ladder (Promega) was used as a marker for DNA size.

The loaded DNA sample was electrophoresed at 100 volts for 45 minutes. The time used in electrophoresis of samples was dependent on agarose concentration of prepared gel. For 2% (w/v) gel concentration, running time of 100 minutes was required for electrophoresis.

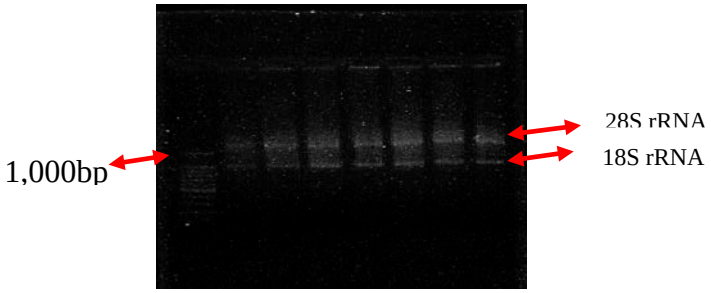


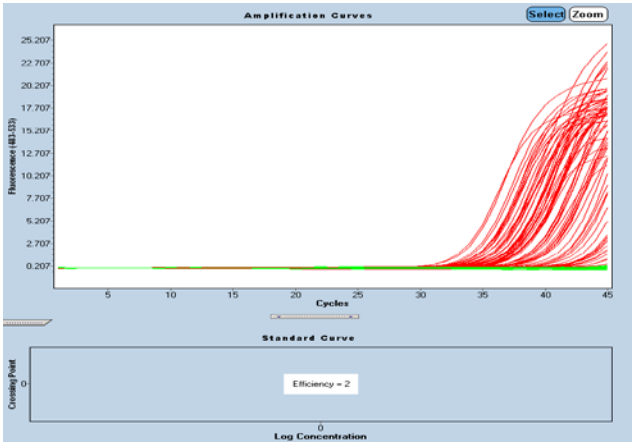
Figure 3.1. Non-denaturing agarose gel electrophoresis gel of chicken skeletal muscle total RNA showing 28 and 18s ribosomal RNA bands of extracted samples visibly intact. Also indicated is the position of the 1000bp DNA marker.

Furthermore, RNA concentration and purity was checked using a Nanodrop® ND-1000 (Thermo Scientific, Wilmington, USA). A 260/280 ratio of ~2.0 was suggestive of relatively high-quality RNA (containing minimal protein contaminants).

3.4.4 RNA integrity check by cyclophilin Q-RT-PCR (Trial 2)

Due to large number of samples and time constraints, RNA integrity check was not done by agarose gel electrophoresis in trial 2. In order to ensure there were no residual genomic DNA in RNA extract solutions, a Q-RT-PCR was carried out on RNA extract samples using cyclophilin primers. This was tested by checking if there was an amplification of a cyclophilin amplicon in samples to be used for Q-RT-PCR analysis. Residual DNA Q-RT-PCR was done as described in section 3.3.7.

a.



b.

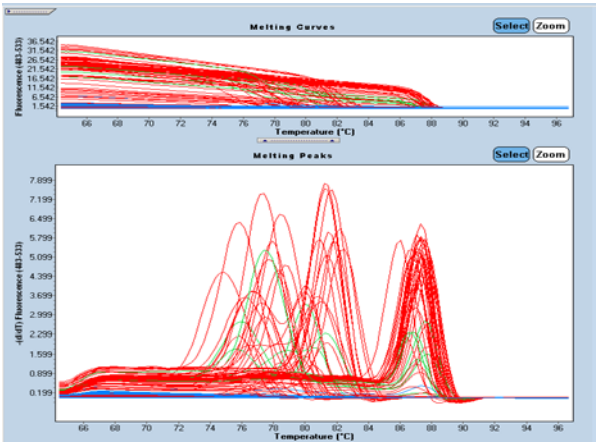


Figure 3.2: Quality assessment of RNA; potential detection of contaminating gDNA. (a). Quantitative PCR amplification curves of RNA extracts with cyclophilin cDNA specific primers. The presence of samples indicating high CP values (indicated on x axis) indicates a lack of genomic DNA present in RNA extracts and thereby potential genomic DNA interference in the Q-PCR. (b). Melt curves with showing several peaks indicating the presence of primer dimers and the absence of cDNA template.

3.4.5 cDNA synthesis (using Roche reagents)

First strand cDNA synthesis was carried out using a cDNA synthesis kit (Transcriptor First Strand cDNA Synthesis Kit, Roche, Burgess Hill, UK). In 96 PCR plates, 500ng (10µl of 50ng/µl) total RNA was added to 2µl of 60µM random hexamer primers and incubated in a PCR machine at 65°C for 10 minutes. The reaction was immediately cooled on ice. Whilst on ice, deoxynucleotide Mix (final concentration 1mM each), 10 U

Transcriptor Reverse Transcriptase and 20U Protector RNase Inhibitor were added to the initial reaction, to a final volume of 20µl. The reaction mixture was gently pipetted mixed and incubated at 25°C for 10 minutes, 55°C for 30 minutes, 85°C for 5 minutes, and then immediately placed on ice. The cDNA was diluted 5-fold to give 100µl "stock" cDNA for Q-RT-PCR and stored at -20°C until analysis. All synthesized cDNA was tested by conducting a single Q-RT-PCR reaction using a validated primer set (cyclophilin) to ensure every sample contained cDNA.

3.4.6 Quantitative RT-PCR

Real time PCR was conducted on a Lightcycler 480® (Roche, Burgess Hill, UK) to assess the relative abundance of specific mRNAs. Reactions were carried out in triplicate on 384 well plates. Each well contained 5µl of cDNA with the following reagents: 7.5µl SYBR green master mix (Roche, Burgess Hill, UK), 0.45µl forward and reverse primers (10µM each; final concentration 0.3µM each) and 1.6µl RNase-free H2O (total volume of 15µl per well). Samples were pre-incubated at 95°C for 5 minutes followed by 45 PCR amplification cycles (denaturation: 95°C for 10 seconds; annealing: 60°C for 15 seconds; elongation: 72°C for 15seconds). A standard curve was produced using serial dilutions (having relative concentrations ranging from (0.08-1)) of a pool of cDNA made from all samples, to check the linearity and efficiency of the PCR reactions. PCR efficiency was approximately 2.0 for all primers indicating an optimum linearity and efficiency of PCR products. Relative transcript abundance was determined using the standard curve and was corrected for either the quantity of housekeeping gene or using oligreen quantification method for total cDNA.

3.4.7 Screening for housekeeping mRNA expression

Housekeeping transcripts are typically constitutive genes that are required for the maintenance of basic cellular function, are expressed in all cells of an organism under normal and patho-physiological conditions and therefore are not influenced by changes in changes in cell/tissue stimulation. In Q-RT-PCR they are used to normalise results and correct for experimental variations. In trial 1, three different primer sets for mRNA transcripts were screened by carrying out a Q-RT-PCR in order to ascertain whether their concentrations were influenced by "treatment" and therefore whether they were suitable to be used as a house-keeping gene. The genes screened were cyclophilin, beta-actin or Glyceraldehyde-3phossphate dehydrogenase (GAPDH). The cDNA solutions were diluted to 1:8 neat cDNA mixture to RNase free water ratio. The neat solution is comprised of the original cDNA synthesized concentration when after incubation in the PCR machine. Samples were afterwards diluted 8-fold. Of the three transcripts screened, cyclophilin gave the best result with a standard curve efficiency of approximately 2, which is the optimum efficiency. In addition, there was

a uniform peak melting point temperature in all samples measured and a uniform level of expression in both muscle types and at all time points. Although GAPDH and beta-actin had close to optimum standard curve efficiency values, beta-actin primers generated more than one peak melting point values. This indicated the detection of other spliced variants or primer dimers, which could lead to inaccurate measurements. GAPDH had significant differences in the level of expression between muscle types across time points as well as a wider variation in the standard replicates when compared to the other two housekeeping genes. Therefore, cyclophilin was adopted as the housekeeping gene used in trial 1.

3.4.8 Optimisation of dilution ratio of synthesised neat cDNA of measured genes.

All genes measured were corrected relative cyclophilin expression with the exception of PHGDH, all synthesised cDNA was made to a 1:8 dilution neat cDNA solution to RNase free water ratio. The CP values of PHGDH were so high that a working standard curve could not be generated at a 1:8 concentration. This is indicative of a low concertation of cDNA template. Therefore, a separate dilution of a 1:4 cDNA neat to RNase free water was prepared in order to generate a working standard curve for PHGDH.

3.4.9 Testing cyclophilin as a housekeeping gene in Ross 308 and Hy-Line *Pectoralis major* and *Peroneus tertius muscles* (trial 2)

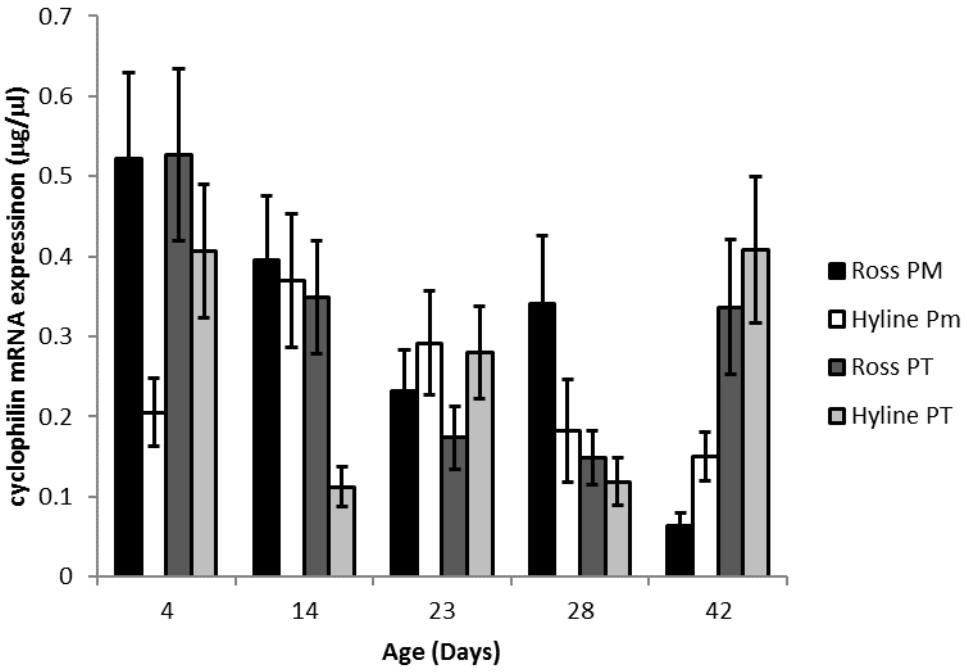


Figure 3.3: Cyclophilin mRNA expression in chicken skeletal muscle over the trial time course (Trial 2). The muscle samples Ross *Pectoralis major* (PM), Hy-Line-PM, Ross *Peroneus tertius* (PT) and Hy-Line-PT at days 4, 14, 23, 36 and 43 (trial 2), n=6,

significant muscle type x time (P=0.007) as well as genotype x time interaction (P=0.002). Values expressed as means ± Standard Error of the mean.

In trial 2, there was a Muscle type x time interaction P=0.007, genotype x time interaction P=0.002, as well as a time effect P<0.001 and muscle type effect P=0.028 in the expression of cyclophilin mRNA between both genotypes and muscle types, thus cyclophilin was unsuitable for use as a housekeeping gene in trial 2, see Figure 3.3.

Like in trial 1 a 1:4 neat cDNA to RNase free water as prepared to measure the mRNA expression of PHGDH. Due to the low CP values in the samples, a standard curve was unable to be generated to accurately measure the expression of PHGDH. These low CP values may be as a result of the low level of expression of PHGDH in the *Pectoralis major* and *Pectoralis major* of Hy-Line chickens (HPM and HPT, respectively) reducing the average CP value of the pooled standard curve containing equal quantities cDNA from muscle samples from Ross *Pectoralis major* (RPM), Hy-Line PM (HPM), Ross *Peroneus tertius* (RPT) and Hy-Line-PT (HPM).

3.4.10 Oligreen quantification of total cDNA (Trial 2)

Due to the broad time course studied for animal husbandry work in trial 2 (from 1 day to 43 day old) quantifying mRNA transcript abundance relative to a housekeeper gene was not possible, due to the interaction with Genotype, age and muscle type (see Figure 3.3). Thus, relative total cDNA concentration was determined using oligreen and mRNA transcript abundance was corrected for total cDNA. Total cDNA quantification was conducted on a Lightcycler 480® (Roche, Burgess Hill, UK) using Oligreen (Invitrogen, Paisley, UK), which has been shown to specifically bind with single stranded DNA molecules (Rhinn et al. 2008). Reactions were carried out in triplicate on 384 well plates. 5µl Oligreen was added to 995µl 1x TE buffer to make the working Oligreen solution. Each well contained 5µl of cDNA plus 5µl Oligreen working solution. Fluorescence was quantified with increasing temperature (constant ramp rate of 0.11°C/s; up to 95°C). The fluorescence at 80°C was plotted against a standard curve, produced using serial dilutions of a pool of cDNA made from all samples, to determine the relative quantity of cDNA. Normalization using the oligreen method was also used for Q-RT-PCR conducted on cDNA derived from Ross 308 and Hy-Line chicken skeletal muscles.

3.5 IMMUNOBLOTTING (WESTERN BLOTTING)

3.5.1 Overview

To achieve an optimal Western blotting result and best possible sensitivity, a series of steps were taken to optimise the method depending on the antibody which was under study. For all antibodies measured, the same membranes and reagents were used for blocking, transfers and imaging. The only variation in reagents were the incubating dilutions of primary antibody mixture used. Two transfer methods were also adopted in the course of this study namely wet and semi-dry electro transfer. Figure 3.4 shows a flow chart of the general procedure.

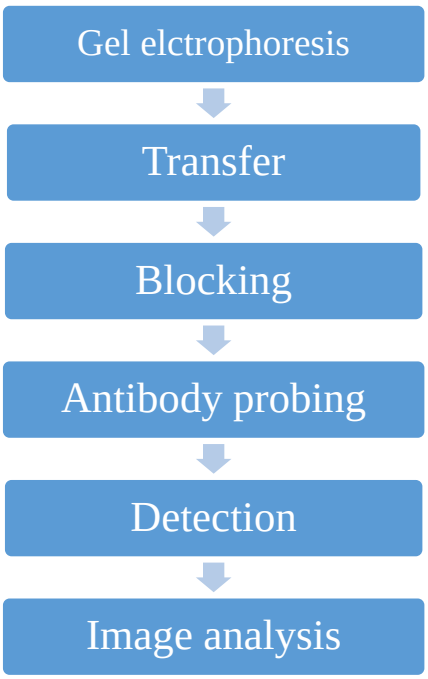


Figure 3.4: Flowchart showing the different steps of Western blotting procedure

3.5.2 Polyacrylamide Gel Electrophoresis

To muscle protein homogenate extracts (200 µl) (as described in section 3.2.2) was added to an equal volume of 2 x SDS (2ml glycerol, 1.25ml 1M Tris/HCL pH 6.8, 4ml 10% (w/v) SDS solution, 0.154g/ml dithiothretol (DTT) and a few grains of bromophenol blue made up to 10ml with water). The extracted proteins were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using either 7.5% mini-protean $^{(R)}$ TGX Stain-Free^{TM} precast gels for small protein sample numbers or 7.5% Criterion^{TM} TGX^{TM} precast gels for larger sample protein numbers (BIO-RAD). Western blotting was carried out using pre-tested and adopted antibodies in our lab found to be compatible with the *Gallus Gallus* species. All protein samples were diluted to give a

minimum 20mg/ml solution in SDS mix, depending on antibody probed. Samples were heated at 100^{O}C for 5 minutes to denature the proteins and centrifuged at 13K xg for 3 minutes at room temperature. Using 4µl, 80µg sample were loaded into each well of the Criterion precast SDS-PAGE gels (Bio Rad) in six duplicates. Also loaded in the gels were protein molecular markers and a standard made up of a pool of all samples. The standard were loaded in triplicates spread across the gel the confirm uniformity of protein blots. The Criterion gels were submerged in 1X Tris-Glycine-SDS PAGE running buffer (0.025M Tris, 0.192M Glycine, 0.1% (w/v) SDS) and run in an electrophoresis tank connected to a power supply for 45 minutes at 200V. After the gels had been run, the casings were dismantled and the gel taken out and submerged in Western blot buffer (400mM Glycine, 25mM Tris-base 5% (v/v) Isopropanol) for 20 minutes at room temperature.

3.5.3 Wet electrophoretic transfer

After gels were run proteins were transferred to nitrocellulose membranes that had been pre-soaked in water for 10 minutes to hydrate, then soaked in western blot buffer. The Western blot cassette was then packed onto the black side of the cassette as follows; On the cathode (-ve) side, a wet sponge pre-soaked in western blot buffer was laid onto the bottom section of the gel cassette followed by two pieces of 3MM Whatman chromatography paper cut to the size of the sponges, also soaked in Western blot buffer. The gel was then placed on top the filter papers and the nitrocellulose membrane (Hybond-C, Amersham Biosciences, and UK) was placed on top of the gel. Another two pieces of Whatman chromatography paper were placed on top of the gel and lastly, on the anode side (+ve) another sponge of the same size, also pre-soaked in Western blotting buffer, and the cassette was closed. The cassette was placed into the Trans-blot tank and transfer buffer was poured into the tank to cover the surface area of the cassette. A block of ice was placed at the back of the tank to prevent overheating. The tank was then connected to the power pack. The gel protein transfer was run for two hours at 200mA with small western blot transfer tanks and 1A for the larger tanks. Upon completion of the transfer, the gel was discarded and the membrane was stained with Ponceau S solution, which is used for a rapid and reversal method for locating protein bands in Western blots, to locate the protein bands and confirm a successful transfer. After staining, the membrane was washed with water and then washed in a 1 X Tris buffer saline (TBS-T) (0.2M Tris, 1.5M NaCl pH 7.5 with 0.05% (v/v) Tween 20) till the stain was washed away.

3.5.4 Semi-Dry electrophoretic transfer

In this method, the Whatman chromatography filter paper cut to size was soaked in Western blot transfer buffer (192 mM glycine, 25mM Tris, 5% (v/v) isopropanol) for 5 minutes. The Semi-Dry blotter supplied by Bio-rad was then opened and the upper plate electrode removed. Two pieces of 3MM filter paper was placed on the plate electrode in the blotter and air bubbles removed. The Nitrocellulose membrane (Hybond-C, Amersham Biosciences UK) was then placed on top of the filter paper, then the gel on top of the nitrocellulose membrane and bubbles were gently rolled out. Another 2 pieces of 3MM filter paper was placed on the metal plate of the electrode and air bubbles rolled out. The upper plate electrode was then place on the blot assembly and using the Semi-Dry blotter the gels were run at 20V for 60 minutes

3.5.5 Blocking and antibody probing

The membrane was then blocked in either a 20ml or 40ml (depending on membrane size) ECL (GE Healthcare, UK) TBS-T blocking solution (2% (w/v) ECL blocking agent in TBS-T buffer) for 1 hour at room temperature. Thereafter the blocking solution was discarded. The membrane was then incubated overnight at $4^{O}C$ in blocking solution containing primary antibody (Table 3.2) of varying concentrations depending on optimum workability of primary antibody. Thereafter, the membranes were washed for over 30 minutes at room temperature with 1 X TBS-T wash buffer solution, which was changed every 5 minutes and then incubated for 1 hour in a 20ml or 40ml secondary antibody at room temperature (ECL anti-rabbit, GE Healthcare, UK 1:50,000 (w/v) antibody to 2% (w/v) ECL TBS-T dilution). After incubation, the membrane was washed again in 1 X TBS-T wash buffer for over 30 minutes at room temperature, while changing the solution every five minutes.

Table 3.2: List of antibodies and manufacturers used for Western blotting

Antibody	Manufacturer	species detected	Produced in	Catalogue no
PHGDH	Sigma prestige antibody	Mouse, Rat, Human	Rabbit	HPA021241
S6RP	Cell Signalling Technology	Mouse, Rat, Human, Monkey	Rabbit	#2217
Phospho S6RP (Ser235/236)	Cell Signalling Technology	Mouse, Rat, Human, Monkey	Rabbit	#2211
EIF4E	Cell Signalling Technology	Mouse, Rat, Human, Monkey, Mink, Zebra fish	Rabbit	#9742
Phospho EIF4E (Ser 209)	Cell Signalling Technology	Mouse, Rat, Human, Monkey	Rabbit	#9741
AKT	Cell Signalling Technology	Mouse, Rat, Human, Monkey, D. Melanogaster	Rabbit	#9272
Phospho AKT (Ser 473)	Cell Signalling Technology	Human, Mouse, Rat, Hamster, D. melanogaster, Bovine, Dog, Pig	Rabbit	#9271

3.5.6 Detection and image analysis

Excess wash buffer was drained from the antibody probed membrane then a working solution containing two enhanced chemiluminescent detection reagents namely, solution A (Luminol) and solution B (peroxide solution) (Amersham ECL prime Western blotting detection kit, UK), mixed in a ratio of 1:1, was added to completely cover the membrane and incubated for 5 minutes at room temperature. ECL is based on the emission of light which occurs when the horse radish peroxidase (HRP) enzyme conjugated to the secondary antibody catalyses an oxidation reaction between the substrate hydrogen peroxide and chemiluminescent chemical luminol. This leads to the production of a chemiluminescent signal showing where the HRP linked antibody is bound to the antigen on the membrane, and the resulting light is detected on film. The membrane was then wrapped in a fresh piece of plastic wrap and air bubbles were gently smoothed out and placed in a hypercassette (Amersham GE healthcare UK) and processed in a dark room. In the dark room an X-Ray (Amersham hyperfilm ECL) was place on top of the membrane and the cassette was covered and left for some time (depending on the antibody). The optimum time adopted was dependent on the background exposure of bands. The cassette was opened and the film was developed in a solution 20% (v/v) Developer solution (Kodak Gbx, Carestream Healthcare Inc, USA) and fixed in a 20% (v/v) solution (Ilford Hypam, Harman Technology UK) using red safe lights. The developed film creates an image (bands) where the antibodies are bound to the targeted proteins on the membrane/blot. Band intensities (protein expression) were thereafter quantified using a Quantity-One Multi-Analyst Imaging Software (BioRad, Hercules U.S.A).

3.5.7 Antibody testing for immunoblotting

Antibodies used for Western blotting in this study were not specifically designed to work in chicken tissues. Therefore, in order to measure the expression of the proteins of interest, experimentation was carried out to investigate the compatibility of those antibodies listed in section 3.5.5 in chicken muscle tissues.

A pool of chicken muscle protein extracted was made and protein expression detected by the individual antibodies measured alongside proteins isolated from C2C12 mouse (*mus musculus*) muscle cell line which was used as a positive control and proteins isolated from sheep muscle as a second test sample. The objective of this experiment was not quantitative but was intended to check if the antibodies being tested detected protein isoforms in chicken muscle tissues. Therefore, the appearance of bands in samples, at the correction molecular weight was taken as sufficient evidence to ascertain whether the antibodies under study were suitable to carry out quantitative expression on the proteins of interest. Figures 3.5, 3.6, 3.7, and 3.8 show blots representing

the expression of Phosphoglycerate dehydrogenase (PHGDH), Ribosomal protein S6 (rps6), Protein kinase B (PKB or AKT) and phospho PKB/AKT respectively in mouse, sheep and chicken muscle protein extracts.

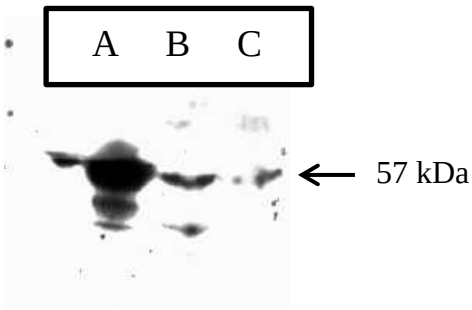


Figure 3.5: The detection of PHGDH protein in chicken skeletal muscle. Lane A, mouse C2C12 myotube cells, Lane B, sheep muscle, Lane C, chicken muscle. 80 µg of protein was loaded per well. Blots were transferred. Indicated is the molecular weight of the detected band. Samples were run on 7.5% mini-protean (R) TGX Stain-Free™ precast gels.

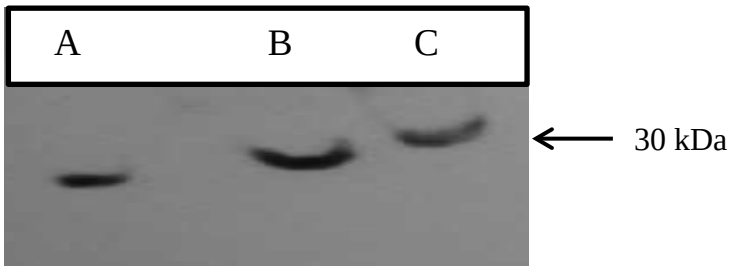


Figure 3.6: The detection of Phospho-ribosomal protein S6 protein in chicken skeletal muscle. Lane A, mouse C2C12 myotube cells, Lane B, sheep muscle, Lane C, chicken muscle. 80 µg of protein was loaded per well. Blots were transferred. Indicated is the molecular weight of the detected band. Samples were run on 7.5% mini-protean (R) TGX Stain-Free™ precast gels.

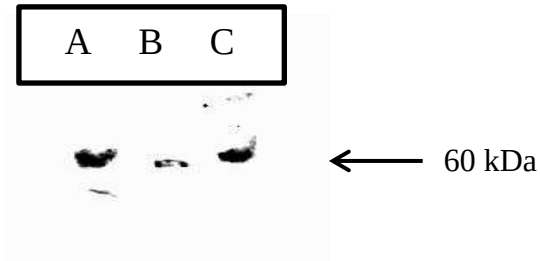


Figure 3.7: The detection AKT protein in chicken skeletal muscle. Lane A, mouse C2C12 myotube cells, Lane B, sheep muscle, Lane C, chicken muscle. 80 µg of protein was loaded per well. Blots were transferred. Indicated is the molecular weight of the detected band. Samples were run on 7.5% mini-protean (R) TGX Stain-Free™ precast gels.

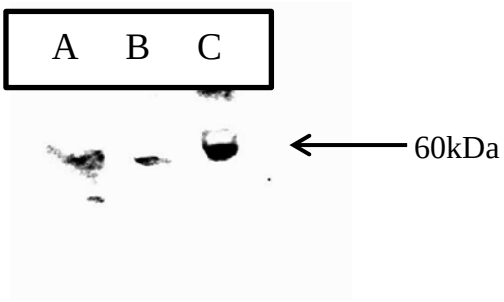


Figure 3.8: The detection of phosphor-AKT protein in chicken skeletal muscle. Lane A, mouse C2C12 myotube cells, Lane B, sheep muscle, Lane C, chicken muscle. 80 µg of protein was loaded per well. Blots were transferred. Indicated is the molecular weight of the detected band. Samples were run on 7.5% mini-protean (R) TGX Stain-Free™ precast gels.

In trial 2 an attempt was made to measure the expression of AKT, P70S6K 4E-BP1 and MAFbx by western blotting. Only anti- P70S6K antibodies were able to detect the appropriate antigen in chicken muscle samples, the other antibodies were unable to detect mouse and chicken isoforms in protein extracts. This could be as a result of loss of efficacy of these primary antibodies, due to change of batch number. Alignments of the primary sequence for the mouse and chicken isoforms for the above proteins indicated a degree of alignment (Appendix 12-15). However, in spite of these similarities the antibodies were unable to detect the chicken isoforms in the samples from trail 2, in many instances the antibodies were unable to detect mouse isoforms even though the manufacturers of the antibodies specified that they were raised against mouse antigens.
In Appendix 16 explanations are given why these antibodies may not detect chicken isoforms.

3.5.9 Stripping procedure for probed membranes

In order to investigate the expression of more than one protein on the same western blotting membrane, primary and secondary antibodies were removed using a stripping buffer. The stripping buffer used was a commercially available blot stripping buffer (Thermo Scientific). The blot was washed with 1x TBS-T or PBS for 10 min at room temperature, then the membrane was immersed in stripping buffer and incubated for 5- 15 min at room temperature, with gentle shaking. The stripping buffer was then removed, and the membrane washed in 1x TBS-T for 15 min, with changes in solution every 5 mins. After that the membrane was blocked in blocking agent for 1 hour, followed by probing with primary antibodies of interest.

3.6 CASEIN ZYMOGRAPHY

3.6.1 Overview

This procedure was used to assay the activity of calcium dependent neutral proteinases, calpain 1 (μ-calpain) and calpain 2 (m-calpain) proteases. The procedure is adapted from that described by Arther and Myles (2000). Upon activation with Ca^{2+} these proteases digest the casein protein (substrate) into short peptide fragments, creating clear bands on an acrylamide gel containing casein that has been stained with Coomassie/amido black dye. This is a non-denaturing procedure taking advantages of mobility differences of the native enzyme isoforms (Raser *et al*., 1995).

3.6.2 Protein extraction and sample preparation.

Ross 308 and Hy-line PM and PT muscle samples stored at -80^{o}C were crushed to powder in liquid nitrogen, and 1g of sample homogenised in 3ml extraction buffer (50mM Tris/HCl (pH 7.5), 5mM EDTA, 200mg/μl AEBSF, 1μg/ml leupeptin, 1μg/ml pepstatin) for 2x30 seconds using a Polytron PT2100 (Kinematica Switzerland). The homogenate was centrifuged at 15,000xg (Beckam Coulter 22R) for 20 minutes at 4^{o}C and supernatants removed into a new tube to which an equal volume of gel sample buffer (125mM Tris/HCl (pH 6.8), 1M DDT, 20% (v/v) glycerol, 0.01% (w/v) Bromophenol blue) was added and mixed prior to gel loading.

3.6.3 Gel preparation, casting and SDS-PAGE

The hand cast gels were prepared from acrylamide and bis-acrylamide monomer solutions. The component solutions were prepared mixed together and poured between two glass plates locked in a casting frame and set on a casting stand. The resolving gel consisted of 2% casein (w/v) copolymerized into 10% separating gel (75:1 acrylamide to bis-acrylamide), 225 mM Tris/HCl, and pH8.8). The casting gel (used to seal plates) was polymerized with 5% (w/v) (37.5:1 acrylamide to bis-acrylamide), 160mM of Tris/HCl pH 6.8, without casein. After the gel solutions were poured between the glass plates, a comb was inserted prior to solidification. The gels were pre-run for 30 minutes at 4^{o}C in electrophoresis buffer (25mM Tris, 125 mM glycine, 1mM EDTA, 1 Mm DTT, pH 8.3) at 125 volts (Biorad power PAC 300). Samples were vortexed and loaded into the gel wells with equal wet weight of tissue equivalent per well, then the gels run under pre-condition for approximately 4 hours.

3.6.4 Ca^{2+} incubation, fixing, staining and band quantification

After electrophoresis, gels were incubated in Ca^{2+}incubation buffer (50mM Tris/HCl pH 7.0, 5mM $CaCl_2$, 10mM DTT) with gentle swirling (Stuart See-Saw Rocker SSL4) at room temperature with 3 changes of buffer over an hour. Following fixing for 20 minutes in 10% (v/v) acetic acid, the gel was then stained in Coomassie/amido black solution (0.2% (w/v) Coomassie blue, 0.2% (w/v) amido black, 10% (v/v), isopropanol 10% (v/v) acetic acid) for 30 minutes and then de-stained with several changes of 10% (v/v) isopropanol, 10% (v/v) acetic acid. Band intensity on zymographs were quantified using a Quantity-One Multi-Analyst Imaging Software (BioRad, Hercules, U.S.A).

3.7 PROTEOSOME ACTIVITY ASSAY

3.7.1 Overview

Proteasome activities were determined in tissues using Promega Proteasome 3-substrate system kit (Proteasome Glo™ Promega Southampton, UK), which are homogeneous, luminescent assays that individually measure the chymotrypsin-like, trypsin-like or caspase-like protease activity associated with the proteasome complex. The Proteasome-Glo™ reagents each contain a specific luminogenic proteasome substrate in a buffer optimized for proteasome activity and luciferase activity. These peptide substrates are Suc-LLVYaminoluciferin (Succinyl-leucine-leucine-valine-tyrosine-aminoluciferin), Z-LRR-aminoluciferin (Z-leucine-argininearginine-aminoluciferin) and Z-nLPnLD-aminoluciferin (Z-norleucine-proline-norleucine-aspartate-aminoluciferin) for the chymotrypsin-like, trypsin-like and caspase-like activities, respectively. Adding a single Proteasome-Glo™ Cell-Based Reagent in an “add-mix-measure” format results in proteasome cleavage of the substrate and generation of a luminescent signal produced by the luciferase reaction. The Proteasome-Glo™ Reagent contains the proprietary thermostable luciferase, Ultra-Glo™ Recombinant Luciferase, and is formulated to generate a stable, “glow-type” luminescent signal that improves performance across a wide range of assay conditions. This coupled-enzyme system, with simultaneous proteasome cleavage of substrate and luciferase consumption of the released aminoluciferin, results in a luminescent signal that is proportional to the amount of proteasome activity in substrate. (Promega technical bulletin TB3460).

3.7.2 Protein extraction from tissue samples

Frozen Ross 308 and Hy-line PM and PT muscle tissues stored at -80^{o}C were pulverized in liquid nitrogen, and 1g of crushed tissue was homogenised in 3 ml of extraction buffer (25 mM 4-(2-hyrodxethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.5), 0.1% (w/v) Triton X-100, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT) and 1 tablet each of protease inhibitor (ROCHE cOmplete protease inhibitor cocktail) and phosphatase inhibitors (ROCHE PhosSTOP per 10ml extraction buffer.) for 3 x 30 seconds at full speed on ice. The homogenate was thereafter transferred to 1.5ml Eppendorf tubes and centrifuged at 15,000xg for 20 minutes at 4^{o}C and supernatant transferred to another set of tubes ready to use samples for the assay. The same procedure was carried out on another set of samples without protease inhibitors in extraction buffer and heated at 100^{o}C to further deactivate any proteasome activity to serve as identical controls. A 50µl aliquot of supernatant protein extract was transferred to another set of Eppendorf tubes, and 950ml of 0.1M NaOH was added to each sample which were then used for protein quantification by Lowry assay.

3.7.3 Measurement of proteasome activity

The proteasome-Glo buffer was thawed and equilibrated with lyophilized Luciferin detection reagent to room temperature. The Luciferin detection reagent is then reconstituted by adding 10ml of the Proteasome-Glo buffer to make the reagent. Then the appropriate mixture was defrosted and equilibrated to room temperature, vortexed briefly and added to the Glo-reagent, mixed and labelled accordingly. The Glo-reagent was incubated at room temperature for 60 mins before use, to allow the removal of any contaminated free aminoluciferin. A ratio of 1:1 of each of the three proteasome variants (Proteasome Glo- reagent 30µl) to sample and control supernatant (30µl) was incubated at room temperature in white 96 well plates and assayed in duplicates for activity with extraction buffer as a blank. The luminescence was measured every 10 mins for 1 hour using FLUOstar Omega spectrometer (BMG LABTECH UK) at wavelength 590nm. The average of the 7 Luminescence readings taken at 0, 10, 20, 30, 40, 50 and 60 minutes were measured relative to unit protein content of samples per µg protein content measured by Lowry assay.

3.8 STATISTICAL ANALYSIS

Statistical analysis was done utilizing statistical software for windows, GenStat 17th Edition (Hernel Hemstead, UK) and Microsoft Excel. In trial 1 a two-way factorial ANOVA was carried out for production and metabolic parameters as well as gene expression. Factors considered included Muscle type and age of animal, with significance level set at $P<0.05$. In trial 2, a three-way factorial analysis of variance was carried out for production parameters, enzyme activity, and gene and protein expression. Factors considered include genotype, muscle type and age interactions. Significance was set where $P<0.05$.

For each muscle tissue in both trials, gene expression was modelled using the following multiple linear regression model:

$$Y_{ij} = \mu_j+(\alpha_j MW_i+\beta_j Age_i)$$

Where Y_{ij} is the predicted expression level of a given gene j in sample i; MW_i, and Age_i are the muscle weight and age of sample i with regression coefficients α_j and β_j for each gene j respectively and μ is the regression intercept for gene j. The model was fitted in excel with the regression function on the data analysis toolpak. Age controlled scatter plots were generated by plotting the calculated (Y_{ij}) with MW_i.

For each gene, a least square approach was used to estimate the regression coefficients. If α_j was significantly deviated from 0, the gene j was considered to be associated with muscle weight (MW). A gene was considered to be up-regulated with MW if $\alpha > 0$ and down-regulated if $\alpha < 0$.

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CHAPTER 4. (TRIAL 1 RESULTS)

Trial 1: Changes in metabolic characteristics of chicken (broiler) muscles during post-natal growth.

4.0 INTRODUCTION

The aim of this study was to investigate molecular and metabolic mechanisms associated with skeletal muscle growth in chickens by comparative analysis of fast and slow growing muscle types within the rapidly growing broiler chicken (Ross 308 broiler). The specific objectives of the study were as follows:

- Measure production parameters associated with skeletal muscle growth in broiler chickens which include whole body weight, breast muscle weight and leg muscle weight (two muscles growing at different rates).

- Measure metabolic parameters associated with muscle growth in broiler chickens, which include protein, glycogen as well as DNA content.

- Expression of genes associated with muscle protein degradation and muscle mass regulation.

- Establish a relationship between the gene expression of glycolytic metabolic enzyme and skeletal muscle growth in chicken.

- Establish a relationship between serine biosynthesis and muscle growth by studying associations between divergent growing PM and PT muscles growth and glycolytic metabolic pathways that can potentially lead to *de novo* serine biosynthesis.

- Characterisation of gene and protein expression of intermediates involved in the mTOR/AKT signalling cascade and to elucidate the relationship with those intermediates and skeletal muscle in two muscles growing at different rates.

- To characterise the expression of genes involved in skeletal muscle cell cycle (Myogenin, MUSTN1 and FGF2R) in the two-divergent growing muscle *Pectoralis major* (PM) and *Peroneous tertius* (PT)

- To determine the relationship between gene expression of LIM domain proteins and muscle growth in the two-divergent growing *Pectoralis major* (PM) and *Peroneous tertius* (PT) muscles.

The production parameters included, total body weight, breast and leg muscle weights. While the metabolic parameters included glycogen content, protein content and DNA content of the leg and breast muscles. Genes expression of glycolytic metabolic proteins α-enolase and GAPDH were measured. Also measured were genes associated with mTOR/AKT pathway namely P70S6K and 4E-BP1, genes in modulating serine biosynthesis namely PHGDH, PSAT and PSAT were also measured. Calpastatin was also measured to have an idea of the calpain proteolytic system is regulated in the differential rowing muscles. Growth factors myostatin and IGF-1R were also comparatively measured. LIM domain proteins CSRP3 and FHL2 were also measured. Other genes involved in the activation and fusion of skeletal muscle satellite cells which include myogenin, MUSTN1 and FGF2R were also comparatively measured.

4.1 TRIAL DESIGN, SAMPLE COLLECTION AND STATISTICAL ANALYSIS

Trial design and sample collection were done as described in section 3.1. While Three-way factorial ANOVA and multi regression analysis were carried out as described in section 3.8. ANOVA of regression output are shown in Appendix 5

4.1.2 GROWTH AND CARCASS WEIGHT PERFORMANCE AND METBOLIC PARAMETERS

In this trial muscle samples were taken from Ross 308 broiler chickens at different points of their growth (days 14, 36 and 43). As well as collecting carcass and muscle weights, glycogen, protein and DNA assays (Chapter 3) were carried out on *Pectoralis major* (PM) and *Peroneus tertius* (PT) which are within the fast-growing breast muscles or relatively slow growing leg muscles respectively.

Table 4.1: Metabolic and production parameters of the *Pectoralis major* (PM) and *Peroneus tertius* (PT) muscles of Ross 308 broiler chickens. n=6

Measurement	Muscle Type	Age (day)			SED[1]	Effect (P value)		
		14	36	43		Muscle	Age	MuscleXage
Animal weight (g)		427.58	2157.5	2895.33	109.9		< 0.001	
Tissue weight (g)	PM[1]	21	149	170	4.3	<0.001	< 0.001	< 0.001
	PT[2]	1	14	12				
% Muscle to total body weight	PM	5	6.9	5.9	0.14	< 0.001	< 0.001	0.002
	PT	0.3	0.7	0.4				
DNA content/tissue (mg/g)	PM	29	40	37	14.80	< 0.001	0.054	0.003
	PT	235	172	244				
Protein/unit tissue (mg/g)	PM	163.4	122.8	151.4	14.10	< 0.001	0.497	0.729
	PT	66	57.6	24.1				
Protein/unit DNA (mg/mg)	PM	6.7	5.1	4.7	1.90	< 0.001	0.226	0.358
	PT	0.3	0.4	0.1				
Glycogen/unit tissue (mg/g)	PM	0.9	3.9	1.2	1.0	<0.001	0.002	<0.001
	PT	1.2	2.7	0.5				
Total protein (mg)	PM	3430	18291	25744	3.60	0.046	< 0.001	< 0.001
	PT	71.5	796.1	296.3				
Total glycogen (mg)	PM	18	578.2	207.7	<0.001	<0.001	<0.001	<0.05
	PT	1.2	38.3	6.3				
Total DNA content (mg)	PM	609	5960	6290	1728	< 0.001	< 0.001	< 0.001
	PT	4935	25628	41480				

[1] *Pectoralis major* (PM)
[2] *Peroneus tertius* (PT)

There was a muscle type and age interaction in tissue weights between and within both muscle types with the PM having the highest value at day 43 (P<0.001) (Table 4.1). The reason for the interaction effect is as a result of PT weight not increasing at day 43 when compared to day 36. There was also an interaction in the proportion of muscle size to total body weight P=0.002 with the highest percentage seen in the PM at day 36. For DNA content per unit tissue weight, there was also a muscle type age interaction between and within the two tissues (p=0.003) with the PT having a higher concentration at all time points with the highest value seen at day 43. For protein content per unit tissue weight there was no muscle x age interaction neither was there any age effect. However, there was a muscle type effect with the PM having a significantly higher value at all time points (P<0.001) (Table 4.1).

There was a muscle type to tissue interaction in total protein content (P<0.001) with the PM having a significantly higher value at all time points with the highest value seen at day 43. There was also a muscle type to age interaction between the PM and PT muscles in DNA content with the PT having a higher DNA content at all time points with the highest seen at day 43 (Table 4.1).

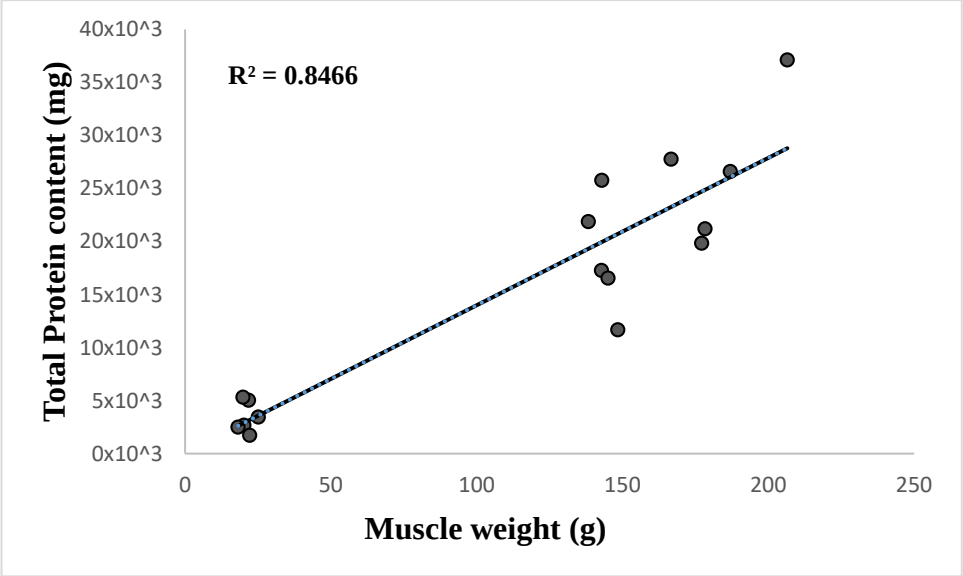

Figure 4.1: Relationship between muscle weight and total protein of Ross 308 *Pectoralis major* at 14, 36 and 43 days post-hatch

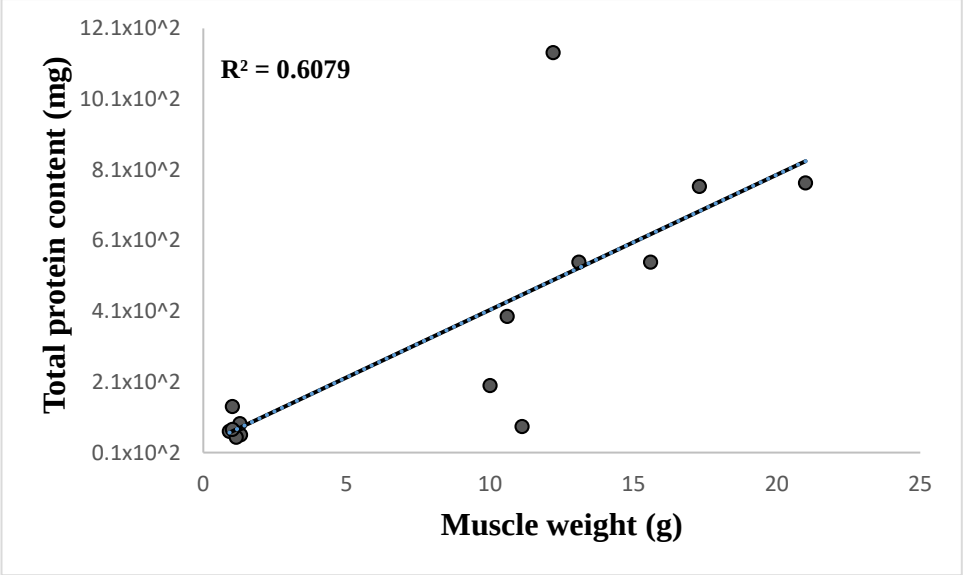

Figure 4.2: Relationship between muscle weight and total protein of Ross 308 *Peroneus tertius* at 14, 36 and 43 days post-hatch

Total protein content of *Pectoralis major* (PM) and *Peroneus tertius (PT)* were significantly positively correlated with their respective muscle with R^2 values of 0.85 and 0.61 respectively, where $P<0.001$ (Figures 4.1 and 4.2). This is an indication that there is a strong association with muscle growth rate in broiler chicken and muscle protein content suggesting that the increase in muscle weight is due to an increase in protein accretion rather than other components of the muscle such as water or glycogen.

4.2 STUDIES ON GENE EXPRESSION

4.2.1 Introduction

Preliminary observations as reported in Section 5.2 on growth and production parameters in the two divergent growing muscles of Ross 308 chicken indicate that there was a faster rate of skeletal muscle growth as well as muscle protein deposition in the faster growing *Pectoralis major* (PM) muscle of the breast when compared to the slower growing leg *peroneous tertius* (PT). These observations also give an indication that the significant difference in muscle protein accretion and muscle growth may be as a result of metabolic and physiological differences at the molecular level in the different muscle types under study. Furthermore, the divergence in muscle growth rate between the PM of the breast and the PT of the leg in chicken provides and interesting model to study the dynamics of how these divergent growth rates are modulated at the molecular level by investigating some specific metabolic and physiological pathways that have previously been associated with the regulation skeletal muscle growth in other animal species. These preliminary observations led to the next

series of experiments in a bid to better appreciate physiological and metabolic mechanisms modulating chicken muscle growth at the molecular level.

Specifically, genes involved in the regulation of protein turnover, glycolytic metabolism, growth factor induced hypertrophy and signal transduction mechanisms modulating protein translation were assessed. In addition, factors involved skeletal muscle satellite cell activation and the subsequent differentiation then fusion of skeletal muscle cells to already existing muscle fibres were also assessed. The expression of a selection of the genes involved in these processes was assessed by quantitative PCR, as summarised in Table 4.2. The objective was to determine whether genes encoding protein involved these mechanisms differed between two muscles PM and PT, that grew at different rates in broiler chickens.

Table 4.2: List of genes measured in *Pectoralis major* or *Peroneus tertius* of Ross 308 broiler chickens and the functions that they participate in.

Gene name	Protein name	Function
CAST	Calpastatin	Protein degradation
IGF1R	Insulin growth factor 1 receptor	Muscle growth factor
GAPDH	Glyceradehyde-3-phosphate dehydrogenase	Glycolytic metabolism
ENO1	α-enolase	Glycolytic metabolism
PHGDH	Phosphoglycerate dehydrogenase	Serine biosynthesis
PSAT	Phosphoserine amino transferase	Serine biosynthesis
PSPH	Phosphoserine phosphatase	Serine biosynthesis
P70S6k	Ribosomal protein S6 kinase	Protein translation
4E-BP1	4E-binding protein 1	Protein translation
CSRP3	Cysteine and Glycine-Rich Protein 3	Satellite cell activation
FHL-2	Four and a half LIM domain proteins 2	Satellite cell activation
MUSTN1	Muscoskeletal Embryonic Nuclear Protein 1	Satellite cell activation
FGF2R	Fibroblast growth factor 2 receptor	Satellite cell activation
MYG	Myogenin	Satellite cell activation

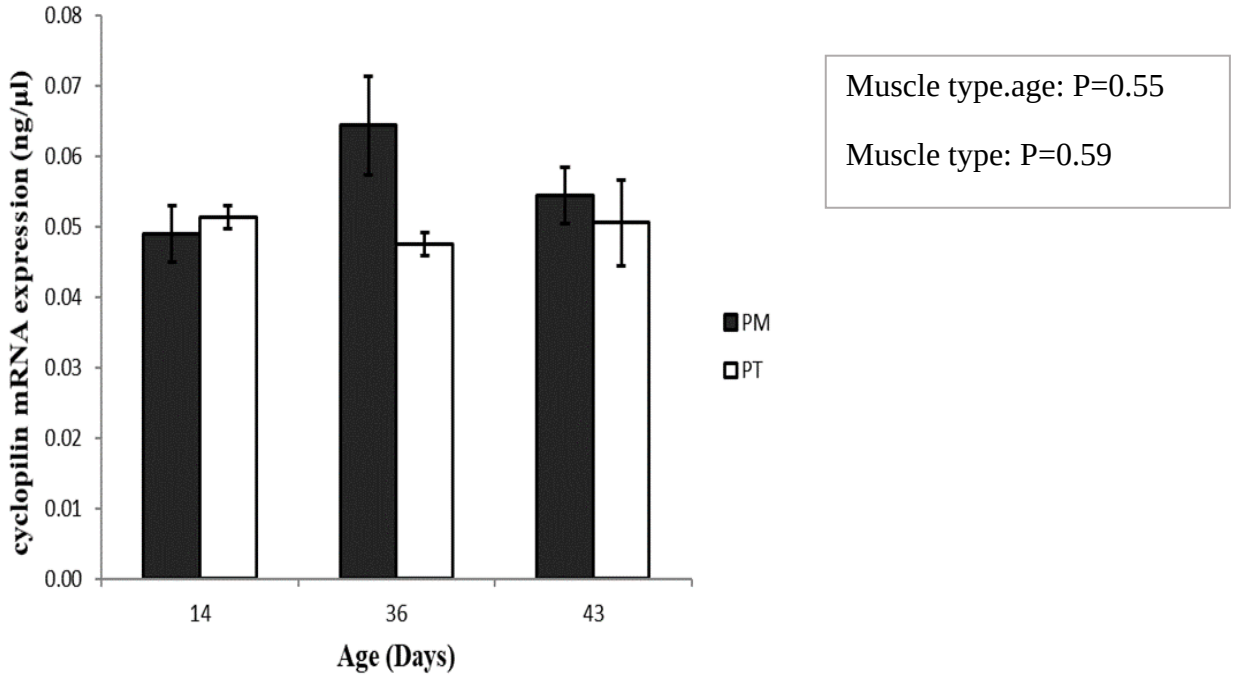

Figure 4.3: Cyclophilin mRNA expression in the PM and PT of Ross 308 at Day 14, 36 and 43 post hatch (trial 2), n=6. Values are quantified as ng RNA equivalents concentrations from light cycler crossing point cycle number. Values expressed as means ± Standard Error of the mean, no significant effects.

As described in Chapter 3, several chicken reference genes that have been reported previously as suitable for correcting for experimental variation. Several of these were measured in all muscle samples to identify the most suitable gene to be used as a reference gene. Out of all genes screened, the most uniformly expressed across all time points with no significant effect on muscle type and age on its expression (P=0.55) and with the best standard curve (an efficiency value of 1.98) was cyclophilin. There was also no effect of muscle type (P=0.59) or age (P=0.51) respectively (Figure 4.3). As a result, this was used to correct the expression of all other genes measured during this study. However, data for expression of all genes were also analysed without correcting for cyclophilin. This was done to further verify the reliability of cyclophilin normalised data. Because samples were normalised to a uniform concentration for RNA used in first strand cDNA synthesis and used at the same concentration for RT-Q-PCR before been corrected by cyclophilin expression. Therefore, the expression profile for both cyclophilin normalised and non-normalised samples which had a similar expression pattern were considered to be acceptable.

4.2.2. Expression of genes associated with muscle protein degradation and muscle mass regulation: calpastatin, myostatin and IGF1R

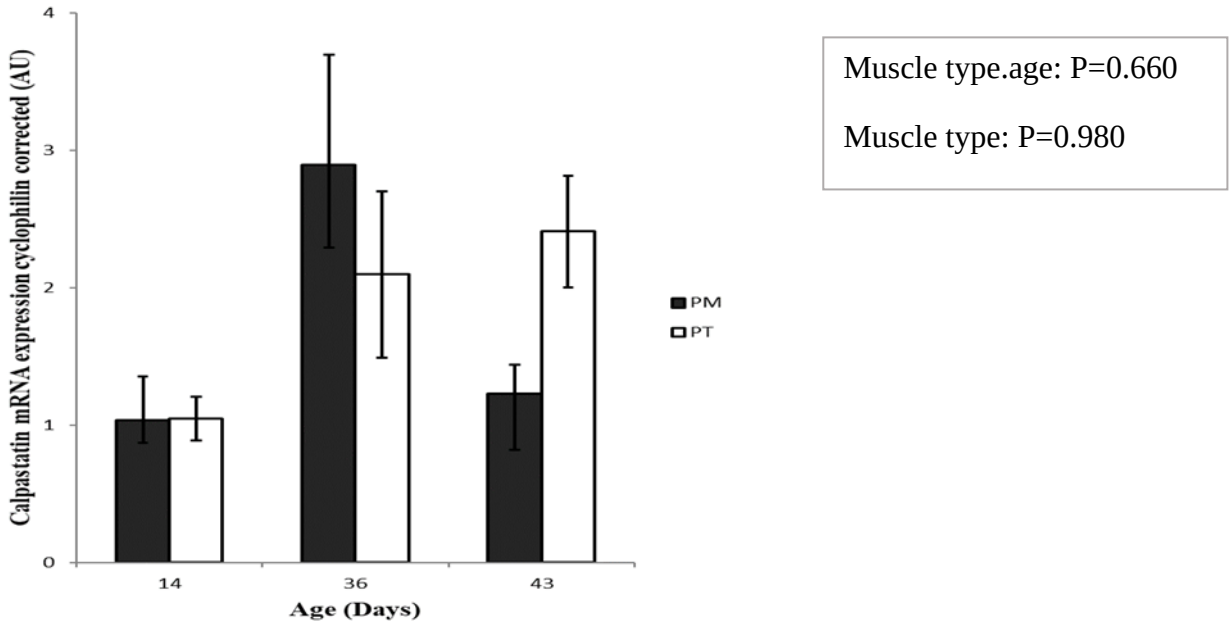

Figure 4.4: Calpastatin cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

For calpastatin gene expression cyclophilin corrected, there was no muscle type x age interaction (P=0.656) neither was there any significant difference between and within the PM and PT at all time points p=0.21 and 0.983 respectively (Figure 4.4). Although the numerical P value were different for calpastatin expression not corrected with cyclophilin, there was a similar expression pattern when compared with cyclophilin corrected expression with no significant muscle type x age interaction (P=0207) nor any significant muscle type effect (P=0.679) or age effect (P=0.207) (Appendix 4).

Myostatin is a negative regulator of skeletal muscle mass determining both muscle fibre number and size. The myostatin pathway is conserved and regulates muscle mass in several animal species (Tobin and Celeste, 2005). In chicken, it has been reported that the expression of myostatin is not inhibited in selected lines of differentially growing chicken neither was there any missense mutation in their coding sequence. (Mott and Iverie, 2002; Schreumann *et al*., 2004). The relevance of these findings in relation to the current study was to test the hypothesis that a difference in mRNA expression of myostatin is not altered in differentially growing chicken skeletal muscles within the same broiler chicken.

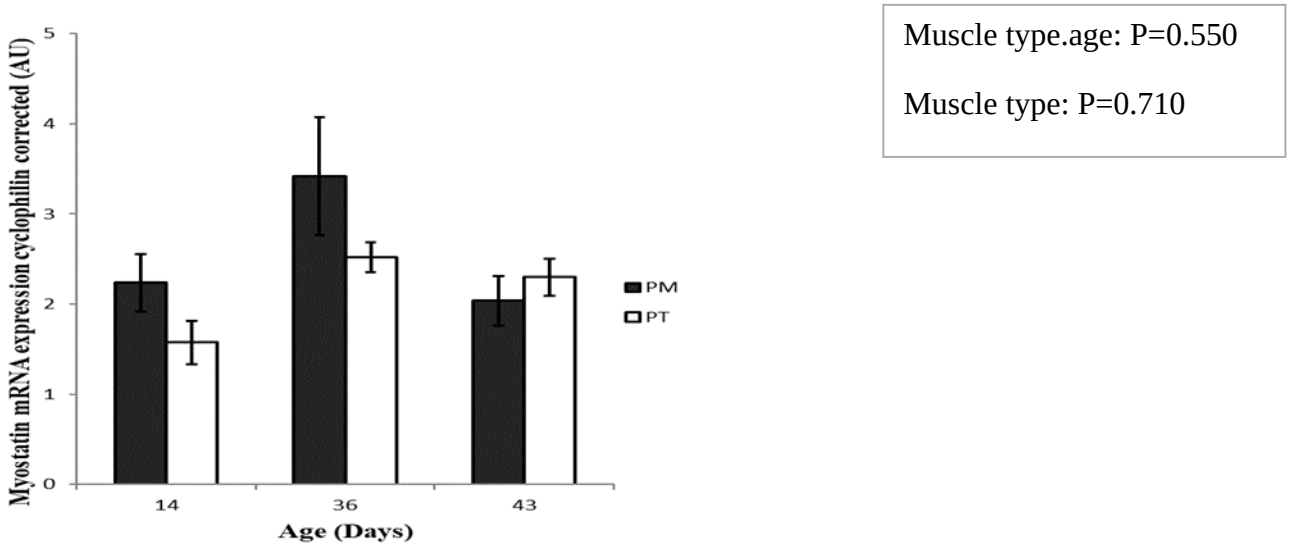

Figure 4.5: Myostatin cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

For myostatin expression (Figure 4.5), there was no interaction between muscle type and age (P=0.55) neither was there any significant difference between and within the PM and PT at all ages (p=0.709 and 0.195 respectively). Although the numerical P value were different for myostatin expression not corrected with cyclophilin, there was a similar expression pattern when compared with cyclophilin corrected myostatin expression also with no significant muscle type to age interaction (P=0.27) nor any significant muscle type (P=0.277) or age (P=0.541) effect between and within the PM and PT at all time points (Appendix 4).

The insulin-like growth factor 1 receptor (IGFIR) is a membrane glycoprotein mediating most biological actions of IGF-1 and IGF-2, which have an important effect on chicken growth, carcass, and meat quality traits (Amilio *et al*., 2003). IGF1R and IGF2R are the two receptor isoforms found in the mammals but only one IGF1R is found in the birds. IGF1R not only regulated the half-life time and activity of IGFs, but also played important roles on the key developmental stage and adult stage such as the cell life cycle, transplantation, metabolism, subsistence, proliferation, and differentiation (Lei *et al*., 2008). IGF-1 regulates muscle mass during development, due to its effect on myogenic cell proliferation and differentiation. As to the role of IGF1 in adult skeletal muscle, several studies indicate that IGF1 can induce hypertrophy and block atrophy (Schiaffino and Mammucari 2011). IGF-1 is mainly produced in the liver and translocated to muscle cells where it takes effect. IGF-1R is however produced in muscles thus the justification for the measurement of its expression.

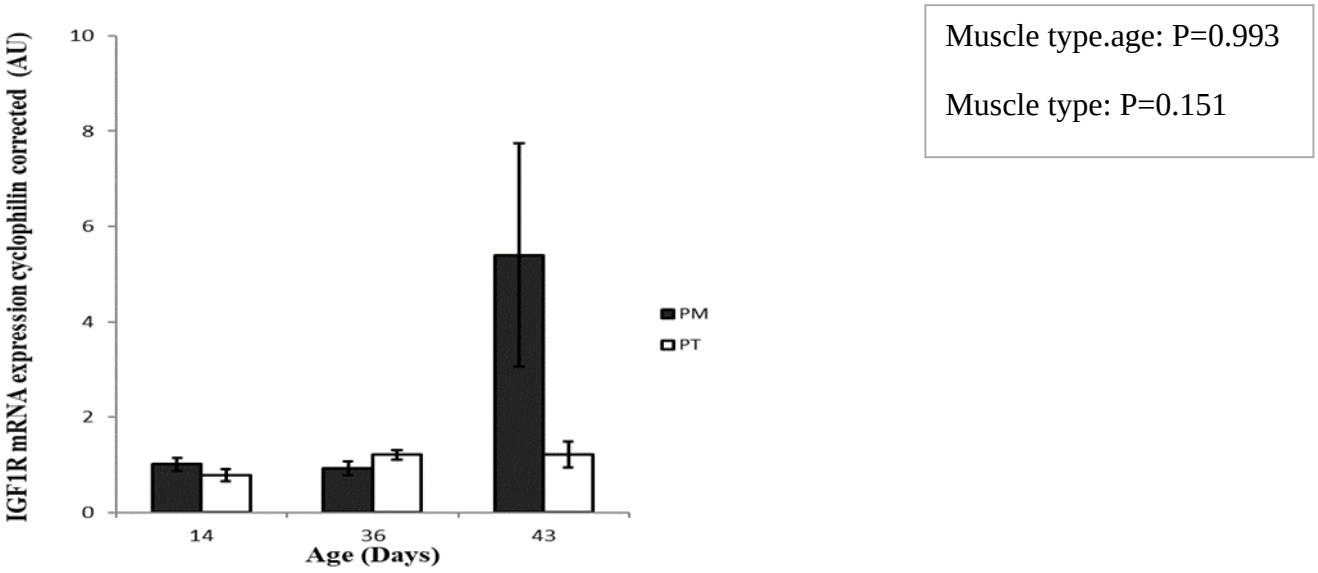

Figure 4.6: IGF1R mRNA cyclophilin corrected expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

For IGF1R expression, there was no muscle type X age interaction (P=0.993) neither was there any significant muscle type or age effect between and within the PM and PT at all ages (p=0.151 and 0.921 respectively, Figure 4.6). There was however a highly variable expression in the PM at day 43 with a large error bar. This was due to an outlier in one of the replicate muscle samples collected from the animal. Although the numerical P value were different for IGF1-R expression not corrected with cyclophilin, there was a similar expression pattern when compared with cyclophilin corrected expression with no significant muscle type to age interaction (P=0.567) nor any significant muscle type (P=0.524) or age difference (P=0.367) between and within the PM and PT at all time points. A highly variable expression in the PM at day 43 with a large error bar was also seen in the mRNA expression of non-corrected IGF1R also due to an outliner in the same replicate muscle sample seen in IGF1R cyclophilin corrected expression corrected. (Appendix 4)

4.2.3. Expression pattern of glycolytic genes regulating glucose metabolism and muscle growth Enolase and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

One of the mechanisms in which skeletal muscle grows is because of skeletal muscle hypertrophy which occurs as a result of an increase in muscle fibre diameter due to the addition of more myofibrils composed of contracting actin and myosin protein filaments to individual fibre bundles resulting in larger muscle motor unit sizes (Aberle and Stewart 1983). These processes are also regulated by some physiological and metabolic mechanisms which in turn are modulated at the molecular level. Type II fibres have an upregulated glycolytic

metabolic capacity when compared to type I slow oxidative fibres due to their higher capacity to generate ATP anaerobically.

One of the objectives of the current study was a comparative measure of the expression of some glycolytic genes, namely glyceraldehyde-3-phosphate dehydrogenase and alpha-enolase whose corresponding proteins catalyse the 6th and 10th step of glycolysis. Measurement of these two genes therefore gives an indication of the glycolytic capacity of the fast and slow growing muscle types of ROSS 308 chicken.

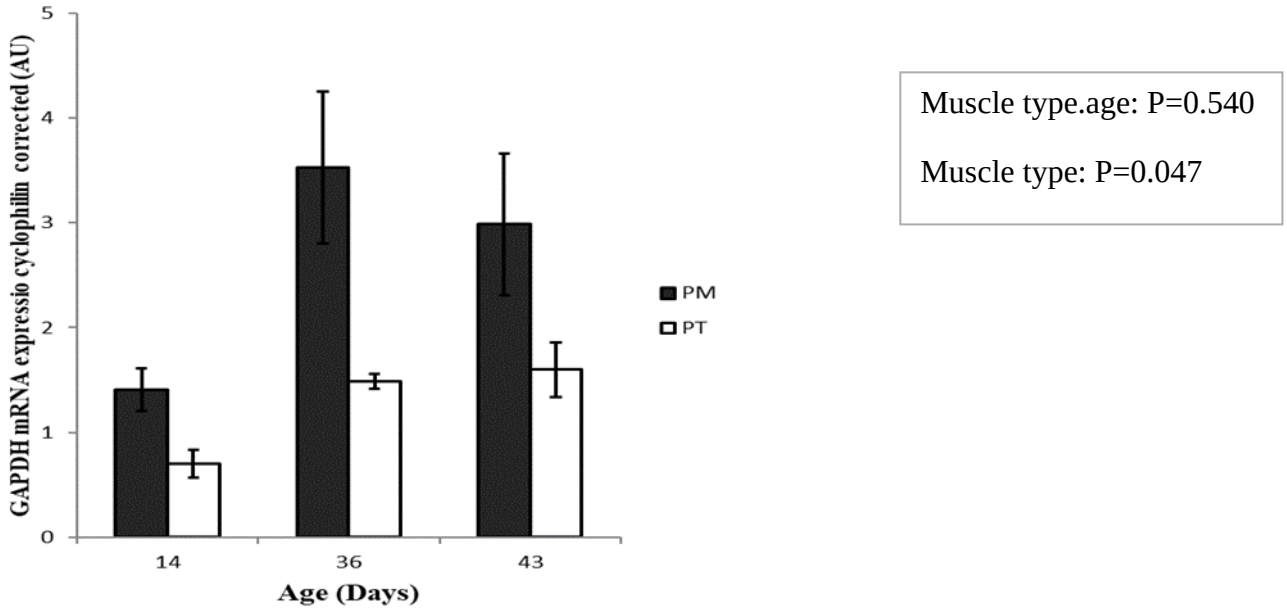

Figure 4.7: GAPDH cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

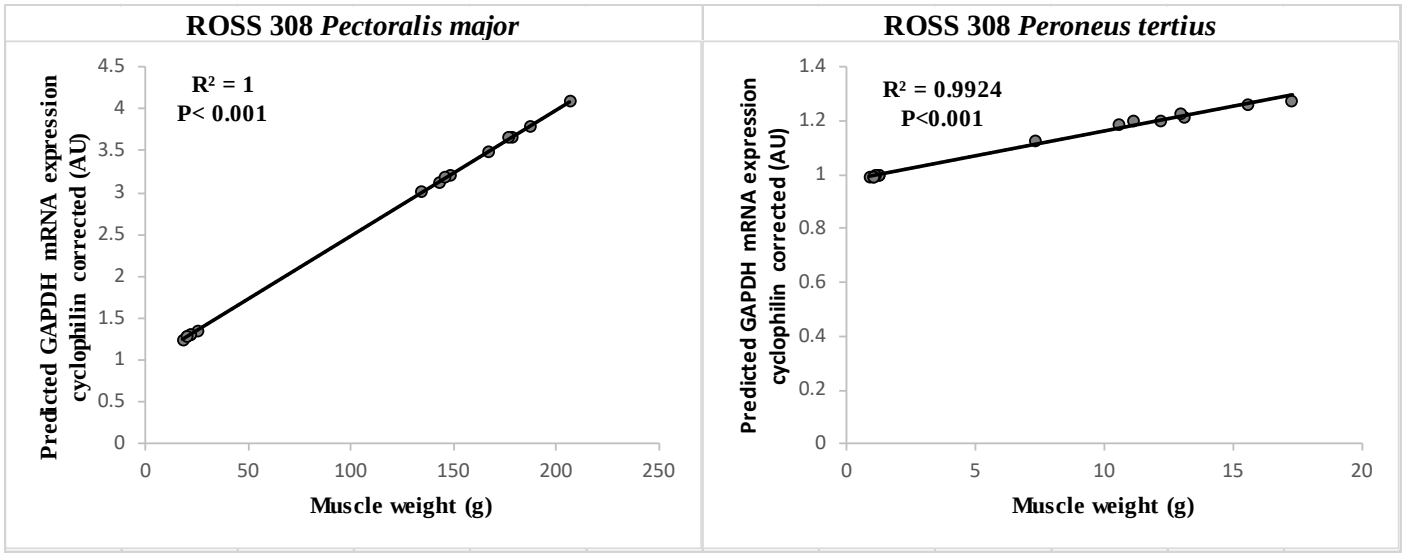

Figure 4.8 Correlated muscle type by muscle weight interaction with age corrected predicted GAPDH mRNA expression (relative cyclophilin expression) of Ross 308 PM and PT muscles at days 14, 36 and 43

For GAPDH mRNA expression cyclophilin corrected, there was no significant interaction effect between muscle type and age P=0.545. However, there was a significant muscle type effect with the PM having a higher expression at all time points when compared to the PT (P=0.047). There was also an age effect (P=0.02) within muscle types with the highest rate of expression seen at day 36 in both the PM and PT. (Figure 4.7). Although the numerical P value were different for GAPDH expression not corrected with cyclophilin, there was a similar expression pattern when compared with cyclophilin corrected expression also having no significant muscle typeXage interaction (p=0.275) and significant muscle type and age effect (p<0.001 and P=0.009 respectively) with the PM having a higher expression at all time points when compared to the PT and the highest rate of expression within muscle types also seen at day 36 in the PM and PT (Appendix 4).

There was also a significant strong positive correlation with age corrected predicted GAPDH mRNA expression (cyclophilin corrected) and muscle weight of the PM where R^2=1 and P<0.001. The same observation was seen in the PT where R^2=0.99 and P<0.001 (Figure 4.8).

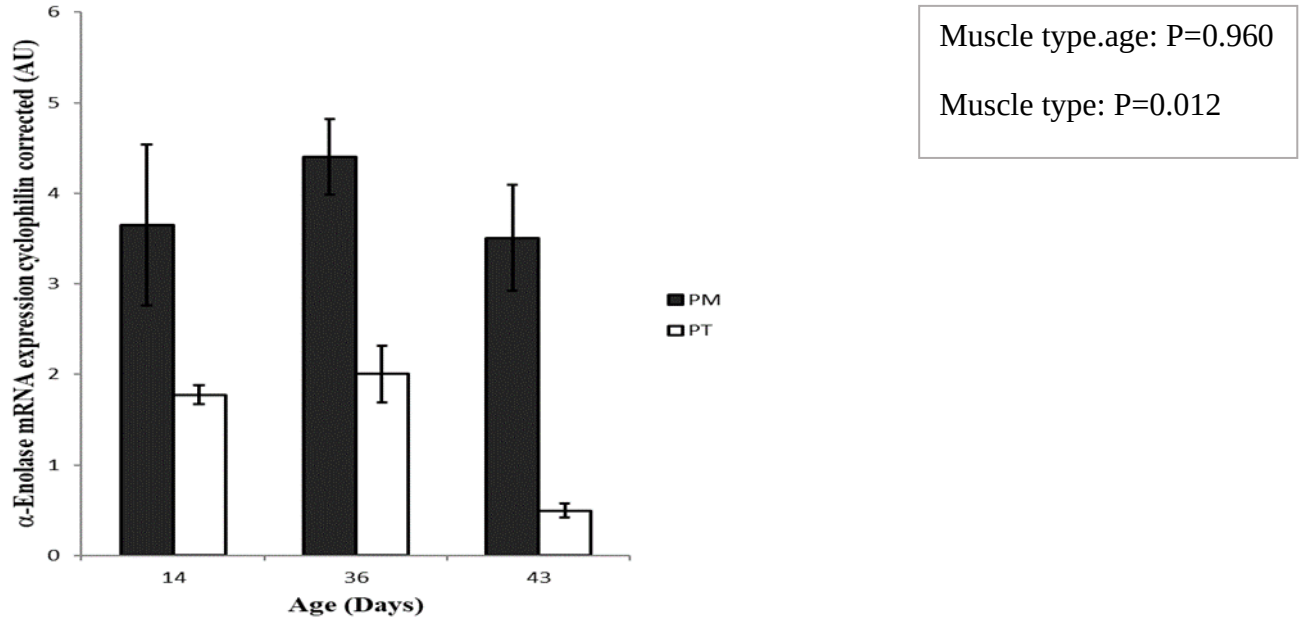

Figure 4.9: α-Enolase cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

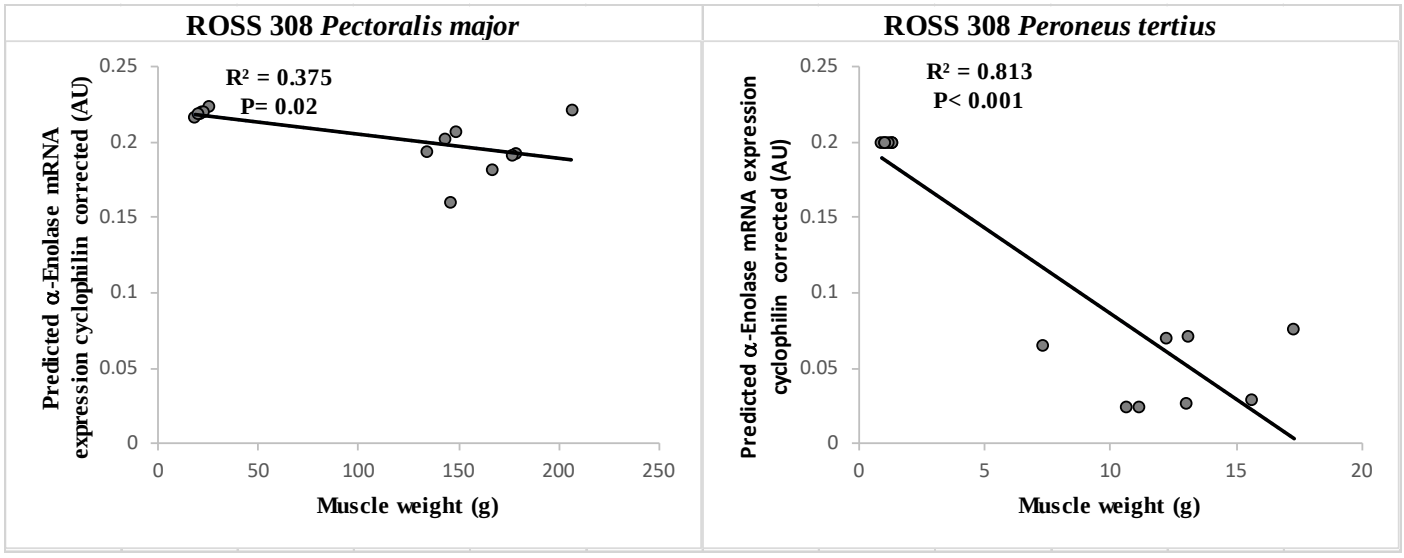

Figure 4.10: Correlated muscle type by muscle weight interaction with age corrected predicted α-enolase mRNA expression (relative cyclophilin expression) of Ross 308 PM and PT muscles at days 14, 36 and 43

For α-Enolase mRNA expression cyclophilin corrected, there was no significant interaction effect between muscle type and age P=0.96. There was also no age effect (P=0.16) within muscle types. However, there was a significant muscle type effect with the PM having a higher expression at all time points when compared to the PT (P=0.012) (Figure 4.9). Although the numerical P values were different for α-Enolase expression not corrected with cyclophilin, there was a similar expression pattern when compared with cyclophilin corrected expression. No significant muscle type to age interaction (P=0.687), nor age effect (P=0.323) was observed. However, there was also a significant muscle type effect (P=0.031) with the PM having a higher expression at all time points (Appendix 4).

There was a significant negative correlation between muscle weight of the PM and age corrected predicted α-enolase mRNA expression (cyclophilin corrected) with R^2 value of 0.375 where P= 0.02. While the PT had a strong statistically significant negative correlation with R^2 =0.81, P<0.001(Figure 4.10).

4.2.4. Expression of genes encoding enzymes catalysing steps in the serine biosynthesis pathway in fast and slow growing muscles of ROSS 308 chicken.

Cell growth and proliferation requires the production of building blocks for the synthesis of cell constituents which include protein, nucleic acids and lipids. Serine and glycine are biosynthetically linked and together provide the precursors for the synthesis of these biomolecules (Locasale *et al*., 2011; Ye, *et al*., 2012 Amelio *et al*., 2014). Serine biosynthesis has also been reported to be upregulated during hyperplastic growth as seen in cancer cells (Possemato *et al*., 2011; Dann and Abraham 2011) However, the interaction between

serine/glycine biosynthesis and cell proliferation is not fully understood. In avian species, myofibre/myoblast number is established post embryogenesis and it is generally thought that the hypertrophy of muscle takes place by the accretion of protein and nuclei originating from the proliferation and fusion of satellite cells (Scheuermann *et al*., 2004). There have been no previous reports on the relationship between serine/glycine biosynthesis and muscle hypertrophy in chicken. However, our laboratory has observed an upregulation in expression of these genes in growth promoter induced skeletal muscle hypertrophy in other species namely pigs and sheep (Parr *et al*.,2015; Al-doski *et al*., 2015; Brown *et al*., 2016). Therefore, one of the objectives of this study was to measure the expression of genes encoding proteins that catalyse the biosynthesis of serine from the glycolytic pathway. We therefore measured the mRNA expression the three enzymatic proteins that catalyse the three step *de novo* biosynthesis of serine namely Phosphoglycerate dehydrogenase (PHGDH), phosphoserine amino transferase (PSAT) and phosphoserine phosphatase (PSPH). See Figure 4.14

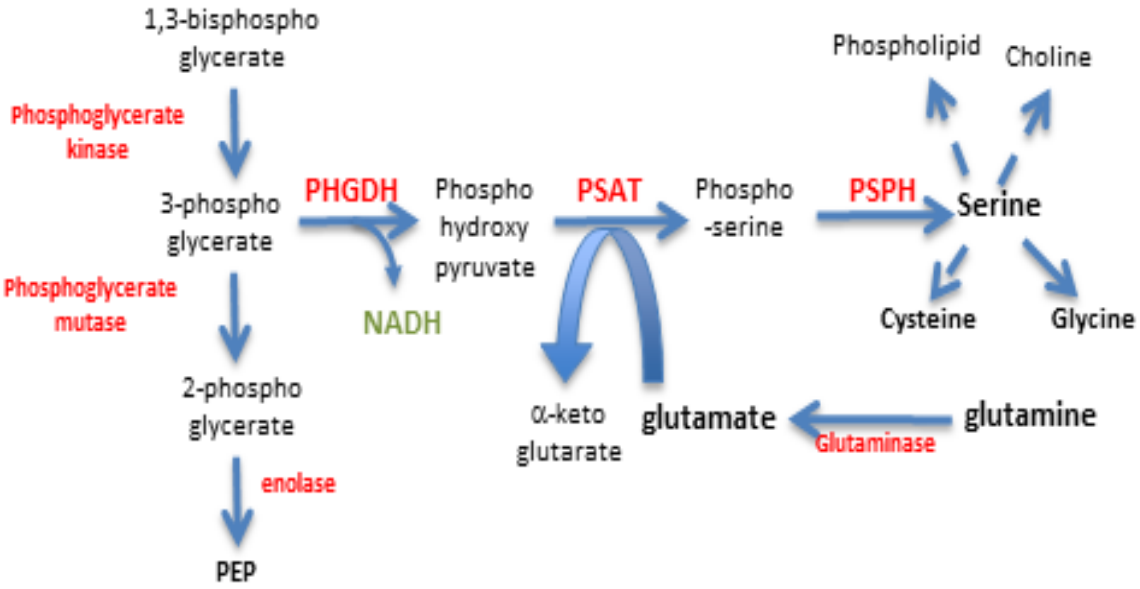

Figure 4.11: Schematic representation of the *de novo* biosynthesis of serine. The first step is the synthesis of Phosphohydroxypyruvate (PPHP) from 3-Phosphoglycerate catalysed by Phosphoglycerate dehydrogenase (PHGDH). PPHP is then converted to Phosphoserine and then finally to serine catalysed by Phosphoerine aminotransferase (PSAT) and Phosphoserine phosphatase (PSPH) respectively. (Parr *et al*., unpublished).

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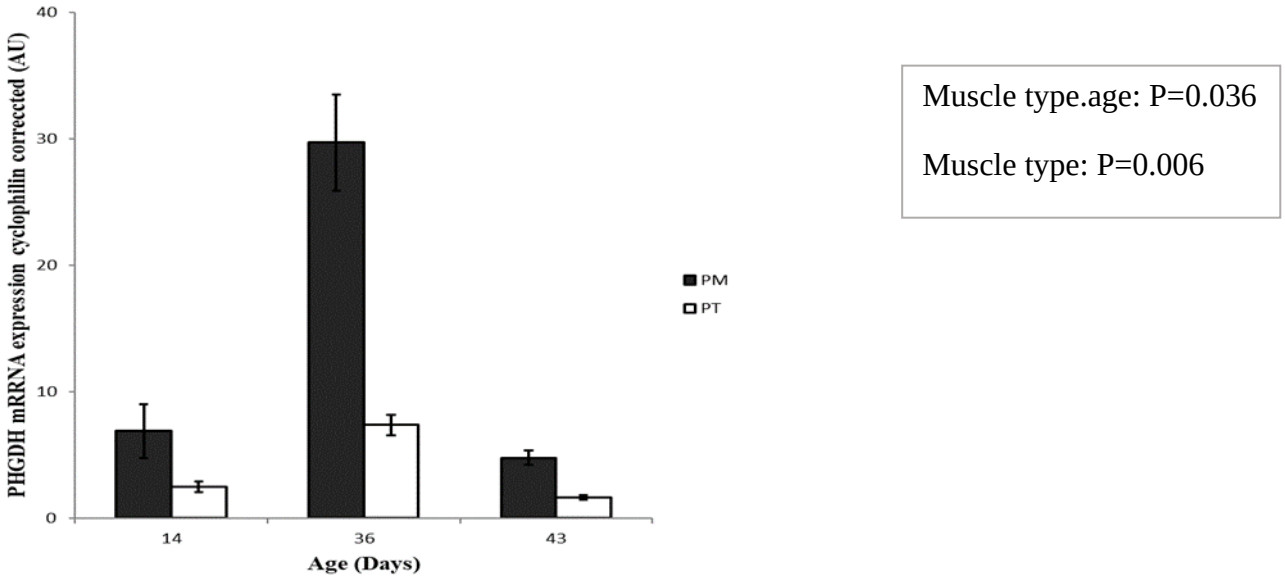


Figure 4.12: PHGDH cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

There was a significant muscle type to age interaction in the mRNA expression of PHGDH cyclophilin corrected (P=0.036) with the PM having a higher expression at all time points with the highest value seen at day 36 in the (Figure 4.12). Although the numerical P value were different for PHGDH expression not corrected with cyclophilin, there was a similar expression pattern when compared with cyclophilin corrected PHGDH having a borderline muscle type to age interaction (P=0.062) and a significant muscle type effect (P=0.005) and age effect (P=0.003). With a higher expression seen in the PM at all time point and peak expression seen at day 36 in both muscles (Appendix 4).

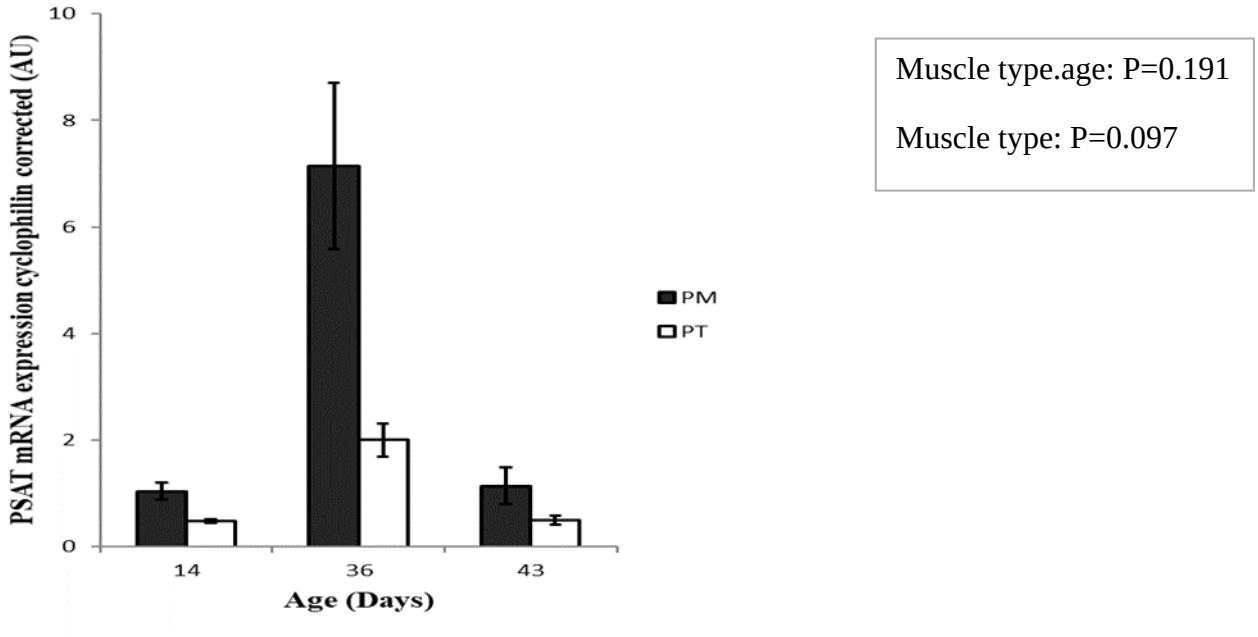


Figure 4.13: PSAT cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

For PSAT cyclophilin corrected mRNA expression, there was no significant muscle type to age interaction (P=0.191). The muscle type effect was also not significant P=0.097, although there was a trend with the PM having a higher expression at all time points. However, there was a significant age effect with the highest expression seen at day 36 in both muscle types (P=0.012). (Figure 4.13). Although the numerical P value were different for PSAT expression not corrected with cyclophilin, there was a similar expression pattern when compared with cyclophilin corrected PSAT having no significant muscle type x age interaction (P=0.089) and a significant muscle type effect (P=0.006) with the PM also having a higher expression at all time points. There was also a significant age effect (P=0.003) with the highest expression see at day 36 in both muscle types (Appendix 4).

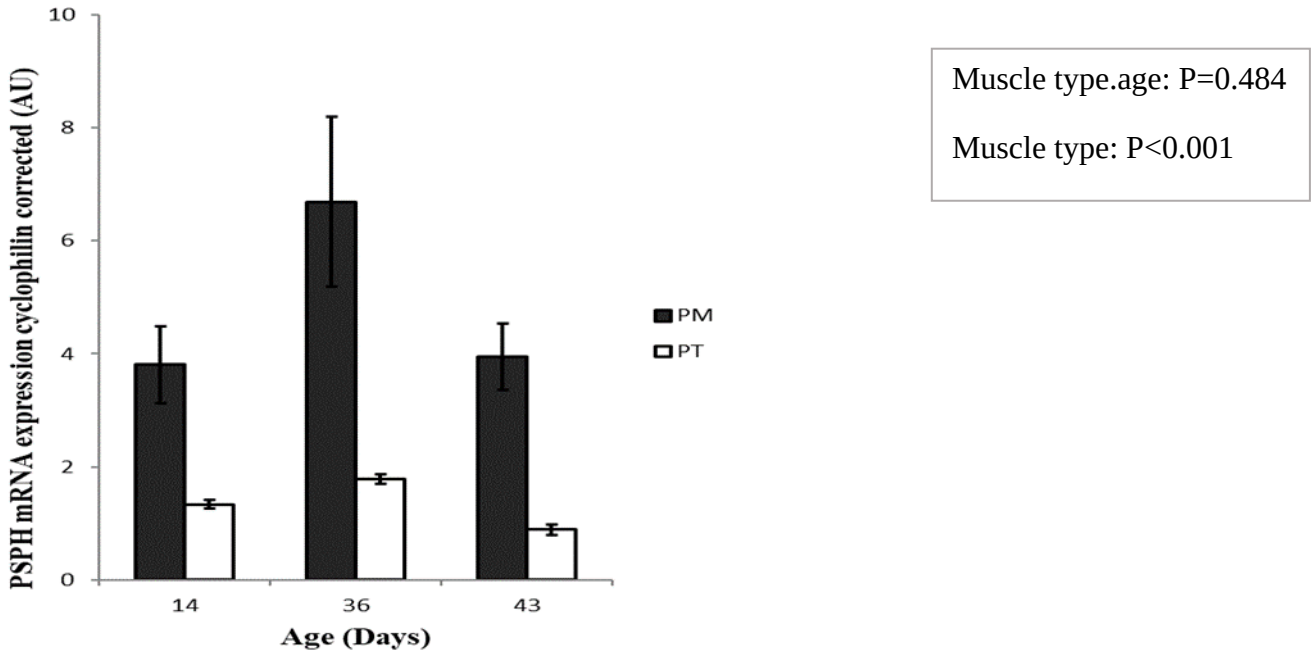


Figure 4.14: PSPH mRNA expression cyclophilin corrected in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

There was no significant interaction between muscle type and age in the cyclophilin corrected mRNA expression of PSPH (P=0.578), neither was there any significant age effect (P=0.176). However, there was a significantly higher expression between muscle type with the PM having a higher expression at all time points (P=0.008). (Figure 4.14). The expression pattern of PSPH non cyclophilin expression pattern also mirrored that of the cyclophilin corrected expression although the numerical P values were different. There was no significant muscle type to age interaction (P= 0.484), neither was there a significant age effect (P=0.263). But there was a muscle type effect (P<0.001) with the PM having a higher expression at all time points (Appendix 4).

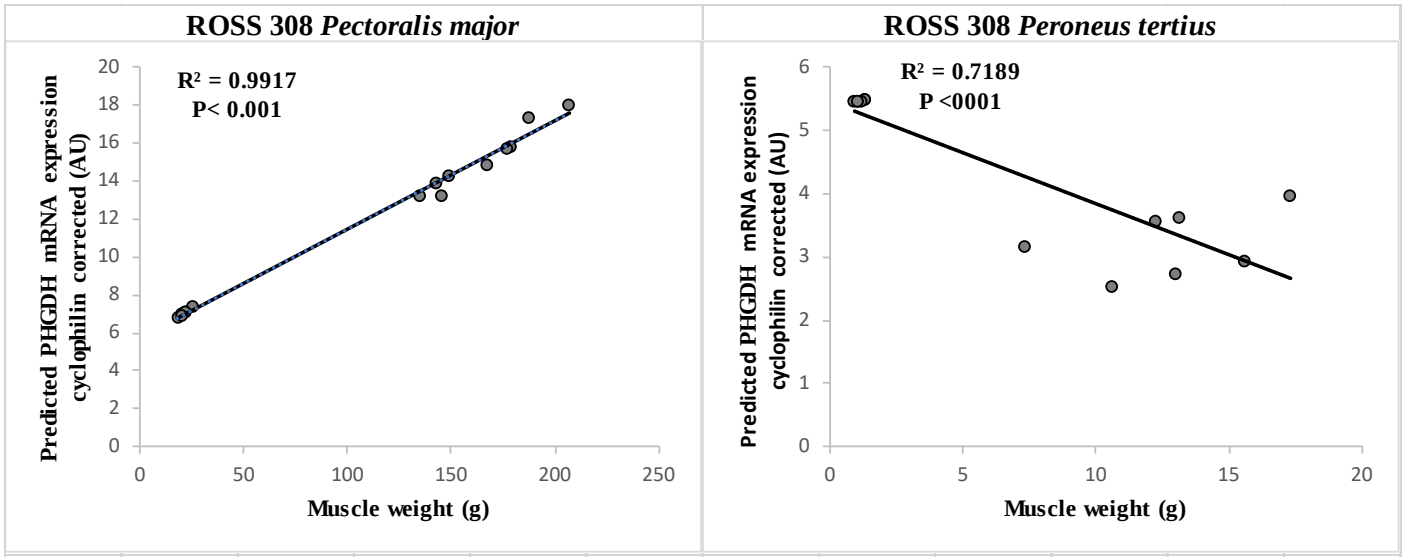


Figure 4.15: Correlated muscle type by muscle weight interaction with age corrected predicted PHGDH mRNA expression (relative cyclophilin expression) of Ross 308 PM and PT muscles at days 14, 36 and 43.

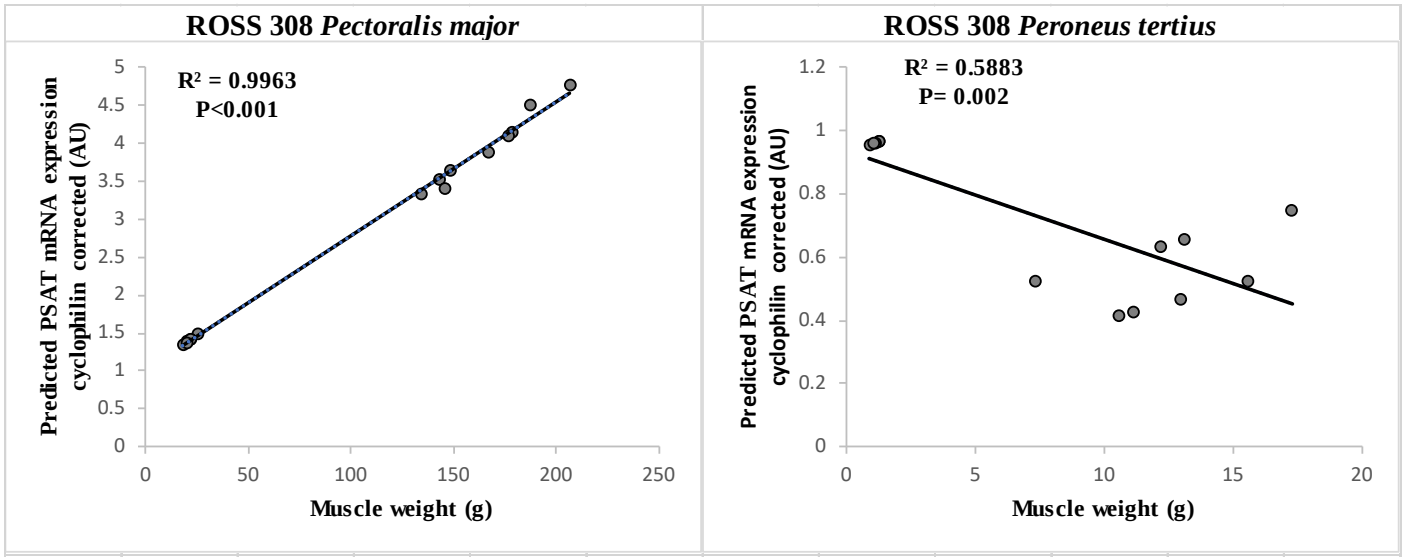


Figure 4.16: Correlated muscle type by muscle weight interaction with age corrected predicted PSAT mRNA expression (relative cyclophilin expression) of Ross 308 PM and PT muscles at days 14, 36 and 43.

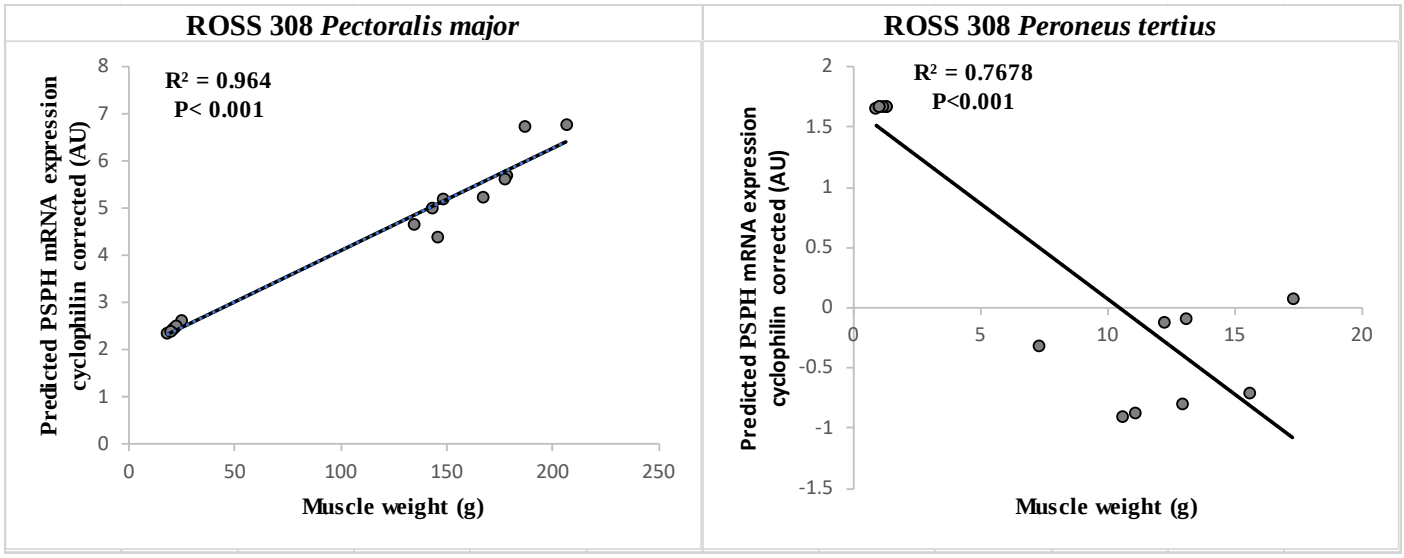


Figure 4.17: Correlated muscle type by muscle weight interaction with age corrected predicted PSPH mRNA expression (cyclophilin corrected) of Ross 308 PM and PT muscles at days 14, 36 and 43

The predicted age corrected mRNA expression (relative cyclophilin expression) of all three serine biosynthetic genes (PHGDH, PSAT, PSPH) were significantly strongly correlated (P<0.001) with the muscle size of the PM with R^2 values of 0.99, 0.99 and 0.96 respectively. However, in the PT muscles, the predicted mRNA of the same three genes expression was significantly negatively correlated with muscle weight with R^2 values of 0.72. 0.59 and 0.77 respectively (P<0.05). The greater level of expression in the three genes of the serine biosynthetic pathway in the larger growing PM indicates that there may be a relationship between skeletal muscle growth and serine biosynthesis in broiler chickens. This effect may be as a result of or may have an influence on the hypertrophic effect as a mechanism of skeletal muscle growth in broiler chickens.

4.2.5. Expression pattern of genes modulating protein translation (downstream AKT/mTOR pathway, P70-S6K and 4E-BP1) in differentially growing Ross 308 broiler muscles

The mTOR pathway is known to regulate protein synthesis through translational capacity and efficiency which leads to an upregulated mRNA translation resulting in increased fibre size. (Zanchi *et al*., 2008) One of the mechanisms by which mTOR controls this process is by the modulation of the activation of the downstream intermediate P70S6 kinase which in turn phosphorylates S6 ribosomal protein. (Nader *et al* ., 2005; Brown *et al*., 1995) Phosphorylation of S6 ribosomal protein correlates with an increase in translation of mRNA transcripts that contain an oligo pyrimidine tract in their 5' untranslated regions. Deng *et al*., (2014) reported an upregulated phosphorylation of mTOR, P70S6K and 4E-BP1 in chick pectoral muscles influenced by an increased dietary leucine. These same observations have also been reported in rats (Kimball and Jefferson 2004). mTOR and its downstream intermediates P70S6K and 4EBP1 have also been reported to upregulate

skeletal muscle protein synthesis initiated by Leucine and other branched chain amino acids by (Anthony *et al*., 2001). Leucine is an essential amino acid that triggers muscle growth when supplemented in the diet. Its mechanism of action is therefore similar to other muscle growth promoting mechanisms.

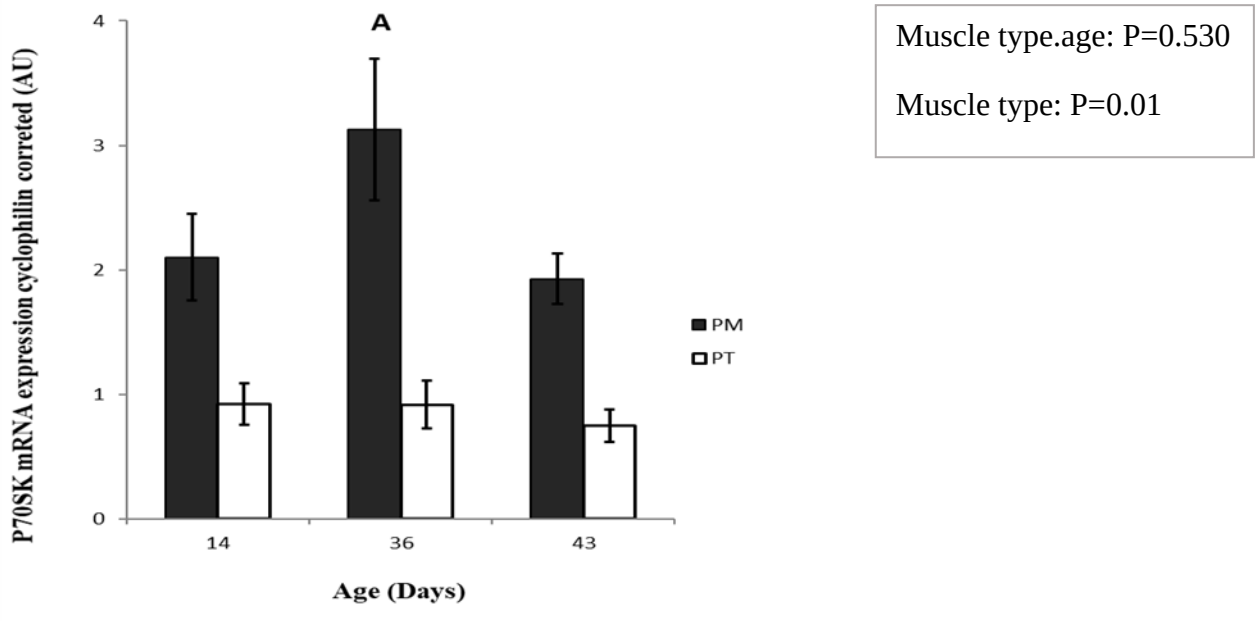


Figure 4.18: P70S6K mRNA expression cyclophilin corrected in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

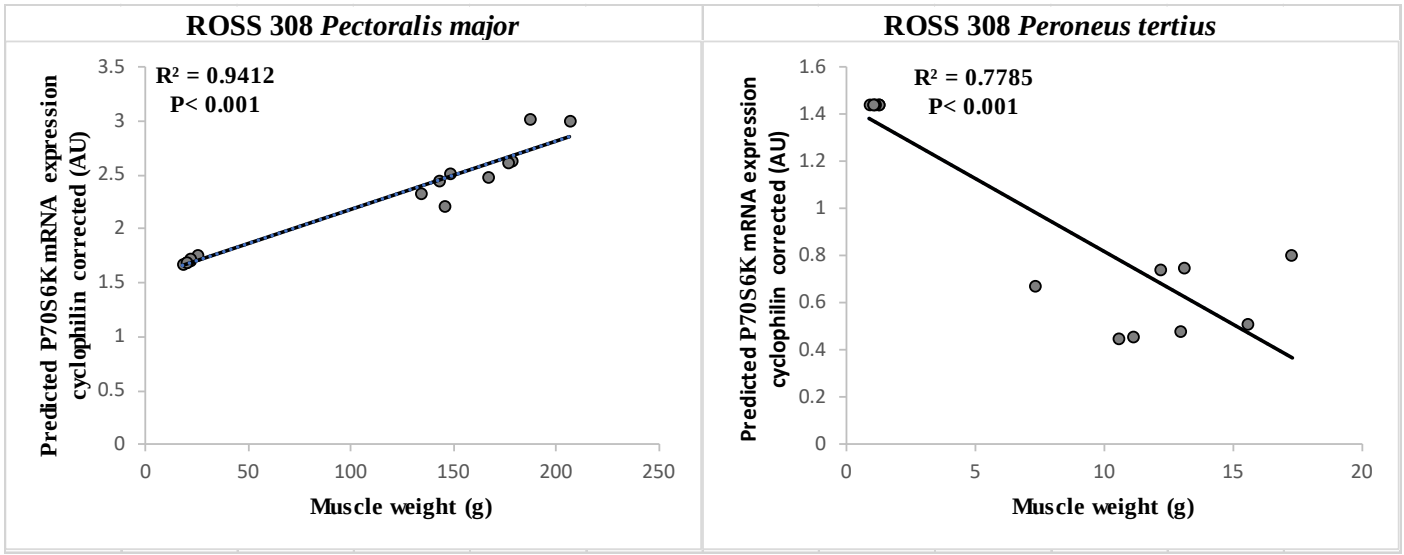


Figure 4.19: Correlated muscle type by muscle weight interaction with age corrected predicted P70S6K mRNA expression (cyclophilin corrected) of Ross 308 PM and PT muscles at days 14, 36 and 43

There were no significant interactions between muscle type and age for the cyclophilin corrected mRNA expression of P70S6K (P=0.53) neither was there any significant ae effect (P=0.359). However, there was a significant muscle type effect with the PM having a higher expression at all time points (P=0.01) (Figure 4.18). Although the numerical P values were different for P70S6K expression not corrected with cyclophilin, there was a similar expression pattern when compared with cyclophilin corrected expression. No significant muscle type to age interaction (P=0.44), nor age effect (P=0.308) was observed. However, there was also a significant muscle type effect (P=0.003) with the PM having a higher expression at all time points (Appendix 4).
This suggests that the rate of protein synthesis is faster in the PM may be as a result of a higher availability of P70S6K transcripts for phosphorylation post translation. As expected, this indicates that there may be a positive relationship between skeletal muscle P70S6K expression and skeletal muscle protein synthesis in chicken. The predicted mRNA expression of P70S6K in the PM had a strong and significant correlation with muscle weight of (R^2= 0.94, P<0.001). However, in the PT, the predicted P70S6K expression had a significant negative correlation with R^2= 0.78 where P<0.001 (Figure 4.19).

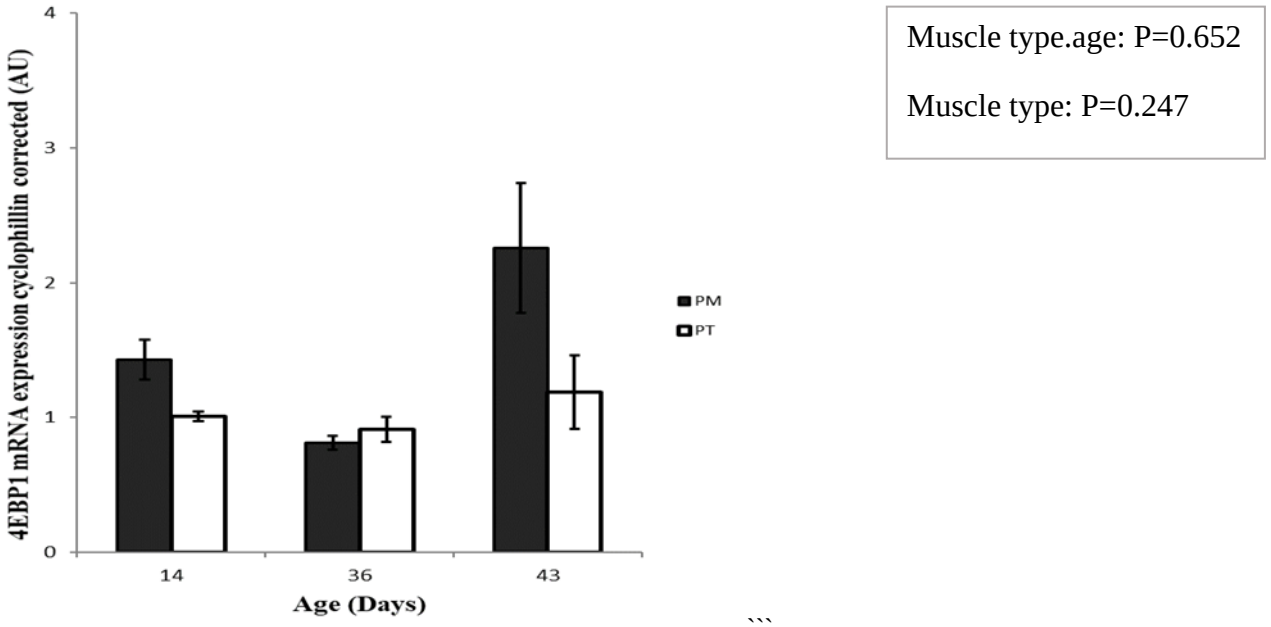


Figure 4.20: 4E-BP1 mRNA expression cyclophilin corrected in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

For 4E-BP1 expression cyclophilin corrected mRNA expression, there was no significant muscle type to age interaction (P=0.652), neither was there any muscle type or age effect where P=0.247 and 0.237 respectively (Figure 4.20). For 4E-BP1 non cyclophilin corrected mRNA expression, a similar expression pattern was seen

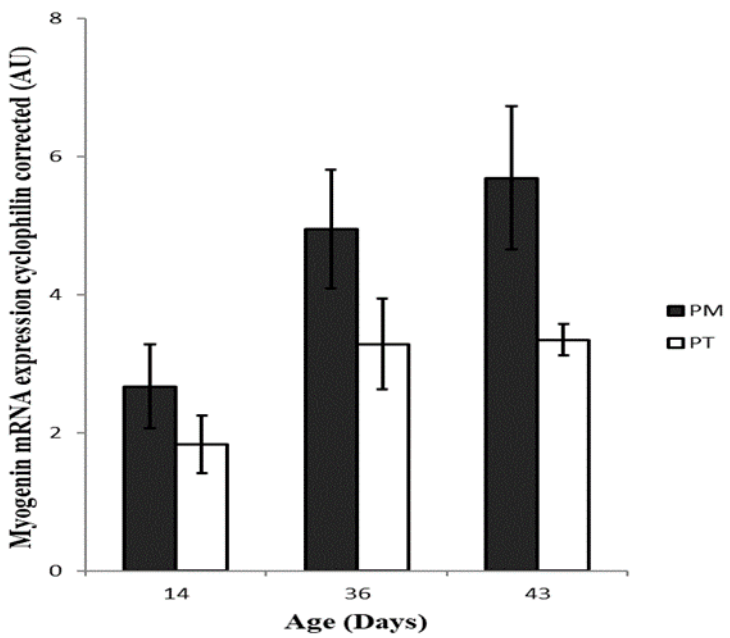
with no significant muscle type to age interaction (P=0.49) and age effect (P=0.49) but there was a borderline muscle type effect (P=0.05) (Appendix 4).

4.2.6 Expression of genes involved in skeletal muscle cell cycle (Myogenin, MUSTN1 and FGF2R) in differential growing skeletal muscles of chicken

MUSTN1 is a muscle development related gene that has been reported to be up regulated during the activation and differentiation of satellite cells and during induced exercise in pigs (Jensen *et al.*, 2012). A higher level of expression of MUSTN1 mRNA has been reported in the thigh muscle chicken when compared to the *pectoralis major* of the breast at 28 days of age (Juan Li *et al*., 2014).

FGFR2 is a receptor for fibroblast growth factor. FGF2 is a potent stimulator of myoblast and satellite cell proliferation, and an intense inhibitor of cell differentiation (Zheng *et al.*, 2009). Thus, plays a minimal role in the differentiation myogenic progenitor cells to muscle fibres. FGFR2 has been reported to be down regulated in the faster growing muscles of broilers when compared to layers (Zheng *et al. 2009;* Kong *et al.*, 2011; Bottje *et al*., 2012). Zheng *et al*., (2009) reported an upregulated expression in the slower growing muscles of layer chicken breast muscles when compared to faster growing broiler breast muscles. The expression of myogenin, FGFR2 and MUSTN1 was comparatively measured between the faster growing PM and slower growing PT in broiler chicken and results compared to what has been reported by Zheng *et al*., (2009).

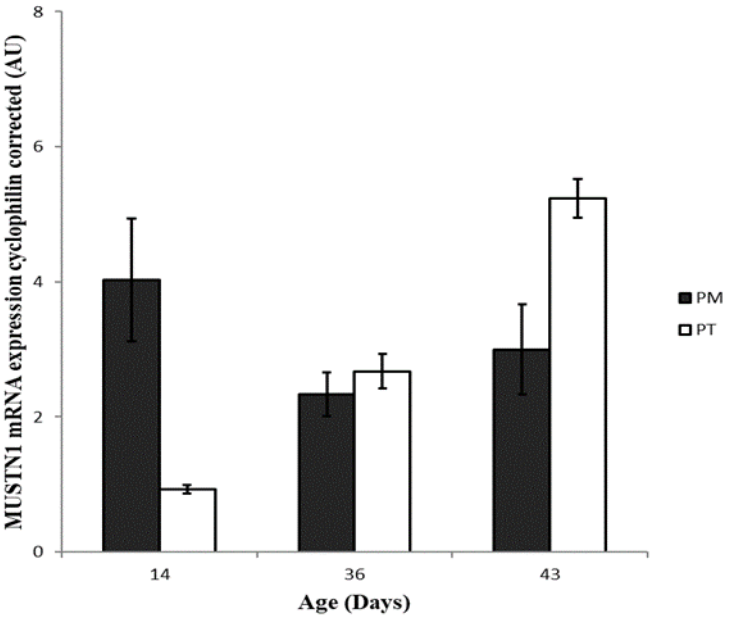
Myogenin is a myogenic regulatory factor that induces progenitor cells to differentiate into specialised myocytes and eventually mature myotubes. This process of myogenesis occurs during embryogenesis and during the activation of quiescent satellite cells to repair damaged mature muscle fibre supporting a form of muscle hypertrophy. The aim of measuring myogenin expression is to have an idea of the activity of satellite cell activation leading to muscle cell differentiation and maturity in the differentially growing muscles of chicken using myogenin as a biomarker.



Muscle type.age: P=0.924

Muscle type: P=0.242

Figure 4.21: Myogenin mRNA expression cyclophilin corrected in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.



Muscle type.age: P=0.032

Muscle type: P=0.713

Figure 4.22: MUSTN1 mRNA expression cyclophilin corrected in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

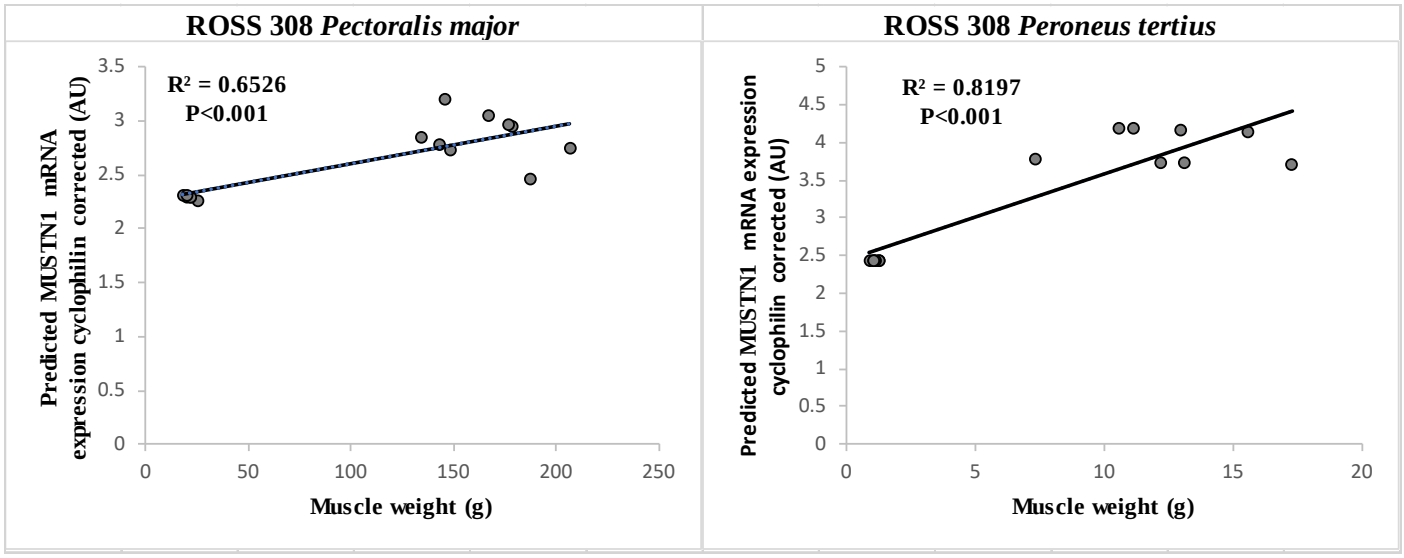


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Figure 4.23: Correlated muscle type by muscle weight interaction with age corrected predicted MUSTN1 mRNA expression (cyclophilin corrected) of Ross 308 PM and PT muscles at days 14, 36 and 43

For myogenin cyclophilin corrected mRNA expression, there was no significant muscle type to age interaction (P=0.924) neither was there any significant muscle type effect between the PM and PT. (P=0.242) there was however a borderline age effect (P=0.06) with an increase in its expression the with age. This observation was more notable in the PM (Figure 4.21). For non-cyclophilin corrected myogenin expression, there was no muscle type to age interaction (P=0.78) neither was there any muscle type nor age effect (P=0.127 and 0.523 respectively) (Appendix 4). While MUSTN1 cyclophilin corrected mRNA expression had a significant muscle type to age interaction P=0.032 with the highest level of expression Seen in the PT at day 43, and an increased expression with age was also seen in the PT (Figure 4.22). Although the P values were different, the mRNA expression pattern of MUSTN1 non-cyclophilin corrected mirrored that of the cyclophilin corrected also having a significant muscle type to age interaction (P=0.025) with the highest expression also seen in the PT at day 43 (Appendix 4). There was also a strong and significant positive correlation with muscle weight and predicted MUSTN1 cyclophilin corrected mRNA expression in the Pm and PT muscles where R^2=0.65 and 0.82 respectively P<0.001.

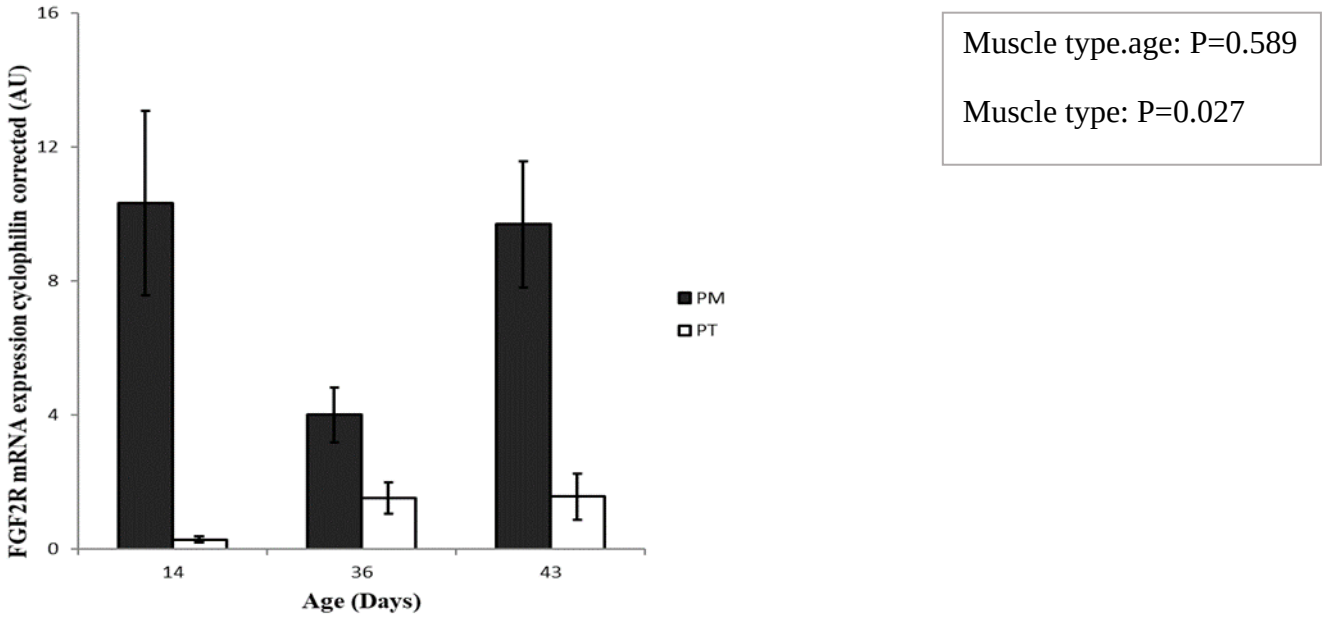

Figure 4.24: FGF2R mRNA expression cyclophilin corrected in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

For FGF2R cyclophilin corrected mRNA expression, there was no significant muscle type to age interaction (P=0.589) neither was there any significant age effect. (P=0.808). However, there was a significant muscle type effect (P=0.027) with the PM having a higher expression at all time points (Figure 4.29). FGF2R non cyclophilin corrected mRNA expression also had a similar expression pattern despite a difference in P values, having a non-significant muscle type to age interaction an age effect (P=0.423 and 0774 respectively). However, there was also a significant muscle type effect (P=0.003) with the PM also having a higher expression at all time points (Appendix 4)

4.2.7 Expression of LIM domain proteins (CSRP3 AND FHL2) in differential growing skeletal muscles of chicken

Zheng *et al.,* (2009) identified 543 differentially expressed genes in the *Pectoralis major* (PM) across five developmental stages after hatching. The genes with the greatest differential expression were those encoding LIM domain proteins, Cysteine and Glycine Rich Protein 3 LIM protein (CSRP3) and four and a half LIM domain protein (FHL2) CSRP3 is a gene that codes for muscle LIM protein (MLP) expressed in skeletal and cardiac muscles (Gehmlich *et al*., 2008). It is known to promote myogenesis and myogenic differentiation (Arber *et al*., 1994). However significantly lower transcripts of CSRP3 have been detected in faster growing broiler breast muscles when compare to that of growing layers at four weeks post hatch (Zheng *et al*., 2009). FHL2 belongs to the four and a half LIM only protein family (Loughna *et al*., 2000). Not much is known about

its function especially in skeletal muscles, but it has been reported to induce differentiation from mouse myoblast to myotubes (Martin *et al*., 2002). Furthermore, a lower level of expression o FHL2 has also been reported in faster growing broiler breast muscles when compared to that of slower growing layers at four weeks post hatch (Zheng *et al*., 2009). The objective of the current study was to determine the expression of these genes in the fast growing (PM) and slow growing (PT) muscles from within the same growing broiler chickens.

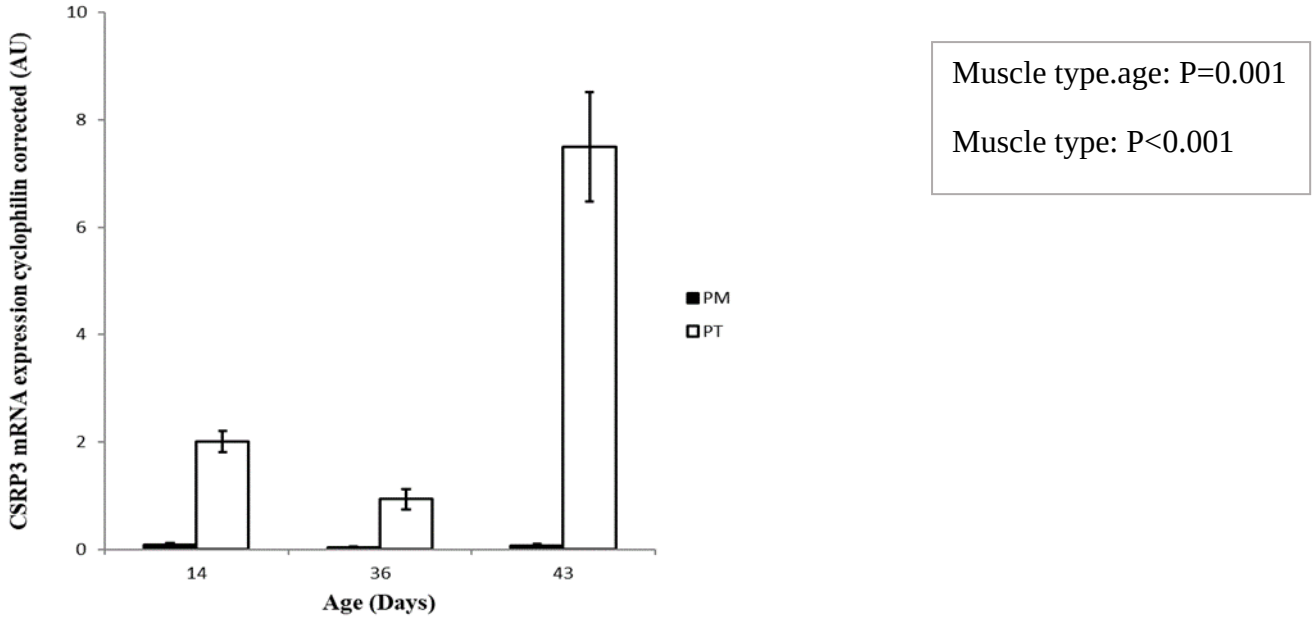

Figure 4.25: CSRP3 mRNA expression cyclophilin corrected in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

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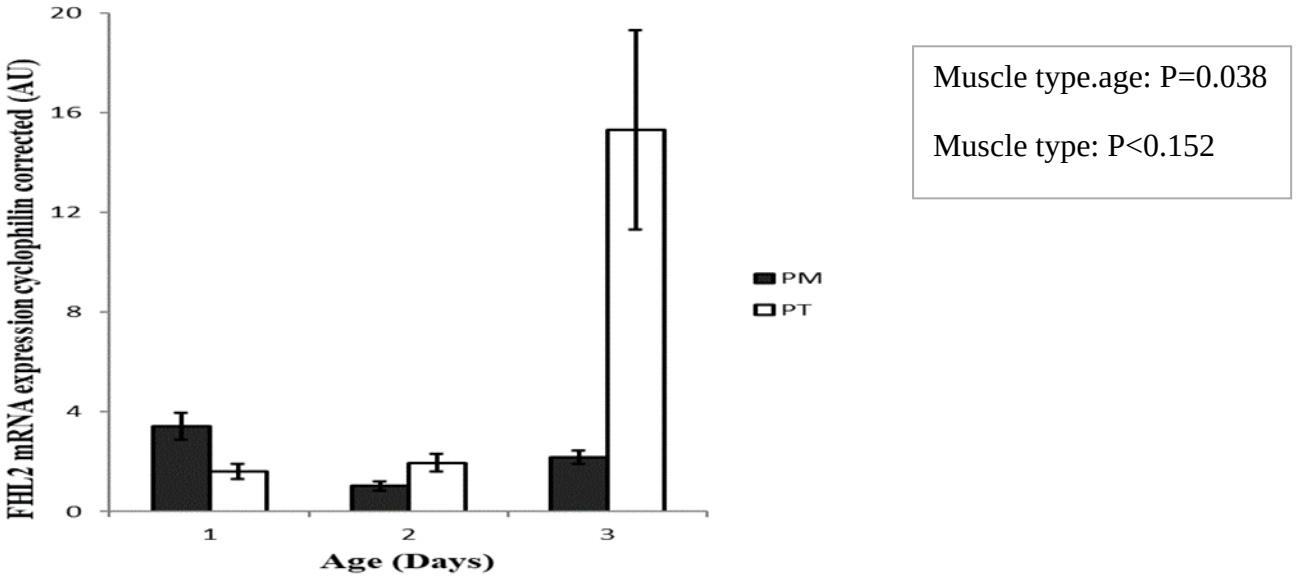


Figure 4.26: FHL2 mRNA expression cyclophilin corrected in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch, n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

There was a significant muscle type to age interaction in the cyclophilin corrected mRNA expression of CSRP3 and FHL2 (P=0.001 and 0.038 respectively) with the highest expression of both genes seen at day 42 in the PT (Figure 4.25 and 4.26). The non-cyclophilin CSRP3 analysed mRNA had a similar expression pattern with the cyclophilin corrected in spite having different P values. There was also a significant muscle type to age interaction (P=0.016) with the highest level of expression also seen in the PT at day 42 (Appendix 4). Non-cyclophilin corrected FHL2 mRNA expression pattern with cyclophilin corrected FHL2 with the highest expression also seen in the PT at day 42. However, this observation was not statistically significant (Appendix 4).

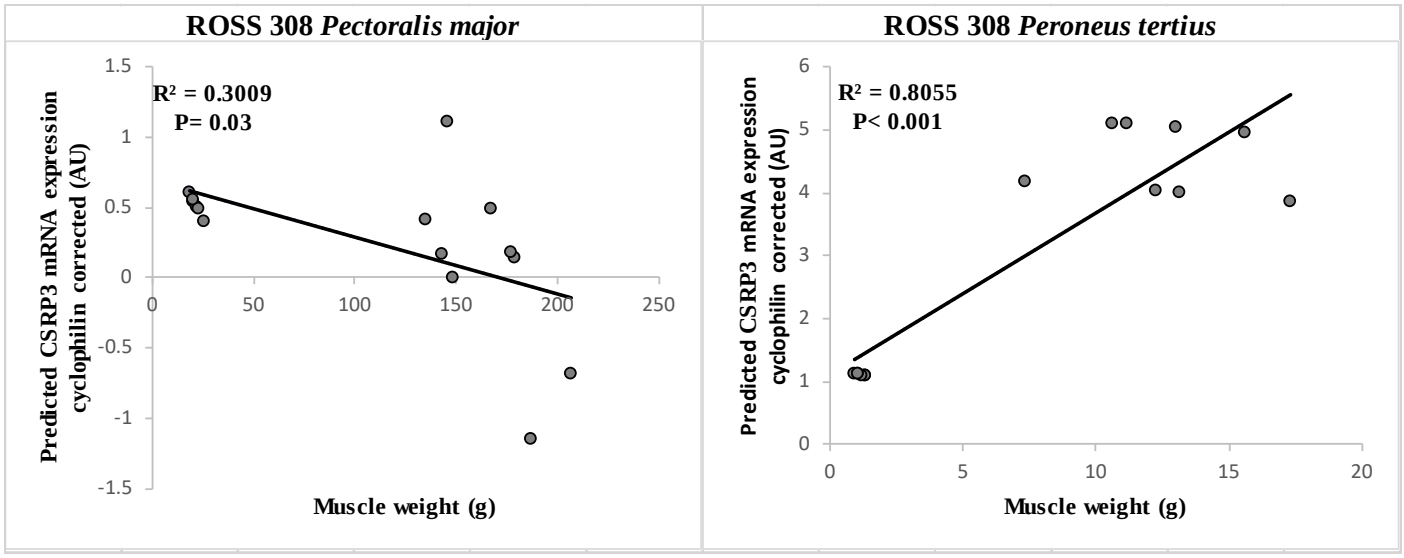


Figure 4.27: Correlated muscle type by muscle weight interaction with age corrected predicted CSRP3 mRNA expression (cyclophilin corrected) of Ross 308 PM and PT muscles at days 14, 36 and 43

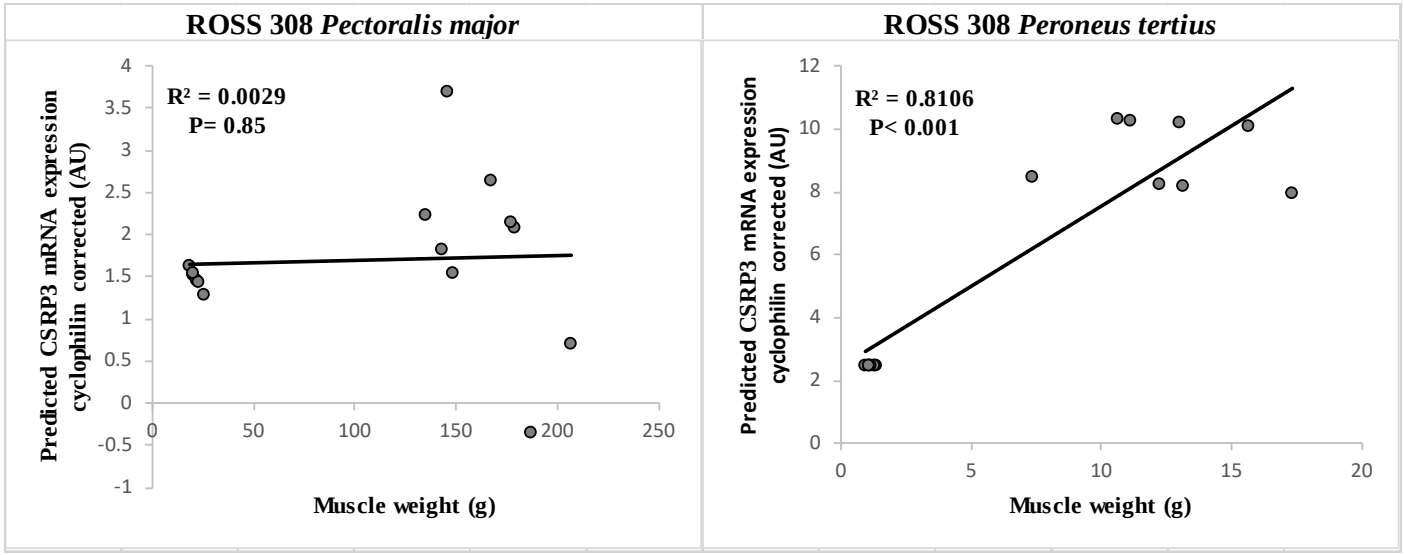


Figure 4.28: Correlated muscle type by muscle weight interaction with age corrected predicted CSRP3 mRNA expression (cyclophilin corrected) of Ross 308 PM and PT muscles at days 14, 36 and 43

There was a significant negative correlation with muscle weight and CSRP3 age corrected predicted mRNA expression having a R^2 value of 0.30 where P=0.03, while the PT had a significant positive correlation with an R^2 value of 0.81 where P <0.001 (Figure 4.27). FHL2 PM expression has no correlation with muscle weight with R^2 value of 0.03 where P=0.85, while the PT had a significant strong positive correlation with an R^2 value of 0.81 where P<0.001 (Figure 4.28).

4.3 SUMMARY

In this trial muscle samples namely, *Pectoralis major* (PM) and *Peroneus tertius* (PT) which are within the fast-growing breast muscles or relatively slow growing leg muscles respectively were taken from Ross 308 broiler chickens at different points of their growth (days 14, 36 and 43) and muscle weights measurements, glycogen, protein and DNA assays were carried out on these muscle samples.

For gene expression studies, cyclophilin was used to correct the expression of all other genes measured during this study. However, data for expression of all genes were also analysed without correcting for cyclophilin. This was done to further verify the reliability of the cyclophilin normalised data. Because samples were normalised to a uniform concentration for RNA used in first strand cDNA synthesis and used at the same concentration for RT-Q-PCR before been corrected by cyclophilin expression. Results showed a significantly higher expression of glycolytic genes GAPDH and α-enolase in the faster growing PM. Also, genes involved in serine biosynthesis PHGDH, PSAT and PSPH also had a significant upregulated expression in the faster growing PM muscles higher expression. For protein synthesis promoting genes, P70S6K had a significant upregulated expression in the faster growing PM but there was no significant muscle type or age effect for 4E-BP1 expression. There was no significant muscle type and age effect in the expression of calpain inhibiting gene calpastatin and muscle enlargement inhibiting myostatin gene. Neither was there any significant muscle type or age effect in the expression of muscle mass regulating gene IGF1R between and within the PM and PT muscles. For genes involved in skeletal muscle cell cycle, there was no significant muscle type or age effect in myogenin expression. But there was a muscle type to age interaction for MUSTN1 expression with the highest expression seen in the PT at day 42. While FGF2R had a significant upregulated expression in the faster growing PM at all time points. LIM domain proteins CSRP3 and FHL2 had a higher expression in the slower growing muscle as well as negative correlations with muscle weight in the faster growing muscles indicating the possibility of those two genes being negative regulators of muscle growth.

This trial served as a pilot study. Further studies were therefore carried out to investigate the expression of target genes and metabolic systems they modulate in more detail in the subsequent trial.

CHAPTER 5. (TRIAL 2 RESULTS)

Trial 2: Changes in metabolic characteristics of muscles during post-natal growth in two distinct genotypes of chickens; broiler (Ross 308) and layer (Hy-Line).

5.0 INTRODUCTION

The purpose of this trial was to simultaneously test the reproducibility of trial one by examining changes in expression of genes associated with muscle metabolism and growth during post-hatch growth in two muscles that had distinct growing characteristics *Pectoralis major* (PM) compared to the *Peroneus tertius* (PT). However, a significant addition to the experiment was the examination of these muscles in cockerels of two genotypes which have differing growth characteristics, a broiler genotype (Ross 308) or a layer genotype (Hy-Line). Results from Trial 1 (Chapter 4) showed that there was a difference in the expression of genes involved in the glycolytic pathway, serine biosynthetic pathway and the mTOR/AKT signalling pathway in the muscle *Pectoralis major* (PM) when compared to the *Peroneus tertius* (PT). It was also observed that LIM domain proteins, CSRP3 and FHL2, that have been reported to regulate satellite cell activation in skeletal muscle cells, had a significantly lower expression in the faster growing and larger PM muscles when compared to the slower growing smaller PT muscles. Other genes previously reported to modulate muscle growth in other animal species were also studied and had no significant effect in their expression between both muscle types resulting in the decision not to investigate them further. Protein and glycogen content because transcription of genes that regulate thee parameters were being measured in this study.

Muscle types comparatively studied were Ross 308 *Pectoralis major* (RPM), Hy-Line *Pectoralis major* (HPM), Ross 308 *Peroneus tertius* (RPT), and Hy-Line *Peroneus tertius* (HPT) at time points of day 4, 14, 23, 35 and 42 post-hatch. Some of the time points were excluded in the examination of some target genes and proteins due to limited availability of resources. Only genes with a significant difference in level of expression from the first trial (Trial 1) were measured in this trial. Furthermore, the mTOR/AKT pathway was further investigated by carrying out protein expression studies on the downstream targets of the mTOR/AKT pathway. Specifically, the expression of total P70S6K in RPM, HPM, RPT and HPT at day 14 and 42, as well as total ribosomal protein S6 (rps6) in the RPM and HPM at day 35. Ribosomal protein S6 (rpS6) is end target of the mTOR/AKT pathway which stimulates protein translation when activated by phosphorylation. This is carried out by phosphorylated P70S6K, which itself has to be phosphorylated via mTOR/AKT pathway. An attempt was made to also measure the expression of other proteins in the mTOR/AKT signalling pathway which include phospho-P70S6K, 4E-BP1 and AKT, but

this was not possible due to the inability to generate protein bands in tested samples on immunoprobed western blots. Metabolic genes, measured by qPCR, included alpha-enolase, beta-enolase, PCK2 which encodes mitochondrial phosphophenol carboxykinase (PEPCK2), Asparagine synthetase (ASNS) and intermediates in serine biosynthesis pathway (PSAT and PSPH), whilst PHGDH protein expression was measured by immunoblotting.

All the above targets were generally associated with the process of protein synthesis. This forms part of the process of protein turnover, the other component being protein degradation. Net accretion of muscle protein could be achieved by increasing protein synthesis or decreasing protein degradation, or by altering both. As the growth rates of PT and PM muscles in Trial 1 had been shown to be significantly different it was decided to examine samples generated from Trial 2 for any differences in specific proteolytic enzyme systems. The first proteolytic system investigated was the calpain system, which has been known to be involved in muscle protein turnover (Goll *et al.*, 2008) and it is inhibited during beta-adrenergic agonist stimulated muscle growth (Higgins *et* al, 1988; Parr *et al*., 2001). The system consists of the ubiquitously expressed micro- and milli-calpain, which are proteinases activated at μM or mM Ca^{2+} concentrations respective, along with a calpain-specific endogenous inhibitor, calapastatin. The second system examined was the proteasome system. This system has been characterised as being involved in protein degradation systems in skeletal muscle (Murton *et al*., 2008). Generally, it is strongly activated in conditions where severe atrophy takes place (Attaix *et al*., 2009; Glass 2005) but has been implicated in the general protein turnover in muscles (Bodine and Baehr 2014). In this system ubiquitin ligases identify proteins for degradation by conjugating ubiquitin to them. Subsequently, these labelled proteins are degraded by the proteasome, a multi-catalytic proteinase consisting of subunits which have differing types of proteolytic activity. In this current study, investigation of the proteasome system studies was carried out by measuring gene expression of ubiquitin ligases muscle atrophy F-box (MAFbx)/Atrogin1 and ring finger 1 (MuRF1) and the different types of proteolytic activities associated with the proteasome, the trypsin, chymotrypsin and caspase -like activities, in the RPM, HPM, RPT and HPT at day 14 and 35. The micro- and milli-calpain activities were also measured by casein zymography in the RPM, HPM, RPT and HPT at days 14, 35 and 42.

In addition, Trial 1 study had identified significant differences in the expression of the LIM domain proteins, therefore gene expression of CSRP3 and FHL2 were also measured in all muscle types at day 4, 14, 23, 35 and 42.

The aim of the current study was to elucidate the mechanisms by which skeletal muscle growth occurs at the molecular level using chickens as a model by investigating various biological and metabolic processes that attenuate muscle growth. The hypothesis was that these processes in fast growing muscles are

regulated by some specific genes the upregulates protein and non-essential amino acid synthesis. The specific objectives of this trial were as follows:

- To measure growth performance and carcass parameters in the two differentially growing genotypes
- Elucidate the effects of metabolic systems modulating glycolytic metabolism, serine biosynthesis, asparagine synthesis and other TCA cycle metabolites on skeletal muscle growth in chicken.
- Investigate the relationship between downstream AKT/mTOR intermediates (P70S6K and rps6) expression and muscle growth.
- Investigate the regulation of muscle growth by proteolytic systems (calpain and proteasome) in chicken.
- Determine the relationship between LIM domain protein (CSRP3 and FHL2) mRNA expression and skeletal muscle growth in the two differential growing chicken genotypes.

5.1 TRIAL DESIGN AND SAMPLE COLLECTION AND STATISTICAL ANALYSIS

Trial design and sample collection were done as described in section 3.1. While Three-way factorial ANOVA and multi regression analysis were carried out as described in section 3.8.

5.1.2 Growth and carcass performance parameters

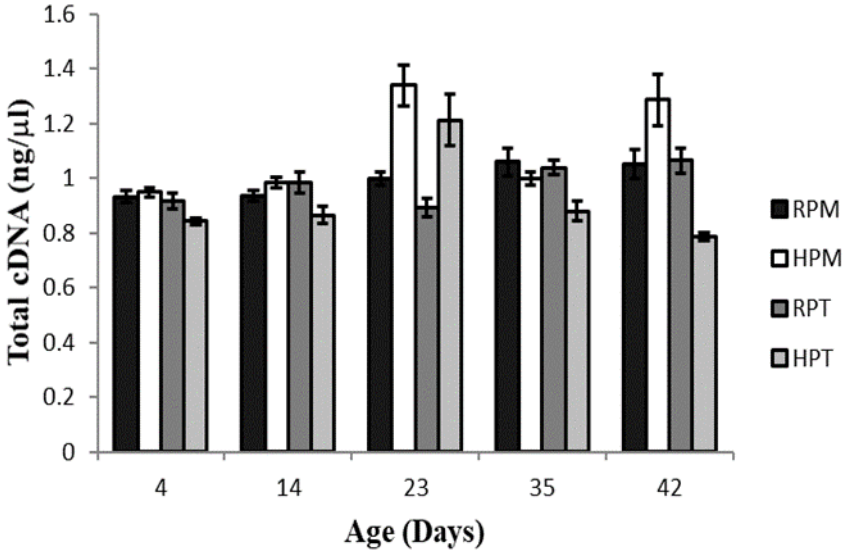
Table 5.1: Total body weight, PM and PT muscle weight and % muscle to body weight of Ross 308 and Hy-Line (Trial 2) at five different time points of day 4, 14, 23, 35 and 42 from trial 1. n=6, Values expressed as means ± Standard Error of the Mean.

Measurement	Animal/Muscle weight (g)	Age (Days)					SED[1]	Effect (P value)						
		4	14	23	35	42		Genotype. muscle.age	Muscle. age	Genotype .age	Genotype .muscle type	Genotype	Muscle type	Age
Animal weight (g)	Ross 308	69.0	240.0	533.0	1216.0	2055.0	32.30	<0.001		<0.001				<0.001
	HY-LINE	50.0	136.0	268.0	525.0	697.0								
Muscle weight (g)	RPM[1]	0.73	7.0	18.0	51.0	99.0	0.75	<0.001	<0.001	<0.001	<0.001	<.0001	<.0001	<.001
	HPM[2]	0.53	3.6	8.0	17.0	24.0								
	RPT[3]	0.42	2.0	4.0	10.0	20.0								
	HPT[4]	0.23	0.85	2.0	4.0	7.0								
%Muscle to total body weight	RPM	1.0	3.0	3.0	4.0	5.0	0.13	<0.001	<0.001	<0.001	<0.001	<.0001	<.0001	<.001
	HPM	1.0	3.0	3.0	3.0	3.0								
	RPT	0.60	0.83	0.75	0.82	0.97								
	HPT	0.47	0.63	0.75	0.76	1.00								

[1]Ross 308 *Pectoralis major* (RPM)
[2]Hy-Line *Pectoralis major* (HPM)
[3]Ross 308 *Peroneus tertius* (RPT)
[4]Hy-Line *Peroneus tertius* (HPT)

Trial design and sample collection were carried out as described in Section 3.1. There was a three-way interaction between genotype X muscle type X age in muscle growth (P<0.001) with the highest value seen at day 42 in the RPM (table 1

5.2 TOTAL cDNA MEASUREMENT BY OLIGREEN QUANTIFICATION



Genotype.muscle.age: P=0.454
Muscle.age: P= 0.524
Genotype.Age: P=0.01
Genotype.muscle: P=0.005
Genotype: P=0.614
Muscle: P<0.001
Age: P=0.007

Figure 5.11: Total cDNA quantification in PM and PT muscles of Ross 308 and Hy-Line at days 4, 14, 23, 35 and 42 (trial 2), n=6. Values expressed as means ± Standard Error of the Mean

To analyse gene expression, it was not possible to utilise a reference gene as these were found to be affected by one or more of the factors being examined in the trial (see section 3.4.9.). Therefore, adjustment for variability in cDNA was made using oligreen which determined the total quantity of cDNA in each reaction.

When quantifying total cDNA by measuring oligreen concentration, as described in section 3.4.10 there was no 3-way interaction between genotype, muscle type and age in total cDNA concentration (P=0.454). Neither was there any muscle type to age interaction (P=0.01). However, there were genotype x age interactions (P=0.01) and genotype to muscle type interactions (P=0.005) (figure 5.1). Despite these observations it was decided to utilise oligreen data to normalise the gene expression data as this reflected any gross changes in cDNA which may have been caused by experimental errors during cDNA synthesis step.

5.3 STUDIES ON METABOLIC SYSTEMS IN CHICKEN MUSCLE.

In this study, expression pattern of metabolic genes which include glycolytic genes α-Enolase and β-Enolase, gluconeogenic gene PCK2, non-essential amino acid synthesis gene Asparagine synthetase (ASNS**)**, as well as genes of enzymes in the serine biosynthetic pathway (PSAT and PSPH) were measured. Protein expression of PHGDH in the RPM and HPM of Ross 308 and Hy-Line genotypes at day 35 was also measured by western blotting.

5.3.1 mRNA expression of glycolytic genes α and β enolase

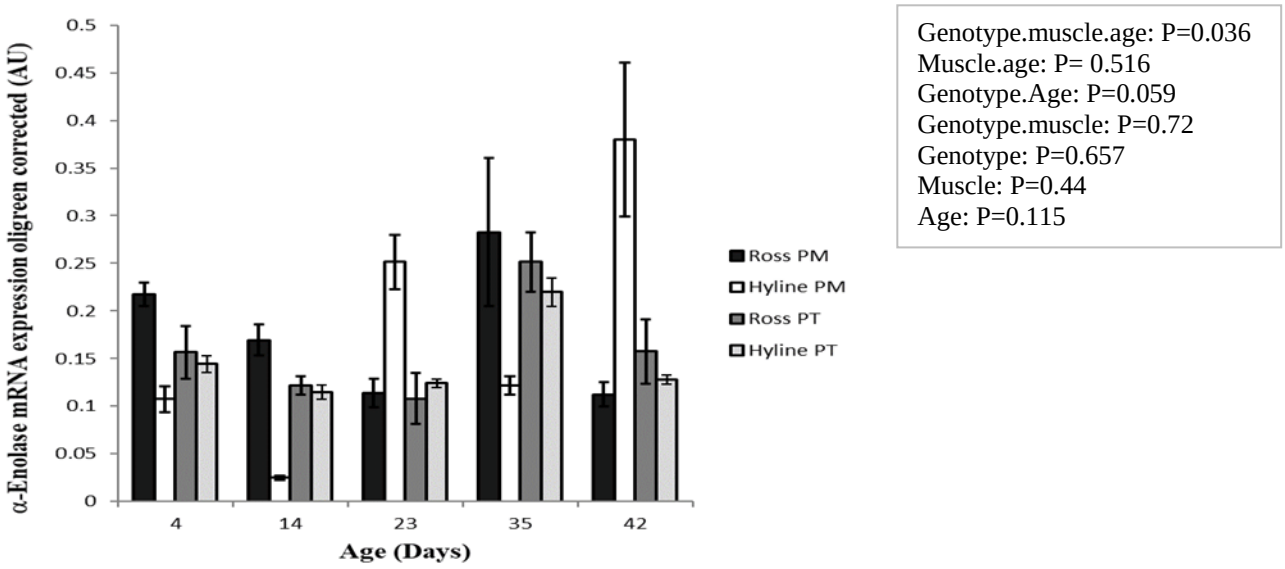
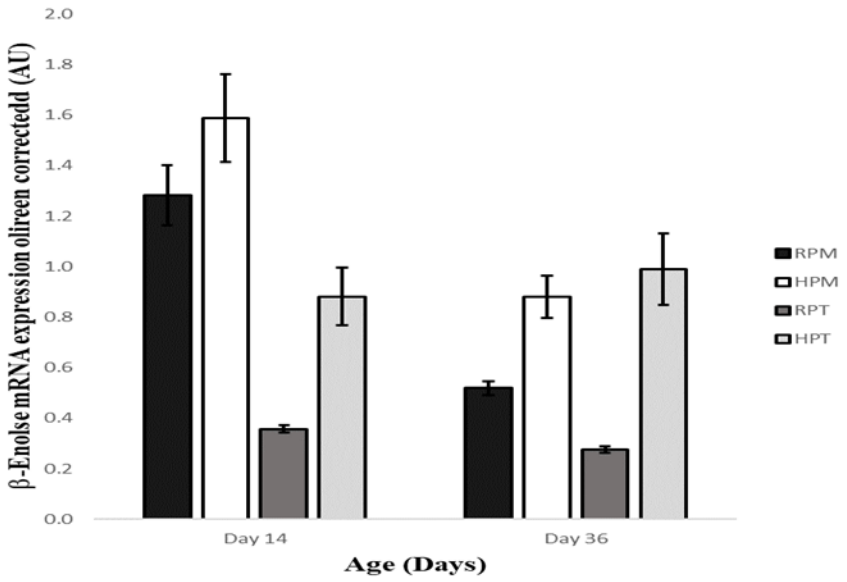


Figure 5.2: The gene expression of α-Enolase mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch. α Enolase mRNA expression relative total cDNA measured by oligreen quantification at Day 4, 14, 23, 35 and 42 (n=6). Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.



Genotype.muscle.age: P=0.279
Muscle.age: P= 0.284
Genotype.Age: P=0.03
Genotype.muscle: P=0.335
Genotype: P<0.001
Muscle: P<0.001
Age: P=0.192

Figure 5.3: The gene expression of β-Enolase mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch. β–Enolase mRNA expression relative total cDNA at Day 14 and 35 (trial 2), n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.

There was a 3-way interaction between genotype, muscle type and age in the expression of α-enolase (P=0.036) with the highest value seen at day 42 in the HPM (Figure 5.2). For β-Enolase expression, there were no interactions but a genotype and a muscle type effect (both P<0.001) with Hy-Line chickens apparently having highest expression whilst PM had higher expression than PT (Figure 5.3).

5.3.2 Gene expression of gluconeogenic gene (PCK2)

Two distinct isoforms of PEPCK enzymes have been identified a cytoplasmic form, PEPCK-C (encoded by *Pck1* gene), and the mitochondrial form, PEPCK-M (encoded by *Pck2* gene) (Yang *et al*., 2009). PEPCK has been well characterised as being involved in gluconeogenesis and TCA cycle function regulating glucose and lipid metabolism, a role that PEPCK-C in the liver is thought to be predominantly responsible for. PEPCK-M has also remained under explored and its role remain relatively unknown. However, it has been reported to cooperate with PEPCK-C in regulating glucose and lipid metabolism in the liver of mouse (Mendez-Lucas et al., 2013). PEPCK-M generates phosphoenolpyruvate (PEP) from oxaloacetate, which can feed into gluconeogenesis and subsequently biosynthetic pathways such as the serine biosynthetic pathway (Lunt and Heiden 2011; Ward and Thompson 2012; Ye *et al.*, 2012) Carbon labelling studies have demonstrated that knockdown of PEPCK-M or use of

the PEPCK inhibitor, MPA, inhibits incorporation of oxaloacetate-derived carbons into serine and glycine. This implicates PEPCK-M as an enzyme capable of redirecting TCA cycle intermediates into the serine/one-carbon/glycine (SOG) biosynthesis pathway (Vincent *et al* 2015). Recent reports, have also revealed a role for PEPCK-M in supporting tumour growth and cell proliferation, particularly in metabolically unfavourable environments (Vincent *et al* 2015; Leither *et al.*, 2015).

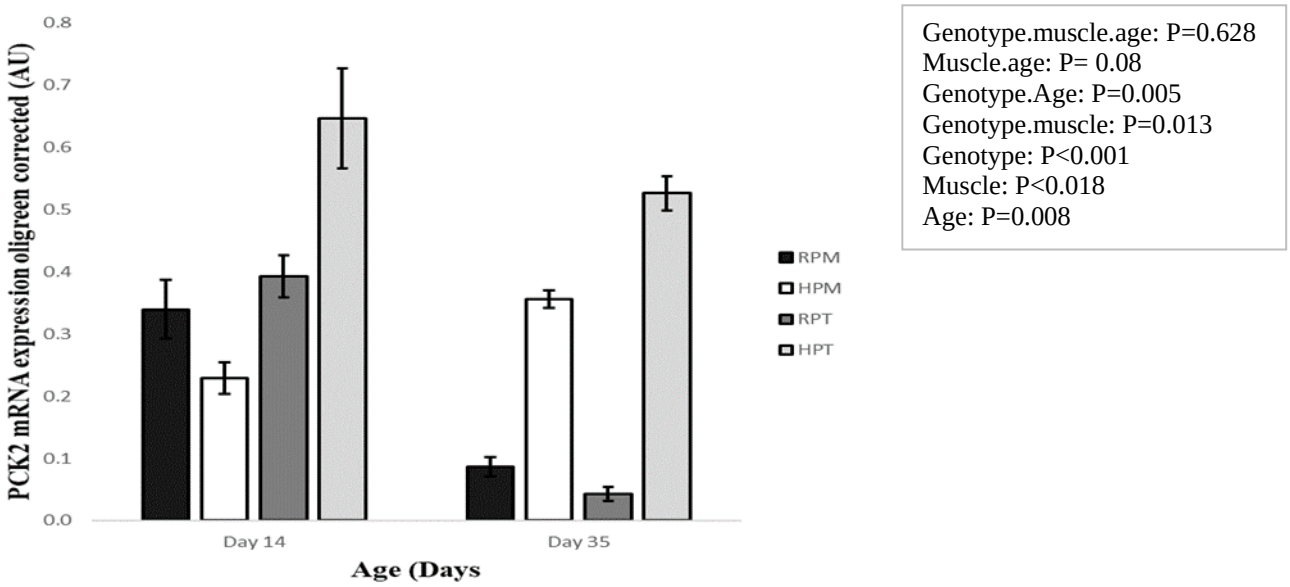


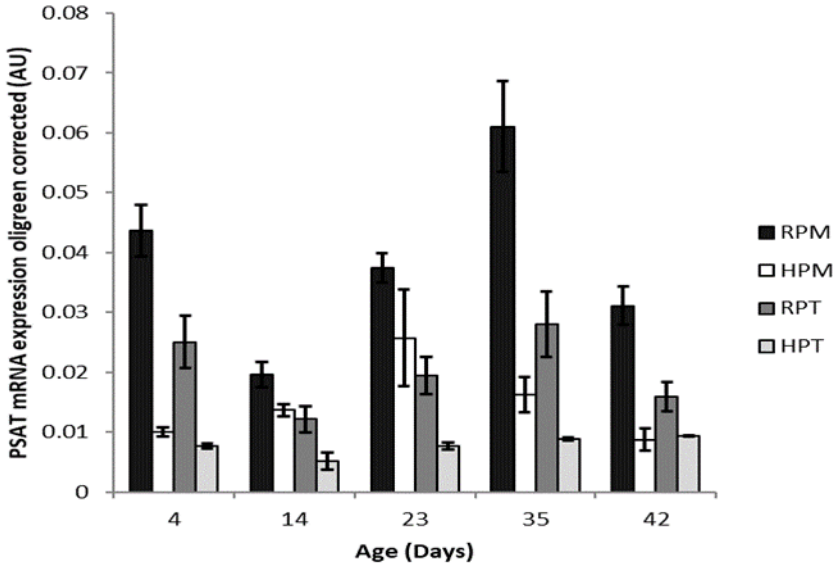
Figure 5.4: The gene expression of PCK2 mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch, PCK2 mRNA expression relative total cDNA at Day 14 and 35 (trial 2) n=6, Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.

For PCK2 expression there was no genotype x muscle type x age interactions. However, there were significant interactions between genotype x muscle type (P=0.013) and genotype x age (P=0.005) with the highest expression seen in the HPM at day 35 (Figure 5.4).

5.3.3 Gene expression of serine biosynthetic genes

Three enzymes catalyse the three-step process of serine biosynthesis namely (PHGDH, PSAT and PSPH) Phosphoglycerate dehydrogenase (PHGDH), catalyses the first step in the serine synthesis pathway by oxidising 3-phosphoglycerate (3-PGA) to 3-phosphohydroxypyruvate (PHP) using NAD+/NADH as a co-factor. Phosphoserine aminotransferase (PSAT) catalyses

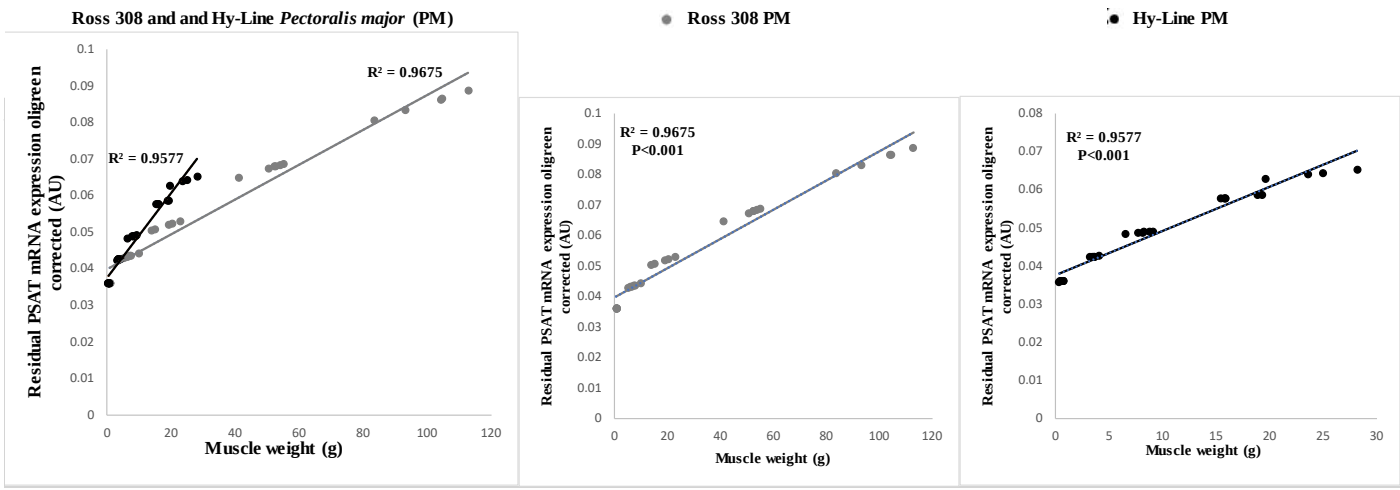
the reaction of PHP to 3-Phosphoserine which is then dephosphorylated by phosphoserine phosphatase (PSPH) to form serine (Sugimoto and Pizer, 1968).



Genotype.muscle.age: P=0.406
Muscle.age: P= 0.746
Genotype.Age: P=0.046
Genotype.muscle: P=0.124
Genotype: P<0.001
Muscle: P=0.002
Age: P=0.06

Figure 5.5: The gene expression of PSAT mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch, PSAT mRNA expression relative total cDNA at Day 4, 14, 23, 35 and 42 (trial 2), n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.

a



b

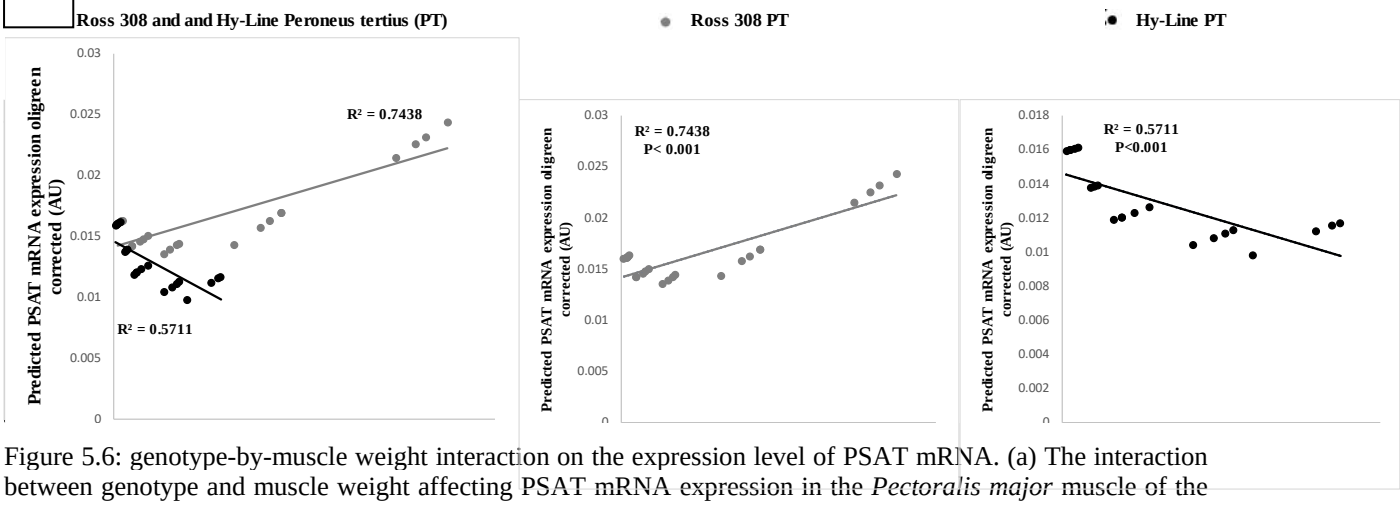
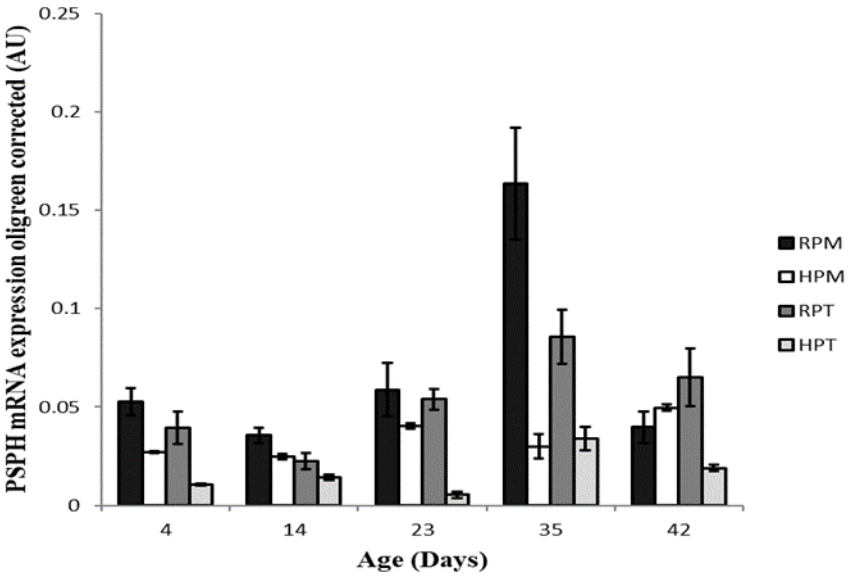


Figure 5.6: genotype-by-muscle weight interaction on the expression level of PSAT mRNA. (a) The interaction between genotype and muscle weight affecting PSAT mRNA expression in the *Pectoralis major* muscle of the breast. (b) The interaction between genotype and muscle weight affecting PSAT mRNA expression in the *Peroneus tertius* muscle of the leg. For each subplot, the larger panel on the left shows all samples, the middle panel shows the Ross 308 samples, and the right panel shows the Hy-Line samples.

For PSAT expression, there was no 3-way interaction between genotype, muscle type and age (P=0.406), neither were there any significant muscle type to age interaction (P=0.75) nor genotype to muscle type interactions (P=0.12). But there was a genotype to age interaction (P=0.046). The highest expression was however seen at day 35 in the in both muscles of the Ross 308 genotype. However, there was a genotype effect with the PM and PT of Ross 308 genotype having a higher expression than their corresponding muscle types of the Hy-Line genotype at all time points (P<0.001). There was also a muscle type effect with the PM having a higher expression when compared to the PT in both genotypes at all time points (P=0.002) (Figure 5.5).

There was a significant positive correlation between muscle weight and PSAT predicted age-controlled mRNA expression in the PM of Ross 308 and Hy-Line genotypes where R^2= 0.97 and 0.96 respectively (P<0.001) (Figure 5.6a). A significant positive correlation was also observed for muscle weight and Ross 308 PT predicted mRNA expression where R^2=0.74 (P<0.001). There was however a significant negative correlation with muscle weight and Hy-Line PT PSAT predicted mRNA expression where R^2=0.57 (P<0.001) (Figure 5.6b).



Genotype.muscle.age: P=0.125
Muscle.age: P= 0.865
Genotype.Age: P=0.018
Genotype.muscle: P=0.918
Genotype: P<0.001
Muscle: P= 0.124
Age: P<0.001

Figure 5.7: The gene expression of PSPH mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch, PSPH mRNA expression relative total cDNA at Day 4,14,23,35 and 42 (trial 2) n=6, Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.

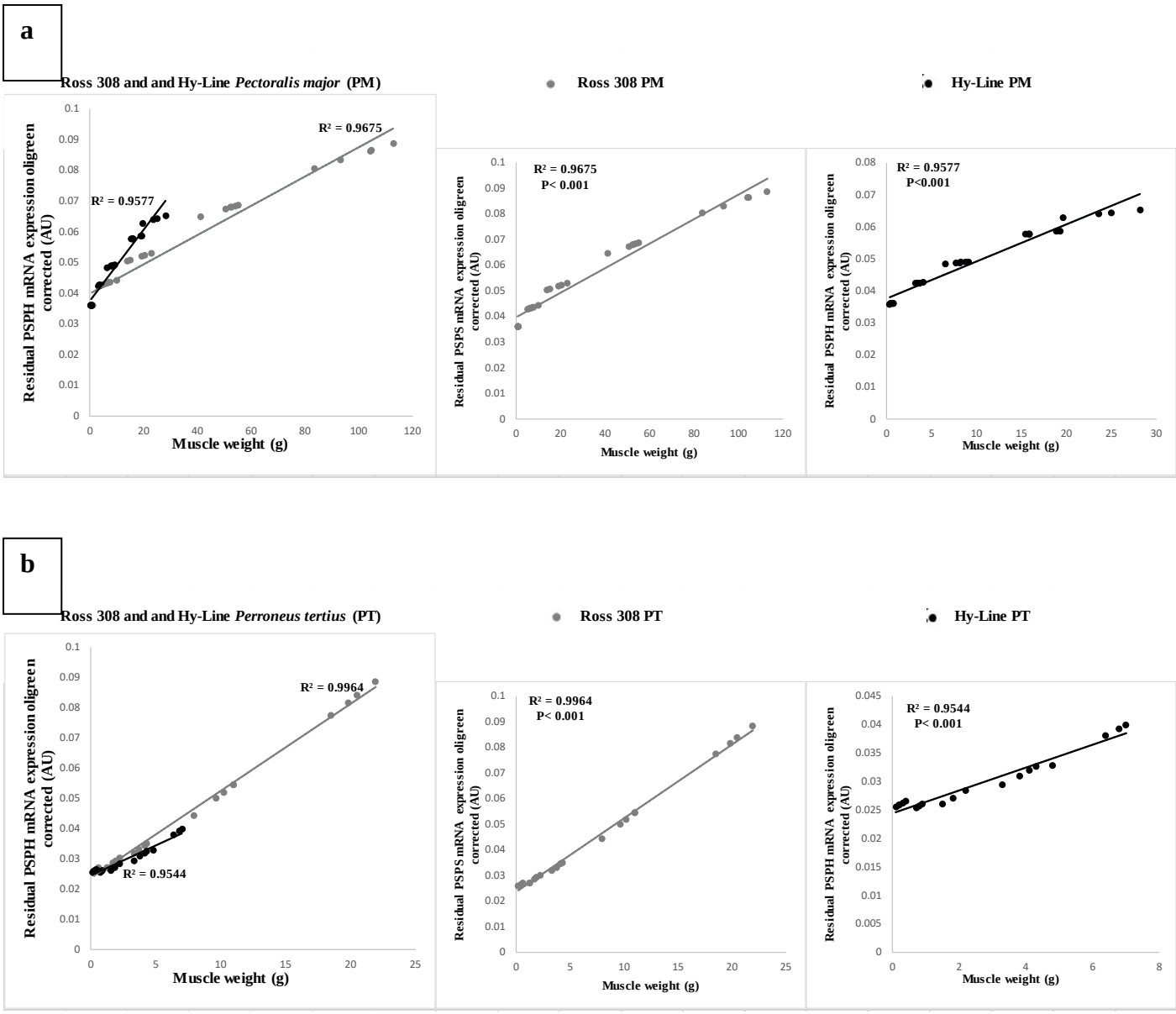


Figure 5.8: genotype-by-muscle weight interaction on the expression level of PSPH mRNA. (a) The interaction between genotype and muscle weight affecting PSPH mRNA expression in the *Pectoralis major* muscle of the breast. (b) The interaction between genotype and muscle weight affecting PSPH mRNA expression in the *Peroneus tertius* muscle of the leg. For each subplot, the larger panel on the left shows all samples, the middle panel shows the Ross 308 samples, and the right panel shows the Hy-Line samples.

For PSPH expression, there was no 3-way interaction between genotype, muscle type and age (P=0.13), neither was there any muscle type to age interaction (P=0.87) nor genotype to muscle type interaction (P=0.92), but there was an interaction between genotype and age (P=0.018). with the highest expression seen in the RPM at day 35 However, there was a genotype effect with the RPM and RPT of Ross 308 genotype having a higher expression than their corresponding muscle types of the Hy-Line genotype at all time points (P=<0.001) (Figure 5.7).

There was a significant positive correlation between muscle weight and age controlled PSPH predicted mRNA expression in the PM of Ross 308 and Hy-Line genotypes where R^2= 0.97 and 0.96 respectively (P<0.001) (Figure 5.6a). A significant positive correlation was also observed for muscle weight and Ross 308 and y-Line PT predicted mRNA expression where R^2=0.99 and 0.95 respectively (P<0.001) (Figure 5.6b).

5.3.3.1 Protein immunoblotting of PHGDH in Ross 308 and Hy-Line *Pectoralis major* muscles at day 35

Due to unsuccessful attempts at measuring the mRNA expression of PHGDH, it was measured at the protein level using immunoblotting technique. Day 35 RPM and HPM muscle samples were assayed for protein expression. The reason for choosing this muscle for sampling at the specific time point was because it was the point at which the largest differential was between the Ross 308 and Hy-Line genotypes in the other genes in the serine biosynthetic pathway (PSAT and PSPH).

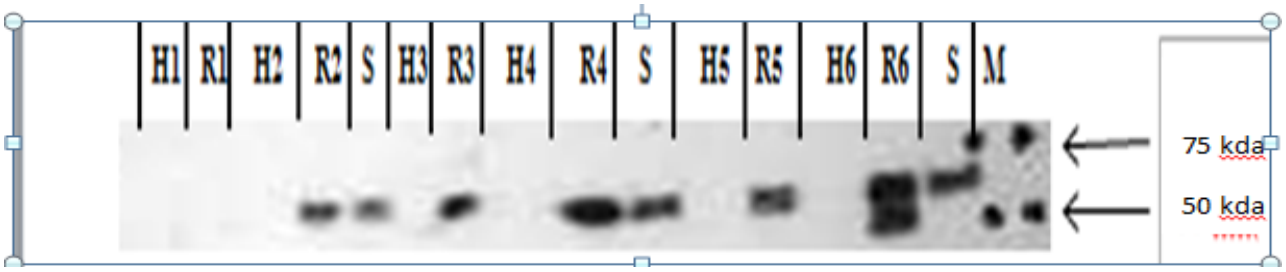


Figure 5.9: PHGDH protein band intensity of individual Ross 308 and Hy-Line PM muscle samples at day 35. Protein load per well =80 µg. Labels with H and R represent replicates of day 35 Hy-Line and Ross 308 pectoralis major muscle protein extracts respectively. S represents standard muscle extracts which constitute a pool of all muscle extracts assayed. While M is the marker with the position of the molecular weight of the bands indicated.

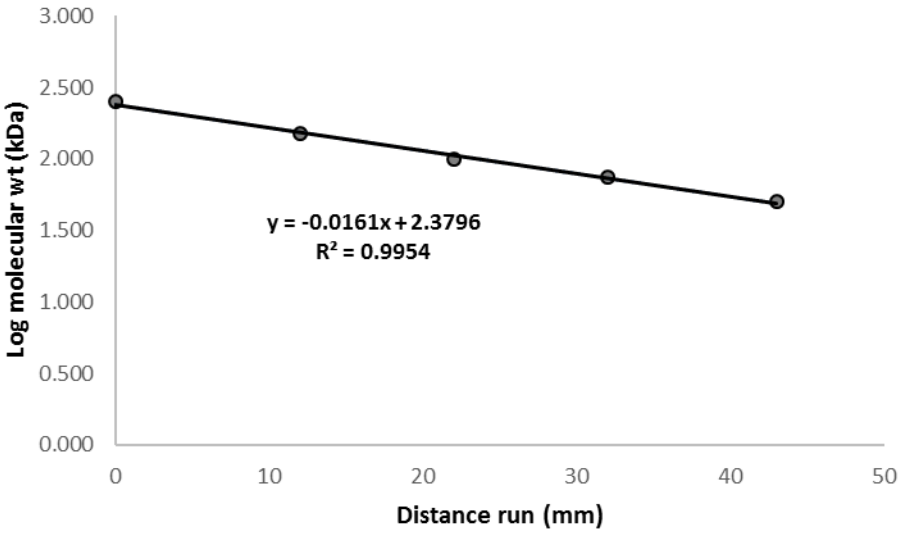


Figure 5.10: Calibration graph indicating the relationship of log molecular weight of marks relative to distance migration for the immunoprobed western blot in Figure 5.9.

Utilising the calibration graph illustrated in Figure 5.10 the molecular weight of the positive band indicated in Figure 5.9 was calculated to be 56.5 kDa which agreed with molecular weight of PHGDH which is 57kDa.

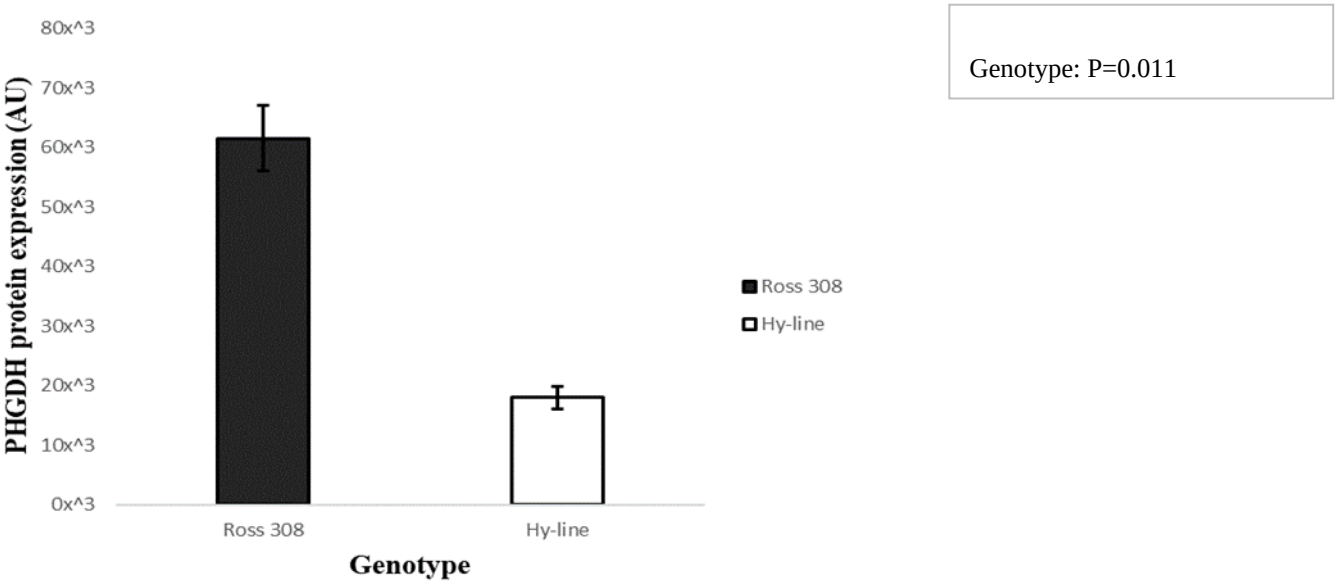


Figure 5.11: The protein expression of PHGDH in the *Pectoralis major* (PM) of Ross 308 and Hy-Line chickens at day 35 post hatch. PHGDH protein expression per unit protein 35 (trial 2), n=6. Values are arbitrarily derived from image analysis software. Expressed as means ±Standard error of the mean.

There was a significantly higher protein expression of PHGDH in the RPM when compared to the HPM at day 35 where P=0.011 (Figure 5.11). Although no bands for PHGDH in the Hy-Line samples was seen in the representative image in Figure 5.9, bands were detected when it was analysed by 1-D analysis software (BIO-RAD).

5.3.4 Gene expression of Asparagine synthetase protein synthetic gene (ASNS)

In this study, mRNA expression of amino acid synthetic gene ASNS was measured in the RPM, HPM, RPT and HPT at day 14 and 35.

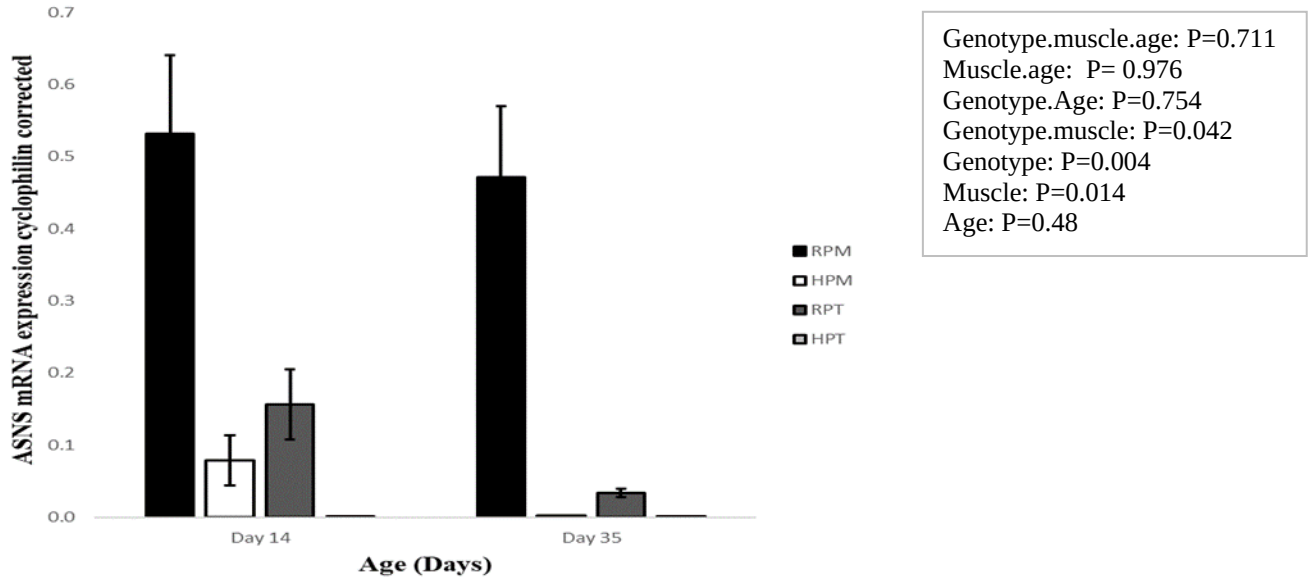


Figure 5.12: The gene expression of ASNS mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch. ASNS mRNA expression relative total cDNA at Day 14 and 35 (trial 2), n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.

For ASNS expression there was no significant 3-way genotype x muscle x time genotype interaction neither were there any genotype x time nor muscle type x time interactions. However, there were significant genotype x muscle type interaction P=0.042, there was also a significant genotype and muscle type effects P=0.004 and 0.014 respectively with the RPM having a higher expression when compared to the HPM and RPT at both time points while the RPT had a higher expression than the HPT at both time points. (Figure 5.12).

5.4 STUDIES ON PROTEIN SYNTHESIS/TRANSLATION

Studies on P70S6k mRNA expression from Trial 1 (Chapter 4) showed an upregulated expression in the faster growing PM of Ross 308 broiler chickens. However, mRNA expression of P70S6K alone is not sufficient information enough to speculate about its role on skeletal muscle protein translation due to its mechanism of action which involves phosphorylation of factors which are downstream of its position in the mTOR/AKT pathway. Phosphorylation of rps6 is by phosphorylated P7OS6K with this phosphorylation taking place as part of the mTOR/AKT signalling pathway. The phosphorylation of rps6 brings about protein synthesis by the stimulation of protein mRNA translation (Fluck 2012).

The purpose of this part of the study was to investigate the difference in expression of P70S6K mRNA in the two divergent growing muscles under study between the two divergent growing chicken genotypes. This study also aimed at investigating the protein expression of P70S6K in all muscle types at days 14 and 42 while the protein expression of the intermediate translational factor rpS6 was measured in the RPM and HPM at day 35. This was done in order to make an informed speculation of the role these two intermediates play in skeletal muscle mRNA to protein translation in chickens.

5.4.1 mRNA expression pattern P70S6K divergent growing muscles of fast and slow growing lines of chicken

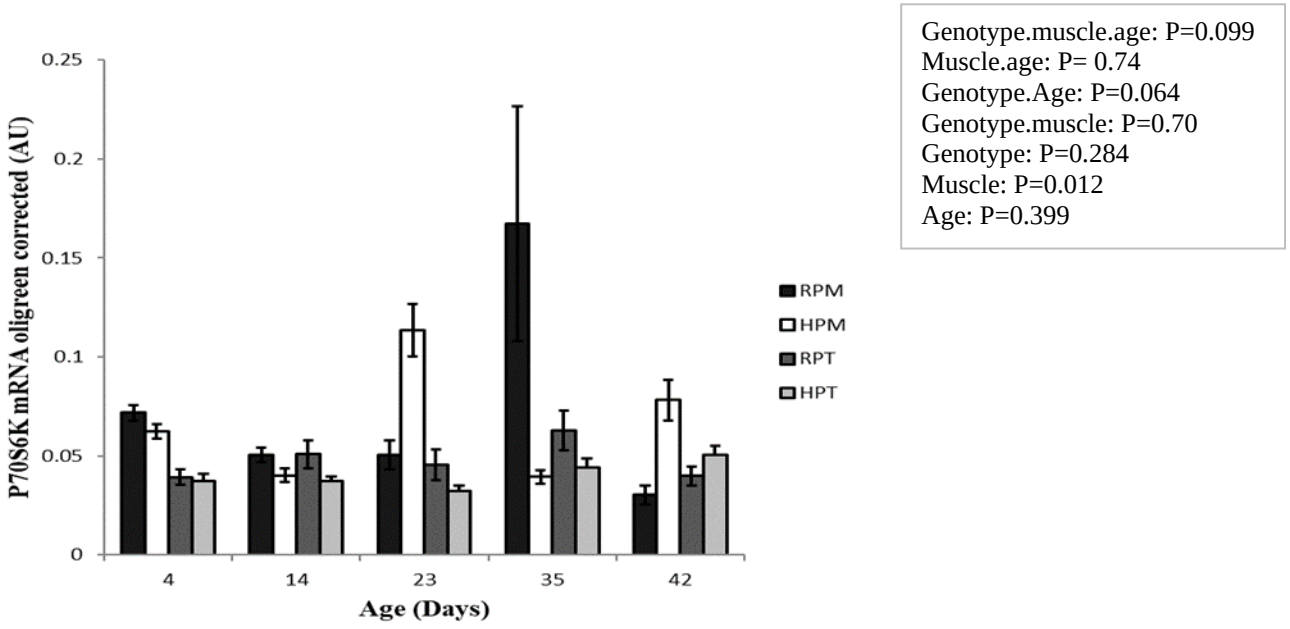
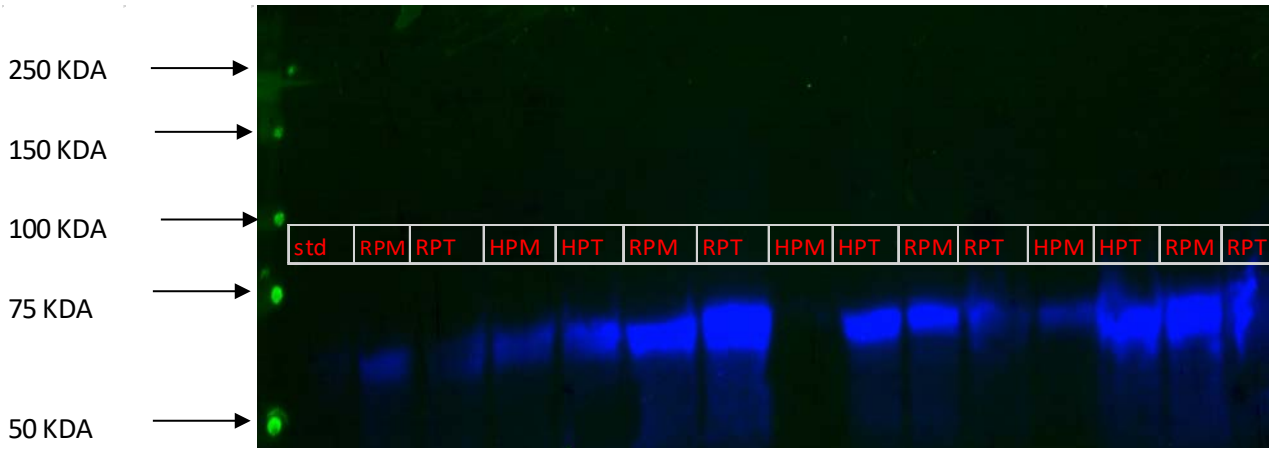


Figure 5.13: The gene expression of P70S6K mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch, P70S6K mRNA expression relative total cDNA at Day

4,14,23,35 and 42 (trial 2), n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.

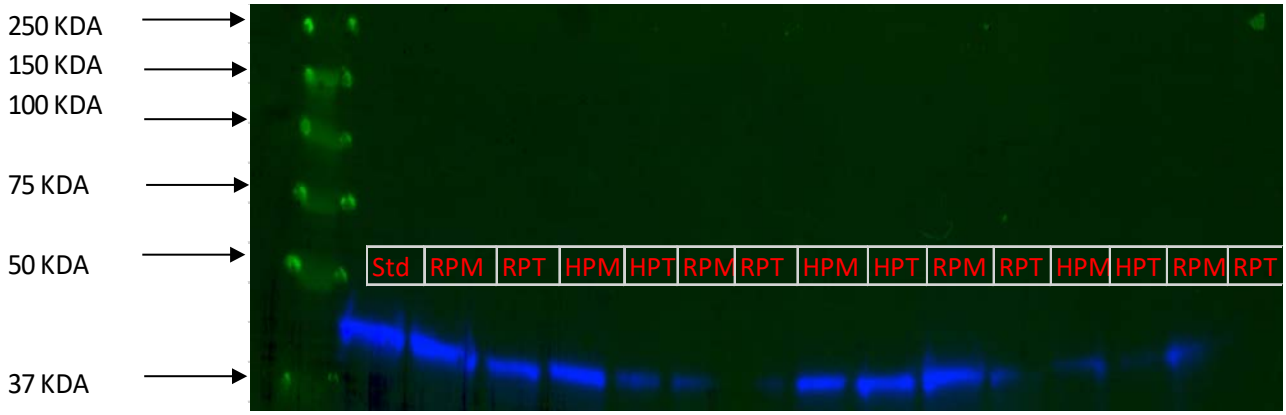
For P70S6K expression, there was no significant genotype x muscle type x age interaction (P= 0.099), neither were there any significant genotype x age interaction (P= 0.064) nor muscle type x age interaction (P=0.74). However, a muscle type effect was seen (P=0.012) with the *Pectoralis major* having a greater rate of expression in both genotypes when compared to the *Peroneus tertius* at most time points (Figure 5.13).

5.4.2 Protein immunoblotting to measure expression of P70SS6K in the RPM, HPM, RPT and HPT of Ross 308 and Hy-Line genotype at day 14 and 42



P70S6K protein band intensity of individual Ross 308 and Hy-Line muscle samples at day 14

M



P70S6K protein band intensity of individual Ross 308 and Hy-Line muscle samples at day 42

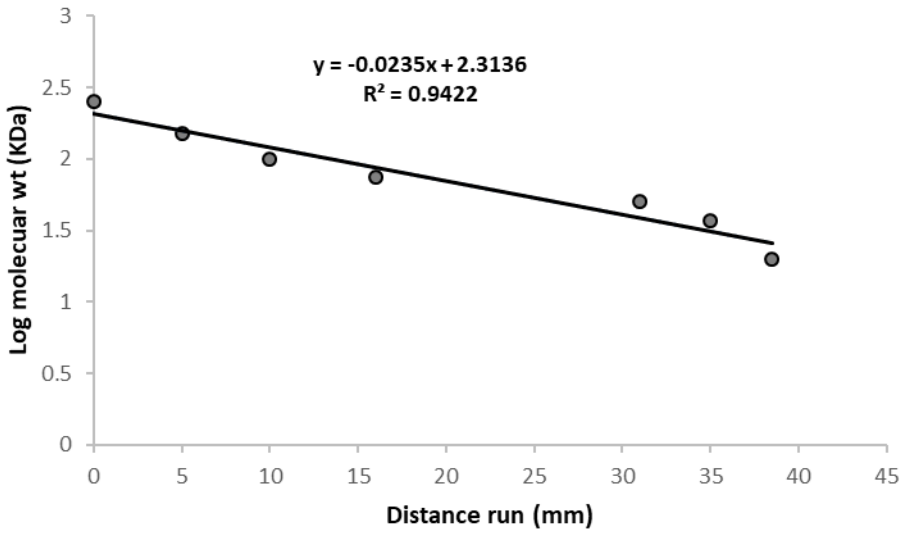
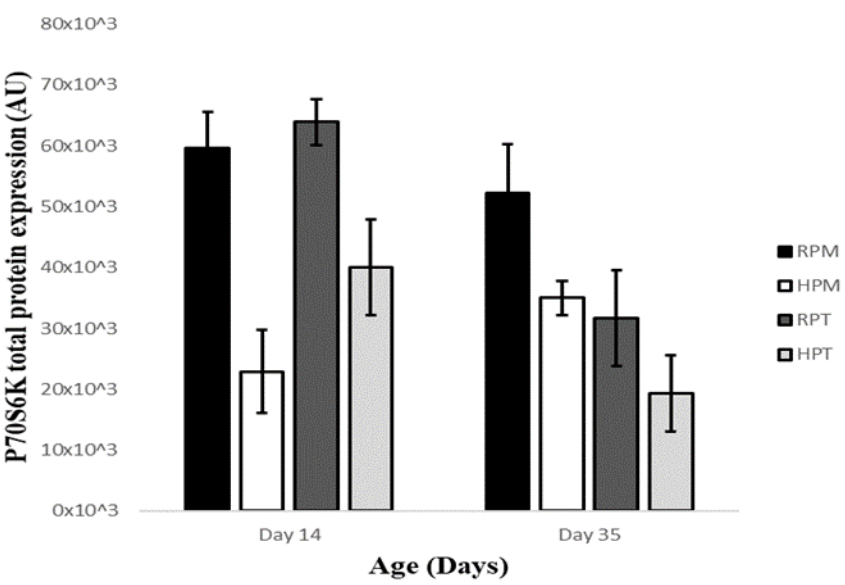


Figure 5.14: The P70S6K protein in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 (R) or Hy-Line (H) chickens at various times post hatch (a). P70S6K protein band intensity of individual Ross 308 (R) and Hy-Line (H) PM and PT muscle samples at day 14 (b) P70S6K protein band intensity of individual Ross 308 (R) and Hy-Line (H) PM and PT muscle samples at day 42. Location where Protein load per well is indicated, 110 µg protein loaded per well, labels with RPM and RPT represent Std represents standard muscle extracts which constitute a pool of all muscle extracts assayed. While M is the marker points indicating the molecular weight of the bands. (c) Calibration graph indicating the relationship of log molecular weight of marks relative to distance migration for the immunoprobed western blot in Figure 5.14a.

Utilising the calibration graph illustrated in Figure 5.14c the molecular weight of the positive band indicated in Figure 5.14c was calculated to be 56.18 kDa which is close to reported predicted molecular weight of P70S6K which is 56kDa.



Genotype.muscle.age: P=0.345
Muscle.age: P=049
Genotype.Age: P=0.748
Genotype.muscle: P=0.663
Genotype: P=0.012
Muscle: P=0.736
Age: P=0.107

Figure 5.15: The protein expression of P70S6K in the *Pectoralis major* (PM) of Ross 308 and Hy-Line chickens at day 14 and 35 post hatch. P70S6K protein expression per unit protein (trial 2), n=6. Values are arbitrarily derived from image analysis software. Expressed as means ±Standard error of the mean.

For P70S6K total protein expression, there was no significant genotype x muscle type x age interaction, neither was there any genotype x muscle type nor genotype x age (P>0.05) interactions. However, there were significant muscle type x age interactions (P=0.049) and a genotype effect (P=0.012). *Pectoralis major* (PM) and *Peroneus tertius* (PT) of the Ross 308 genotype having a higher expression when compared to the corresponding muscle types in the Hy-Line genotypes (Figure 5.15).

5.4.3 Protein immunoblotting of rps6 in Ross 308 and Hy-Line PM Muscles at day 35

The PM muscles of the Ross 308 and Hy-Line genotypes were used for rps6 comparative protein expression measurement because they appeared to be the muscles that had the highest level of expression of genes that have been associated with muscle growth during these studies. Furthermore, 70S6K had the highest level of gene expression measured the RPM at the same time point, day 35 (see Figure 5.13)

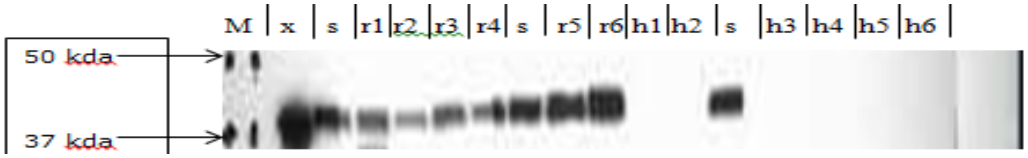


Figure 5.16: rps6 protein band intensity of individual Ross 308 and Hy-Line PM muscle samples at day 35. Protein load per well =80 µg. labels with H and R represent replicates of day 35 Hy-Line and Ross 308 pectoralis major muscle protein extracts respectively. S represents standard muscle extracts which constitute a pool of all muscle extracts assayed. While M is the marker with the position of the molecular weight of the bands indicated.

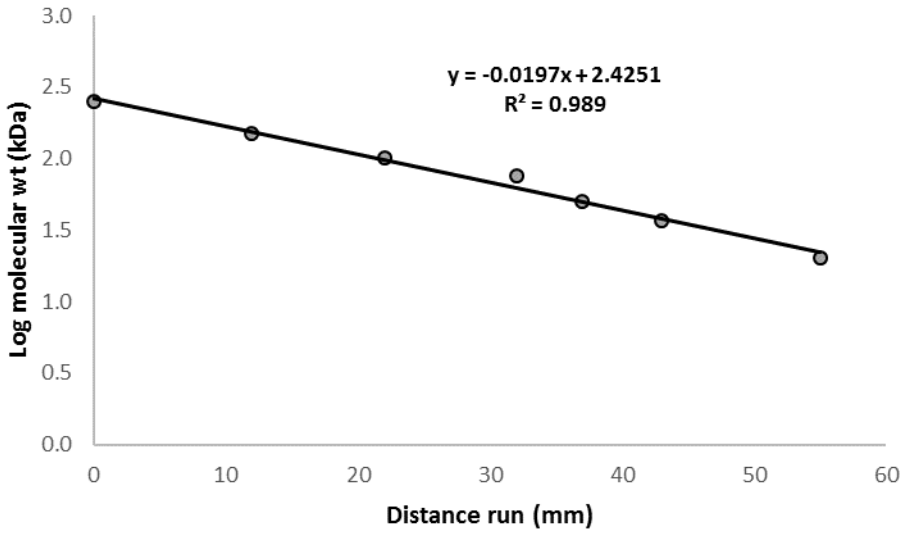


Figure 5.17: Calibration graph indicating the relationship of log molecular weight of marks relative to distance migration for the immunoprobed western blot in Figure 5.16.

Utilising the calibration graph illustrated in Figure 5.17 the molecular weight of the positive band indicated in Figure 5.16 was calculated to be 28 kDa which is close to reported molecular weight of PHGDH which is 32kDa.

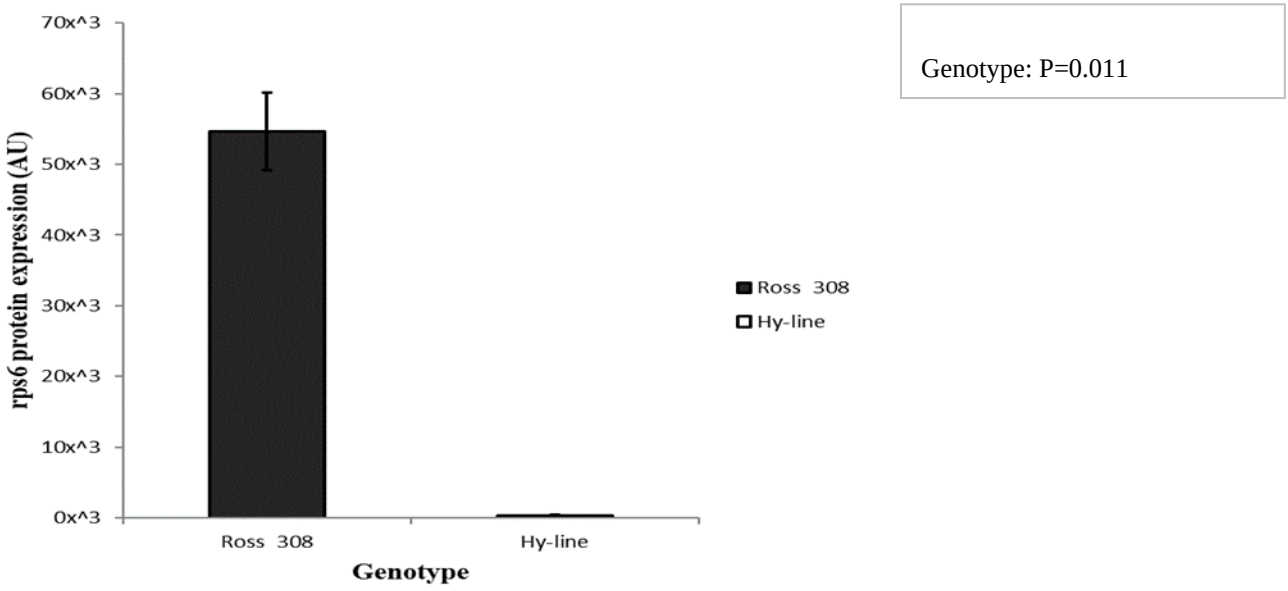


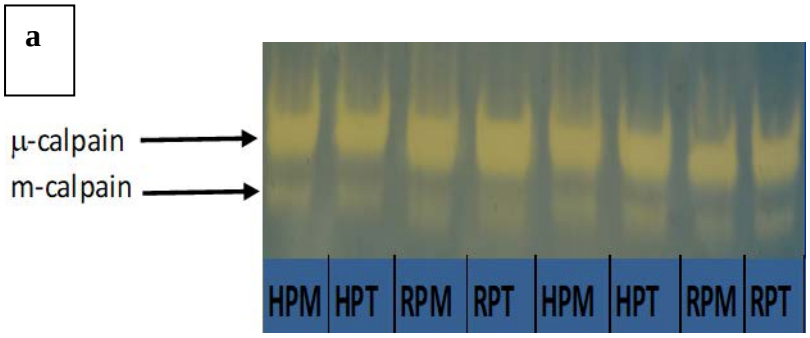
Figure 5.18: Mean protein expression at day 35 of rpS6 in the PM of Ross 308 and Hy-Line genotype. n = 6 for each genotype values expressed as means ± standard error of the mean.

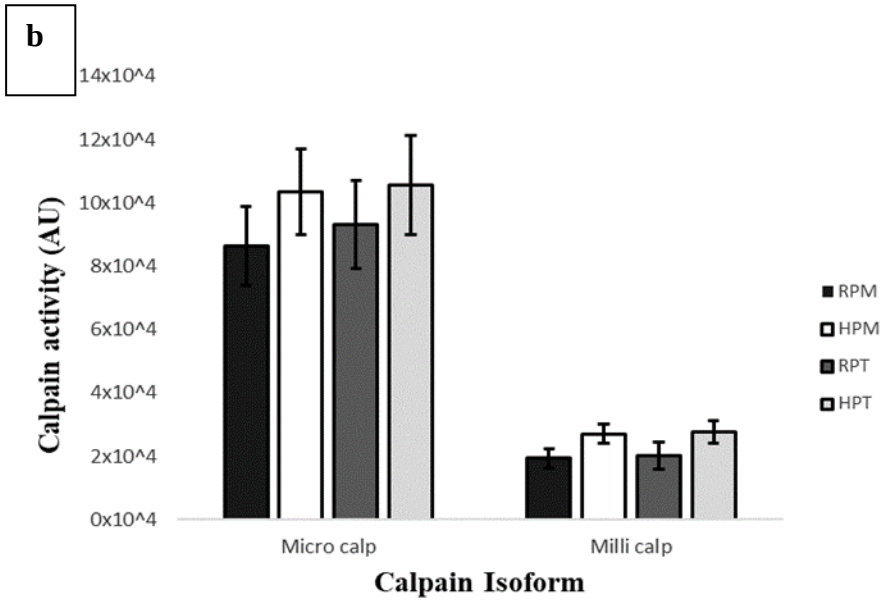
As shown in figure 5.13, there was significantly higher rpS6 protein expressed in Ross 308 chickens compared Hy-Line at day 35 (P=0.011). There seemed to be no apparent expression of rps6 in the slower growing Hy-Line PM muscle, but this was not the case in the Ross 308 PM although the same amount of protein of both muscles was loaded in the wells of the gels used. This is likely due to the low-level expression of rps6 protein in HPM when compared to the RPM. This means that in order to get a reading for the expression of rps6 protein in the HPM, a greater concentration of HPM protein should be loaded. This is an indication that skeletal muscle growth in chicken is stimulated by the synthesis and phosphorylation of the intermediates P70S6K and rps6 through the mechanism of skeletal muscle protein mRNA translation.

5.5 STUDIES IN PROTEOLYTIC SYSTEMS IN CHICKEN SKELETAL MUSCLE

Skeletal muscle contains four proteolytic systems that could be involved in protein turnover the lysosomes, the calpains, the caspase system, and the ubiquitin proteasome system (UPS) (Murton *et al*., 2008). The contribution of lysosomal proteases to metabolic turnover of myofibrillar proteins is probably small. Due to their low pH optima as cathepsins are not active at the PH of cell cytoplasm. Furthermore, Inhibitors of lysosomal proteinases were reported to fail to diminish the release of N-methylhistidine which is an amino acid only found in actin and myosin on its release during proteolysis (Bradford *et al*., 1986). It is also unlikely that the caspase system displays significant activity in normal-functioning muscle cells, since the caspases are activated by events that initiate apoptosis (Goll *et al*., 2008). Therefore, the calpain proteolytic system and the UPS are likely to be the main proteolytic pathways involved in metabolic turnover of myofibrillar proteins in such a way that calpains initiate the process by disassembling and releasing myofilaments, which would be later degraded to small peptides or amino acids by the UPS (Huang and Forsberg 1998).

5.5.1 Calpain zymography measuring calpain activity in all muscle types across genotypes at day 14, 35 and 42.





Genotype.muscle.isoform: P=0.913
Muscle.isoform: P= 0.843
Genotype.isoform: P=0.697
Genotype.muscle: P=0.902
Genotype: P=0.241
Muscle: P=0.786
isoform: P<0.01

Figure 5.19: Micro- and milli-calpain activity in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 (R) or Hy-Line (H) chickens at day 14 a). Representative calpain zymography of Ross 308 (R) and Hy-Line (H) PM and PT muscle samples at day 14. Position of micro-calpain (μ-calpain) and milli-calpain (m-calpain) are indicated Band intensity indicates calpain activity. Protein load per well =150 μg. (b). Calpain activity derived by measuring band intensity at Day 14 samples of the RPM, HPM, RPT and HPT muscles (Trial 2), n=6. Values are arbitrary quantified from Gel-doc image analyser, expressed as means ±Standard error of the mean.

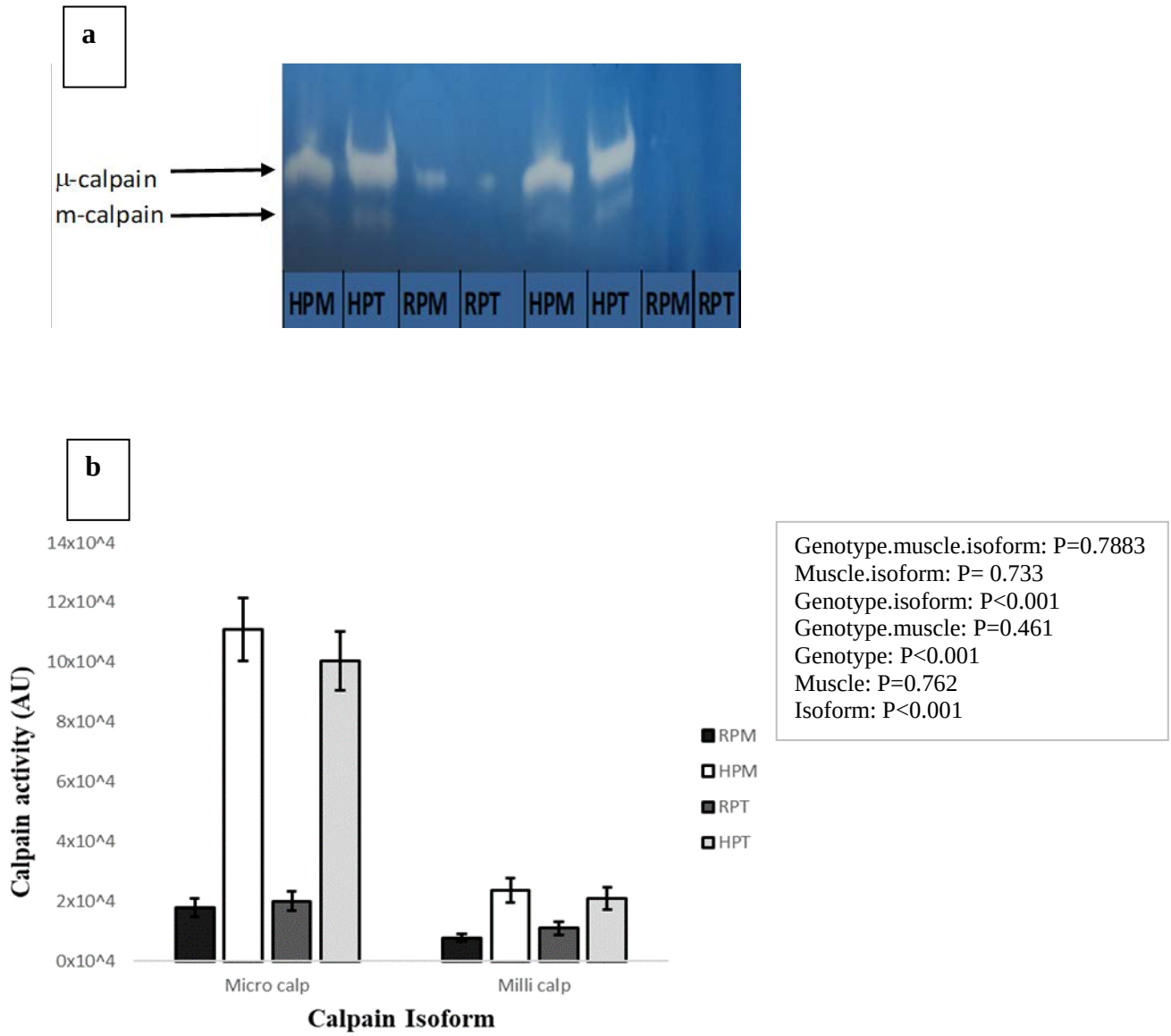


Figure 5.20: Micro and mill calpain activity in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 (R) or Hy-Line (H) chickens at day 35 a). Representative calpain zymography of Ross 308 (R) and Hy-Line (H) PM and PT muscle samples at day 35. Position of micro-calpain (μ-calpain) and milli-calpain (m-calpain) are indicated Band intensity indicates calpain activity. Protein load per well =150 μg. (b). Calpain activity derived by measuring band intensity at Day 35 samples of the RPM, HPM, RPT and HPT muscles (Trial 2), n=6. Values are arbitrary quantified from Gel-doc image analyser, expressed as means ±Standard error of the mean.

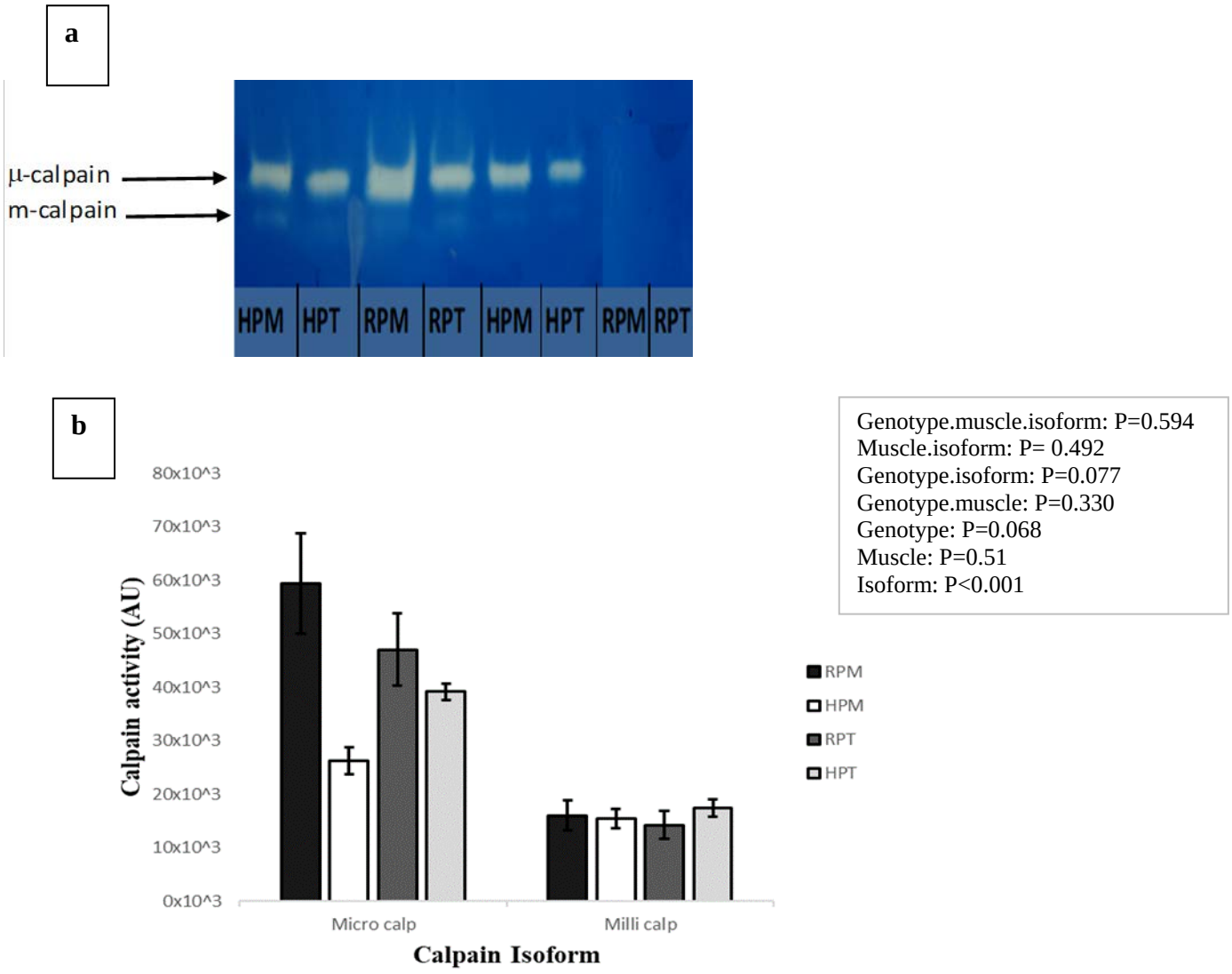


Figure 5.21: Micro and milli calpain activity in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 (R) or Hy-Line (H) chickens at day 42 a). Representative calpain zymography of Ross 308 (R) and Hy-Line (H) PM and PT muscle samples at day 35. Position of micro-calpain (μ-calpain) and milli-calpain (m-calpain) are indicated Band intensity indicates calpain activity. Protein load per well =150 μg. (b). Calpain activity derived by measuring band intensity at Day 42 samples of the RPM, HPM, RPT and HPT muscles (Trial 2), n=6. Values are arbitrary quantified from Gel-doc image analyser, expressed as means ±Standard error of the mean.

For calpain activity, at day 14 there was no significant genotype x muscle type x isoform type interaction neither were there and significant genotype x muscle type, genotype x isoform nor muscle type x isoform interactions (P>0.05) in the activity of micro and milli-calpain in the RPM. HPM, RPT and HPT. However, there was a significant isoform effect P<0.001 with the micro-calpain having a higher expression in all muscle types across genotypes when compared to milli-calpain (Figure 5.19b).

At day 35, there was no significant genotype x muscle type x isoform interaction (P=0.788). Neither were there any significant genotype x muscle type nor genotype x isoform interactions

(P>0.05) However, there was a genotype x isoform interaction with the Hy-Line genotype having a higher calpain activity than the Ross 308 genotypes in both the *Pectoralis major* (PM) and *Peroneus tertius* (PT) muscles (Figure 5.20b). While at day 42, there was no significant genotype x isoform x muscle type interactions. Neither were there any genotype x muscle type, genotype x isoform nor muscle type x isoform effects (P>0.05). However, there was a significant isoform effect P<0.001 with the micro-calpain having a higher expression in all muscle types across genotypes when compared to milli-calpain (Figure 5.21b).

5.5.2 Examination of Proteasome proteolytic enzyme activities in differential growing muscles at day 14 and day 35

The proteasome system proteolytic activity is mediated via the proteasome which is a multi-catalytic proteinase consisting of subunits which have differing types of proteolytic activity. These activities are classified according to their similarities to well established proteinases which are the trypsin, chymotrypsin, and caspase -like activities. Therefore, these activities were measured in the RPM, HPM, RPT and HPT at day 14 and 35.

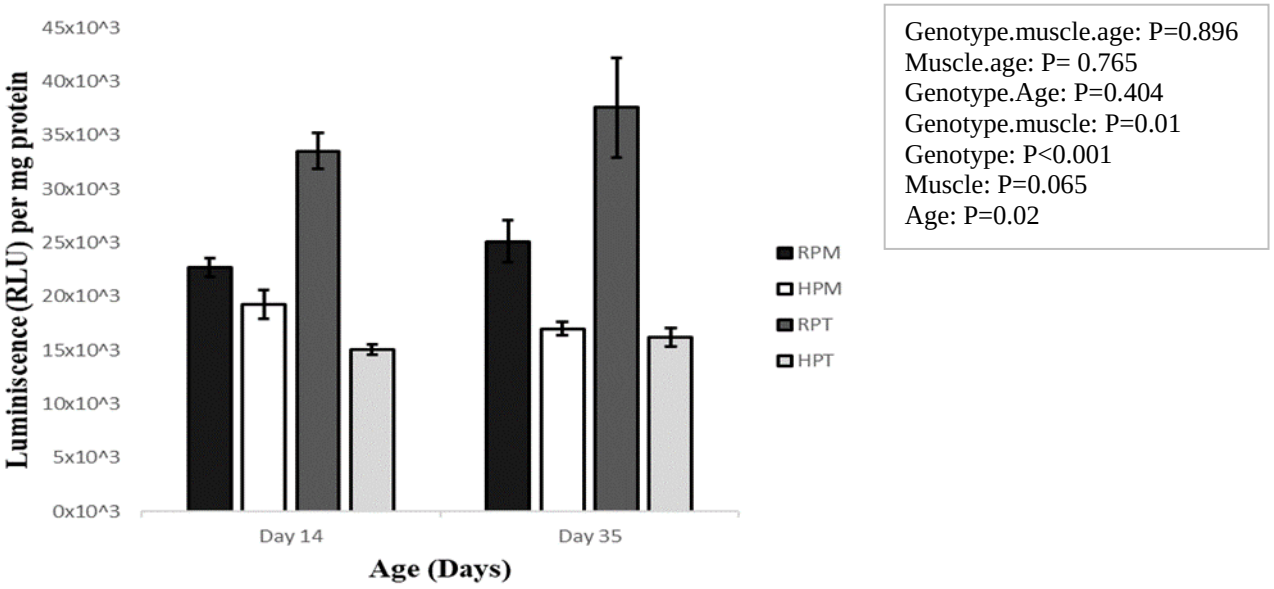
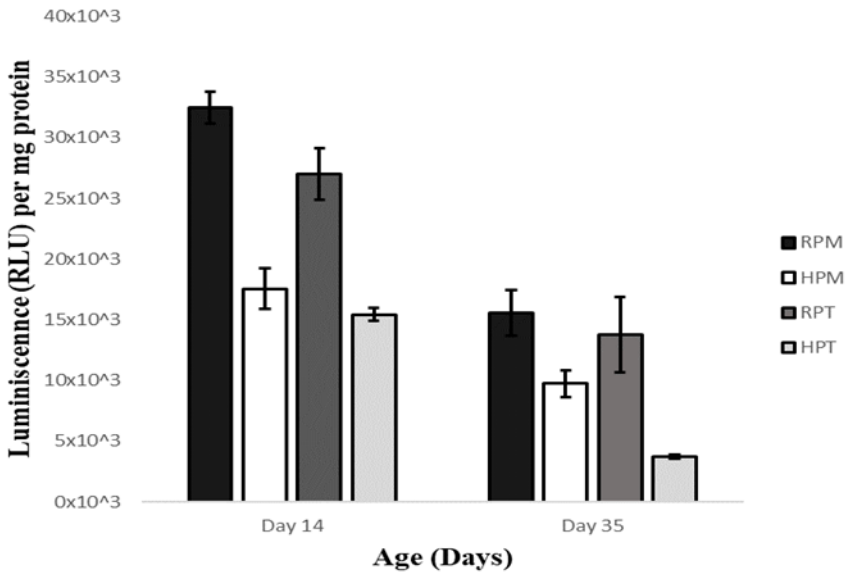
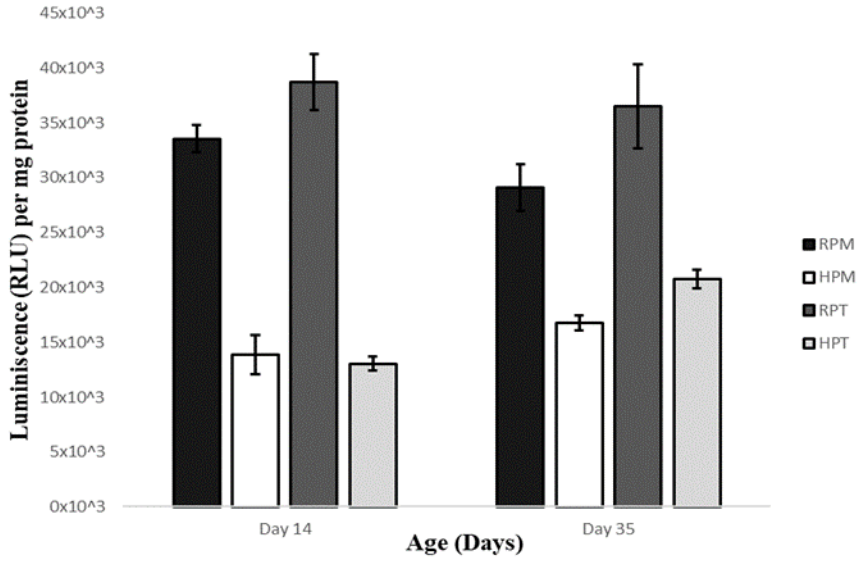


Figure 5.22: Proteasome Trypsin-like protease activity in in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 (R) and Hy-Line (H) chickens at day 14 and 35 post-hatch. Luminescence readings were measured at 10-minute intervals and the average of the 7 Luminescence readings taken at 0, 10, 20, 30, 40, 50 and 60 minutes were measured relative to unit protein content of samples per µg protein content. (According to manufactures directions). Values are expressed as means where n=6, ±Standard error of the mean.



Genotype.muscle.age: P=0.847
Muscle.age: P= 0.774
Genotype.Age: P=0.179
Genotype.muscle: P=0.603
Genotype: P<0.001
Muscle: P=0.065
Age: P<0.001

Figure 5.23: Proteasome Chymotrypsin-like protease activity in in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 (R) and Hy-Line (H) chickens at day 14 and 35 post-hatch. Luminescence readings were measured at 10-minute intervals and the average of the 7 Luminescence readings taken at 0, 10, 20, 30, 40, 50 and 60 minutes were measured relative to unit protein content of samples per µg protein content. (According to manufactures directions). Values are expressed as means where n=6, ±Standard error of the mean.



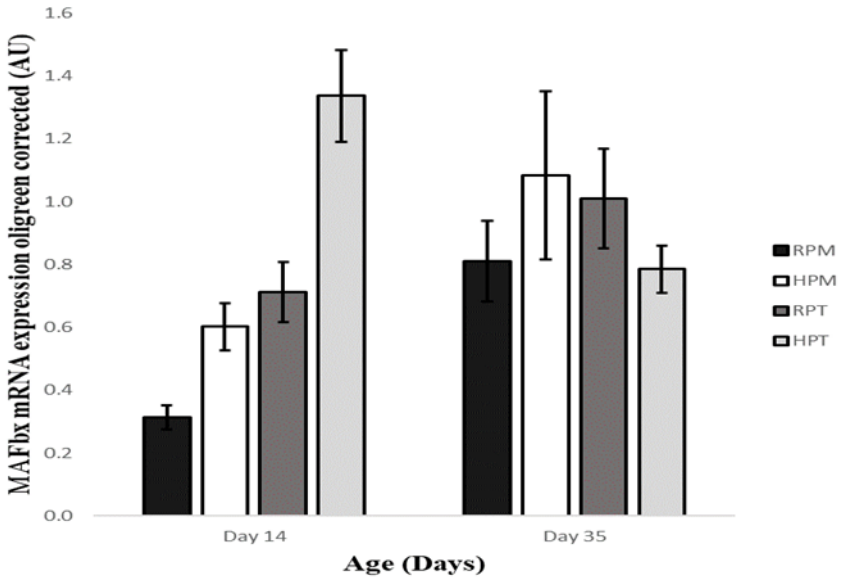
Genotype.muscle.age: P=0.84
Muscle.age: P= 0.50
Genotype.Age: P=0.12
Genotype.muscle: P=0.38
Genotype: P<0.001
Muscle: P=0.17
Age: P=075

Figure 5.24: Proteasome Caspase-like protease activity in in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 (R) and Hy-Line (H) chickens at day 14 and 35 post-hatch. Luminescence readings were measured at 10-minute intervals and the average of the 7 Luminescence readings taken at 0, 10, 20, 30, 40, 50 and 60 minutes were measured relative to unit protein content of samples per µg protein content. (According to manufactures directions). Values are expressed as means where n=6, ±Standard error of the mean.

For trypsin, chymotrypsin and caspase – like activities, there were no significant genotype x muscle type x age interactions, neither were there any, genotype x age nor muscle type x age (P>0.05). But there was a genotype x muscle type interaction for trypsin-like activity (P=0.01) but no significant interactions in the other 2 assays. However, there was a significant genotype effects P<0.001 in all three assays with the muscles from Ross 308 having a higher activity when compared muscles from Hy-Line at both time points (see Figures 5.22-5.24). For chymotrypsin-like activity, there was also an age effect (P<0.001 respectively) with a higher activity in all muscle types of both genotypes at day 14 when compared to day 35 (Figure 5.23).

5.5.3 mRNA expression of ubiquitin ligases muscle atrophy F-box (MAFbx) and muscle ring finger protein 1 (MURF-1)

Within the proteasome system ubiquitin ligases identify proteins for degradation by conjugating ubiquitin to them, subsequently the proteasome degrades these labelled proteins. In muscle the two predominant ubiquitin ligases muscle atrophy F-box (MAFbx)/Atrogen- and ring finger 1 (MuRF1) are utilised to target proteins for degradation**.** Therefore, the gene expression of these ligases was assessed.



Genotype.muscle.age: P=0.93
Muscle.age: P= 0.05
Genotype.Age: P=0.938
Genotype.muscle: P=0.346
Genotype: P=0.867
Muscle: P=0.088
Age: P=0.987

Figure 5.25: The gene expression of MAFbx mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch, MAFbx mRNA expression relative total cDNA at Day 14 and 35 (trial 2,) n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.

For MAFbx mRNA expression, there were no significant genotype x muscle type x age interaction (P= 0.93). Neither was there any significant genotype x muscle nor genotype x time

interaction (P>0.05). However, there was a borderline muscle type x time interaction P=0.05 in the expression of MAFbx mRNA with the RPT and HPT having a higher expression than the RPM and HPM respectively at day 14. At day 35, the RPT also had a higher expression than the RPM while the HPM had a higher expression when compared to the HPT thus the interaction effect (Figure 5.25).

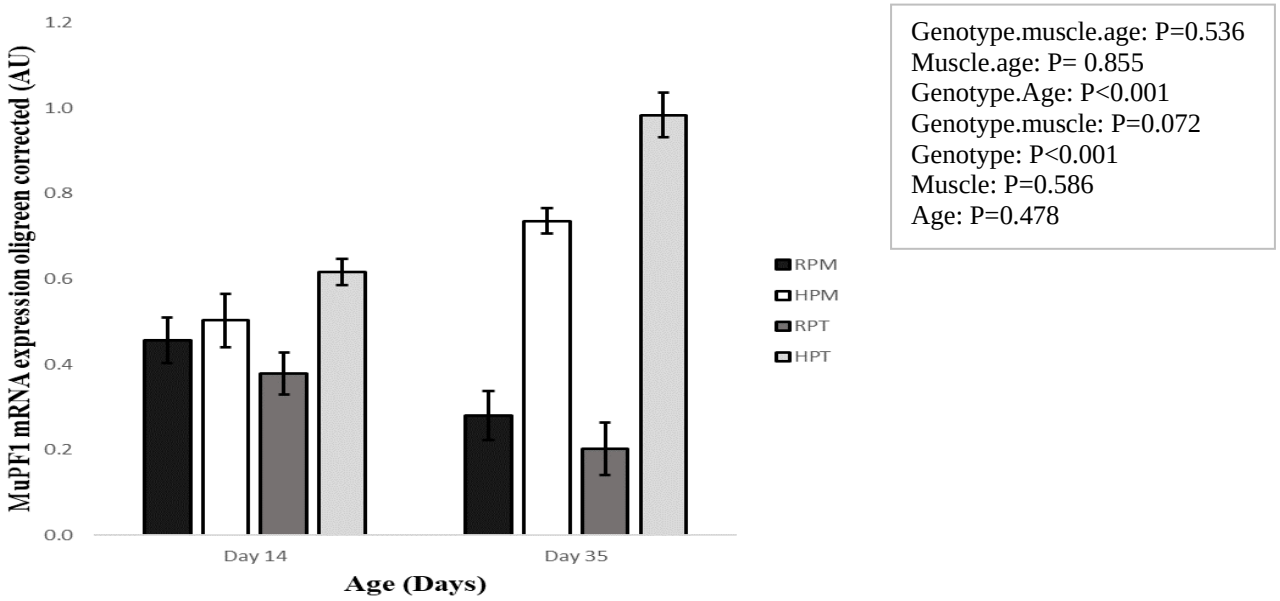
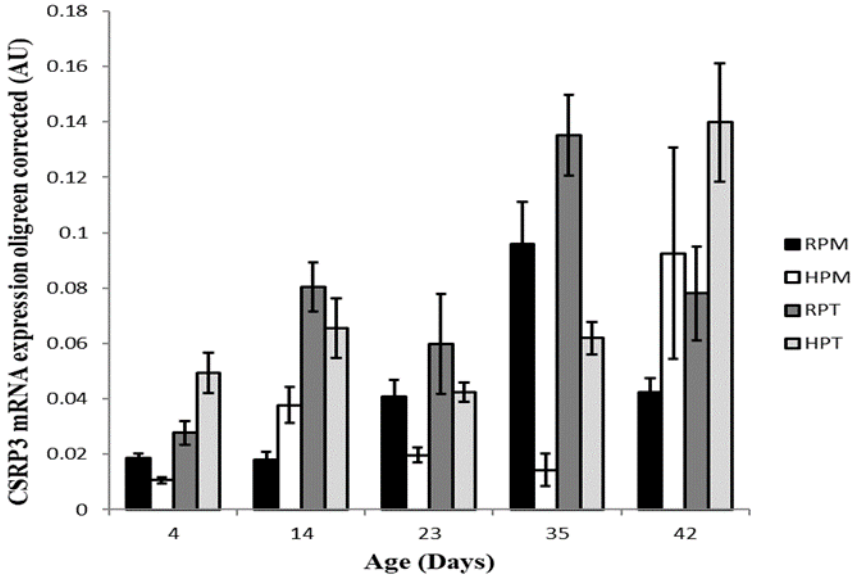


Figure 5.26: The gene expression of MuRF1 mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch, MuRF1 mRNA expression relative total cDNA at Day 14 and 35 (trial 2), n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.

For MuRF1 mRNA expression, there was no significant genotype x muscle type x age interaction (P=0.536). Neither was there any significant genotype x muscle nor genotype x time interaction (P>0.05). However, there was a significant genotype x age interaction (P<0.001) in the expression of MuRF1 mRNA with the HPM and HPT having a higher expression of the RPM and RPT at day 14 when compared to the expression of the same muscle types in the same genotype in day 35. While the HPM and HPT had a higher expression at day 35 when compared to the same muscles at day 14 (Figure 5.26).

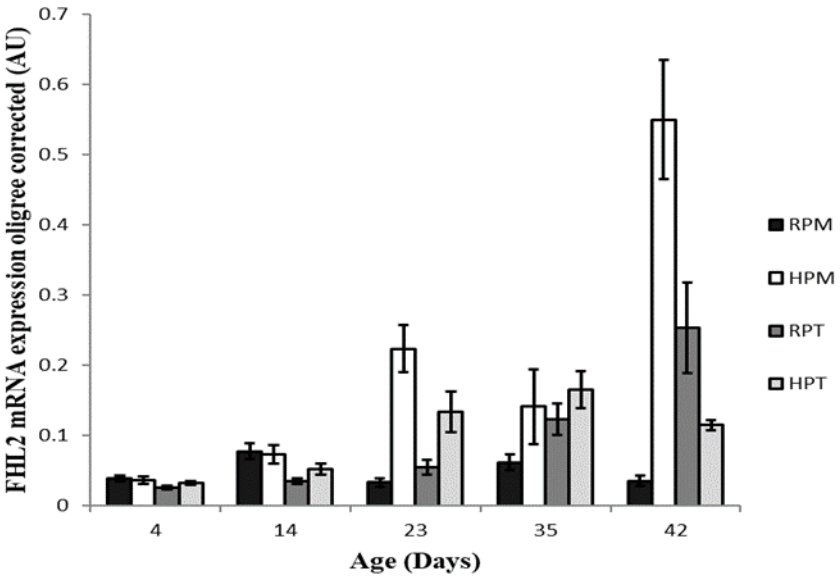
5.5.4 EXPRESSION PATTERN OF LIM DOMAIN PROTEINS (CSRP3 AND FHL2) IN A FAST AND SLOW GROWING LINE OF CHICKEN (ROSS 308 AND HY-LINE)



Genotype.muscle.age: P=<0.801
Muscle.age: P=0.658
Genotype.Age: P<0.001
Genotype.muscle: P=0.727
Genotype: P=0.355
Muscle: P<0.001
Age: P<0.001

Figure 5.27: The gene expression of CSRP3 mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch, CSRP3 mRNA expression relative total cDNA at Day 4,14,23,35 and 42 (trial 2), n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.

For CSRP3 expression, there was no interaction between genotype, muscle type and age (P=0.80), neither was there any genotype to muscle type interaction (P=0.73) nor muscle to age interaction (p=0.66), but there was a genotype to age interaction (P<0.001), with the highest expression seen in the HPT at day 42. There was also muscle type effect within genotypes with the RPT and HPT having a higher expression than the RPM and HPM respectively (P<0.001) (Figure 5.27).



Genotype.muscle.age:P=<0.001
Muscle.age: P=0.132
Genotype.Age: P=0.004
Genotype.muscle: P<0.001
Genotype: P<0.001
Muscle:P=0.097
Age: P<0.001

Figure 5.28: The gene expression of FHL2 mRNA in *Pectoralis major* (PM), *Peroneus tertius* (PT), in Ross 308 and Hy-Line chickens at various times post hatch, FHL2 mRNA expression relative total cDNA at Day 4,14,23,35 and 42 (trial 2), n=6. Values are arbitrary quantified concentrations from light cycler crossing point cycle number, expressed as means ±Standard error of the mean.

For FHL2 expression, there was a 3way interactions between genotype, muscle type and age (P<0.001) with the highest expression seen at day 42 in the HPM (figure 5.28)

5.6 SUMMARY

This study was a continuation of studies from the previous section, where the expression of genes suspected to modulate skeletal muscle growth in chicken were compared in differentially growing muscles at different time points. In this study, metabolic, proteolytic and protein synthesis systems were studied in more detail in chicken with a different variable of genotype added to all parameters. Interactions were thereby between genotype, muscle type, and age. Like the previous study production parameters included total body weight, *Pectoralis major* weight of the breast and *Peroneus tertius* weight of the leg muscles and these were compared between genotypes Ross 308 and Hy-Line. The expression of genes measured in this study were to an extent dependent on results derived from observations from the previous study. However, more genes were measured to give a more detailed analysis on factors modulating skeletal muscle growth in chicken. Metabolic enzymes α-enolase and β-Enolase as well as gluconeogenic enzyme PEPCK2 had irregular expression patterns in the divergent growing muscles under study, thus the effect of anaerobic respiration and gluconeogenesis and the extent to which these processes modulate muscle growth in chicken could not be ascertained

by those parameters. Metabolic factors shown to be upregulated faster growing muscles include serine biosynthetic enzymes PHGDH, PSAT and PSPH as shown in the upregulated expression of PSAT and PSPH mRNA expression and PHGDH protein expression in faster growing chicken muscles. This presumably increased the muscle to increase the synthesis of non-essential amino acid serine but also the biosynthetic that are derived from serine such as choline and the associated phospholipids. In addition, there was an increase in ASNS expression which presumably increases the capacity to synthesise the non-essential amino acid asparagine, which is only required for protein synthesis. Parameters skeletal muscle mRNA protein translation of protein synthesis and which include P70S6K gene expression, as well as protein expression of P70S6K and rps6 associated with mTOR/AKT pathway, generally showed an upregulated expression in the faster growing muscles under study. An attempt was also made to further study the mTOR/AKT pathway by measuring the protein expression of other intermediates in the pathway which include 4E-BP1 and AKT by western blotting but unfortunately the primary antibodies were also not compatible with chicken muscles.

An attempt was also made to measure calpastatin protein expression but unfortunately the primary antibodies were also not compatible with chicken muscles. However, Proteolytic systems studied which include the calpain proteolytic system activity, proteasome activity (trypsin-like, chymotrypsin-like and caspase-like activities) and mRNA expression of ubiquitin ligases MAFbx and MURF1 gave some insight into the mechanistic pattern proteolysis modulates skeletal muscle growth in chicken which is discussed in further detail in Chapter 6. LIM domain proteins CSRP3 and FHL2 were also measured and generally had a higher level of expression in the slower growing muscles.

CHAPTER 6 (DISCUSSION)

6.0 (MOLECULAR AND METABOLIC MECHANISMS REGULATING GROWTH IN DIVERGENT GROWING CHICKEN MUSCLES.

The hypothesis investigated in this research was that the accelerated growth rate phenotype seen in fast growing genetically selected chicken muscles was accentuated by metabolic modifications that impacts growth by the increment of protein accretion. During this research work, one of the objectives of the initial study in trial 1 was to characterise glycolytic metabolic gene expression in divergent growing muscles of chicken. The study highlighted that the faster growing PM muscles displayed an increased mRNA abundance of glycolytic genes in comparison to the slower growing PT. For the first time, this study also reveals that in fast growing chicken muscles, there is an upregulated mRNA expression of the three enzymes involved in serine biosynthetic pathway (PSAT, PSPH and PHGDH). Which suggests that glycolytic intermediates are being used for the synthesis of non-essential amino acids in the faster growing chicken muscles. The upregulated expression of the AKT/mTOR intermediate P706K was also seen in the fast-growing muscle supporting the wealth of information detailing its role in protein synthesis. The growth factors myostatin and IGF1R had no differential expression between muscle types. LIM domain protein and other genes involved in satellite cell activation and cell cycle were also measured. There was an upregulated LIM domain protein and MUSTN1 mRNA expression in the slower growing muscles while FGF2R which also plays a role in protein synthesis was upregulated in the faster growing muscles.

In trial 2, there was an upregulated muscle type expression of glycolytic enzyme β-enolase in the fast-growing muscles which supports the notion that glycolytic metabolism is generally upregulated in fast growing muscles as observed in Trial 1. Serine biosynthetic genes PSAT and PSPH and protein expression of PHGDH as well as protein expression of AKT/mTOR intermediates P70S6K and ribosomal protein s6 were also upregulated in the faster growing muscles supporting Trial 1 reports. In this trial, the calpain and the proteasome proteolytic systems were also examined. Another novel finding was the observed higher activity of the three proteolytic sub units of the proteasome system in faster growing chicken muscles in contrast to the slower growing muscles suggesting that the proteasome serves a basic housekeeping function as an amino acid recycling machine, rather than being just a waste

disposer. LIM domain proteins also had a higher expression in faster growing muscles in comparison to slower growing muscles in this trial supporting observations seen in trial 1.

6.1. DIFFERENCES IN BETWEEN MUSCLES AND CHICKEN GENOTYPES IN PRODUCTION AND MUSCLE COMPOSITION PARAMETERS

As might have been expected in Trial 1 the *Pecctoralis major* (PM) was found to have a faster growing rate proportional to body weight than *Peroneus tertius* (PT) in the broiler chickens (Ross 308) which were examined. The larger PM tissue size was likely due to the presence of larger muscle fibres which characterised by higher level of expression of fast glycolytic adult myosin heavy chain isoforms. The PM had a higher total protein content when compared to the PT at all time points. This may be because of a greater rate of protein accretion in the faster growing PM muscles. This agrees with earlier observations where a greater rate of protein deposition has been reported in the PM of a faster growing line of chicken when compared to their slower growing layer type chicken counterpart (Tesseraud *et al.*, 2000). The larger amount of DNA in the PT when compared to the PM is an indication of a larger myonuclear number per unit tissue weight, as muscle cells are multinucleated. While the higher protein quantity per unit tissue, as well as per unit DNA content in the PM muscle compared to the PT is an indication that the PM has a higher protein retention capacity and a larger Myonuclear domain (MND), which is. The theoretical volume of cytoplasm within the myofibril regulated by the gene products of a single myonucleus (Teixeira and Duarte., 2011).

Although there were no significant differences in glycogen content per unit tissue between PM and PT at all time points, when measured as total tissue content, the PM had a significantly higher glycogen content at all time points, due to the larger tissue size of the PM. It is important to note that within PM and PT per unit tissue and DNA content corrected there was no difference in protein content at days 36 and 43 but there was a significant reduction in glycogen content per unit tissue at day 43 in both muscles. These observations suggest that the reduction in glycogen content at day 43 in the PM and PT muscles may have influenced net total protein synthesis as the birds grew older in all muscle types. The reduction in glycogen could possibly be due to the utilisation of glucose as a source of ATP and a precursor of non-essential amino acids for muscle protein synthesis as well as *de novo* synthesis of serine and its metabolites. As

the seen glycogen content reduction was coincident with the time point where the highest PM weight was measured.

In Trial 2, where the broiler Ross 308 (R) and the layer Hy-Line (H) genotypes were compared there was the expected muscle type and genotype effect observed, with the PM been larger than the PT in both genotypes and the PM and PT of Ross 308 being larger than those of Hy-Line at all time points. Unfortunately, protein and glycogen were not measured in this trial. However, proteolytic and glycolytic systems investigated gave some insight on the metabolic properties in the muscles studied in the trial.

6.2 DIFFERENCES IN BETWEEN MUSCLES AND CHICKEN GENOTYPES IN METABOLIC SYSTEMS

6.2.1. Gene expression of glycolytic metabolic genes (GAPDH, α–enolase and β–enolase)

In Trial 1, expression of GAPDH and α-enolase was higher in PM compared to PT. This may be as a result of greater proportion of fast type II fibre types, as these fibres have a higher glycolytic metabolic capacity when compared to type I slow oxidative fibres. Skeletal muscle hypertrophy occurs as a result of an increase in muscle fibre diameter (Baechle and Earle 2008). It has been reported that when comparing broilers to the slower growing muscles of layers they had larger diameter type I and II myofibres and greater apparent myofibre number; the proportion of type II red myofibres decreased and that of type II fast twitch white myofibres increased during growth (Aberle and Stewart 1983).Quantitative analysis of fibre type composition demonstrates that, in broilers, the *Pectoralis major* (breast muscle) is composed of 99% glycolytic type 2B fibres, whilst the *Bicep femoris* (leg) had a more heterogenous fibre type distribution, with a larger population of oxidative type 1 fibres (Cooke *et al*., 2003).

In Trial 2 a muscle x genotype x time interaction effect observed in α-enolase expression as a result of the unexpected higher level of expression in the slower growing HPM when compared to the RPM at days 23 and 42 cannot be easily explained. This is probably due to the irregular expression pattern in the PM and PT of both Ross 308 and Hy-Line genotypes at the five-time points measured. Enolase is a key glycolytic enzyme that catalyses the dehydration of 2-phosphoglycerate to phosphoenolpyruvate, in the last steps of the catabolic glycolytic pathway

(Pancholi, 2001). In humans although α-enolase may be found in virtually every tissue as it is not the muscle specific glycolytic enolase isoform (see review by Diaz-Ramoz *et al*., 2012). β-enolase is the predominant enolase isoform found in muscles and has also been reported to have a higher expression in fast muscle fibres (Mareco *et al*., 2015; Merkulova *et al*., 2000). This may serve as an explanation as to why a significantly higher expression of β-enolase in the RPM and HPM was seen when compared to the RPT and HPT at both time points of day 14 and 35 measured in trial 2. Furthermore, in both Ross 208 fast growing broilers and slow growing Leghorn their PM has been reported to be composed of 99-100% fast white skeletal muscle fibre types, whilst the *Ileotibialis lateralis M. semimembranosus* of the thigh had a significantly lower composition of fasting white fibres ranging from 32-36% fast white fibres and approximately 65% slow red fibres (Branciari *et al*., 2014).

6.2.2 Gene expression of gluconeogenic gene PEPCK-M (PCK2)

A recent study by Brown *et al*., (2016) reported that PCK2 gene expression increased in *Longissimus Dorsi* (LD) pigs of pigs treated with growth promoting anabolic agent beta-adrenergic agonist, pig implicating it in skeletal muscle biosynthetic metabolic pathways. A similar expression pattern has also been observed in sheep LD muscle treated with beta-adrenergic agonists (Parr *et al*., unpublished data). However, in the rapid growing muscles of chickens the highest expression of PCK2 was seen in the slowest growing HPT muscle at both the time points measured, day 14 and 35. Thus unlike beta-adrenergic agonist stimulated muscle growth, fast growing chicken muscles do not have an associated increase in PEPCK-M.

6.2.3 Expression of genes encoding protein enzymes catalysing the serine biosynthetic pathway in differential growing skeletal muscles of divergent growing chicken genotypes

Brown et al (2016) and others (Lunt and Heiden, 2011; Ward and Thompson, 2012; Ye *et al.,* 2012) have proposed that an increase PEPCK-M serves a role to enable increases in intermediates that can be used in biosynthetic pathways such as serine synthesis. Although mRNA encoding PEPCK-M was not increased upregulated expression of the genes involved in the *de-novo* biosynthesis of serine was seen in the in the faster growing PM muscles of Ross 308 in both Trial 1 and 2, suggesting that there may be an association with an upregulated

serine biosynthesis and muscle hypertrophy. In chicken the potential relationship between muscle hypertrophy and serine biosynthesis is unknown as no previous work has been done showing an association between upregulated serine biosynthetic potential and faster growing chicken muscles. However, in both pigs and sheep beta-adrenergic agonist stimulated hypertrophy of fast type fibres is associated with an upregulated expression of PHGDG, PSAT and PSPH (Al-Doski *et al*., 2015, Brameld *et al*., 2015, Parr *et al*., 2015 ;). The genotype to age interaction effect for PSAT expression in Trial 2 was due high PSAT Ross 308 chickens (irrespective of muscle) particularly at day 35 and 42 (Figure 5.7). Interestingly in the fast growing and largest muscle, the Ross PM, there was a significantly positive correlation between muscle weight and PSAT gene expression. Although for Ross 308 PT there was a positive relationship it was not significant (Figure 5.8). In Trial 2, PSPH expression had a similar pattern with that of PSAT. Interestingly, like PSAT, the highest level of expression was seen at day 35 in both the RPM and RPT. Muscle weight and PSPH expression were also correlated at days 14, 23 and 35.

A further indication of the co-ordinate activation of the serine pathway in the rapidly growing Ross 308 PM was indicated by the significantly higher protein expression of PHGDH in this muscle from this genotype compared to the Hy-Line chickens at day 35 post hatch. These results give an indication that the expression of PHGDH is consistent with the other two enzymes in the serine synthesis pathway. This agrees with reports where gene and protein expression were upregulated with growth promoter induced muscle hypertrophy in sheep and pigs (Al-Doski *et al*., 2015; Brameld *et al*., 2015). An upregulated serine biosynthesis has also been seen in cancer cells where molecular expression of the enzymes that catalyse the pathway have been reported to be associated with hyperplastic growth (Posemato *et al*., 2011). In cancer cells, there is a metabolic switch from oxidative phosphorylation to aerobic glycolysis, which is used for anabolic pathways, described as the Warburg effect (Tong et al., 2009). This metabolic similarity with hyperplastic cancer cells may serve as foundation to give an explanation as to why the expression of genes involved in anaerobic glycolysis and serine synthesis pathway was seen in the faster growing chicken muscles.

The relationship between serine biosynthesis and muscle hypertrophy is not well understood, however it may have a similar process elucidated in hyperplastic growth as seen in cancer cells. One of such processes is mediated by transcriptional factors associated with the endoplasm reticulum (ER) mediated amino acid response (AAR) such as ATF4. For genes activated by this response which include asparagine synthase, and cationic amino acid transporter CAT-1 (SLC7A1), which alongside genes encoding enzymes involved in the serine biosynthetic

pathway which have also been reported to have increased expression in beta-adrenergic agonist induced growth in pigs (Parr *et al*., 2015). Skeletal muscles have an ER known as sarcoplasmic reticulum (SR) which plays a critical role in muscle contraction and maintenance of muscle homeostasis (Rayavarapu *et al*., 2012). SR/ER stress occurs in times of amino acid deprivation due to the accumulation of unfolded or misfolded proteins in the SR/ER which in turn elicits the unfolded protein response (UPR). The UPR then leads to the degradation of these unfolded proteins (Obeg *et al*., 2006; Crawford *et al*., 2011). Explained in more detail at sections 6.3.1.1 and 6.4.2**)**. Possibly increasing the availability of intracellular amino acids to meet protein synthesis demand. Further studies would however be required to elucidate the mechanistic details of how SR/ER stress response transcriptional factors interact with serine biosynthesis to bring about the upregulated rate of protein synthesis seen in the faster chicken skeletal muscle in this study.

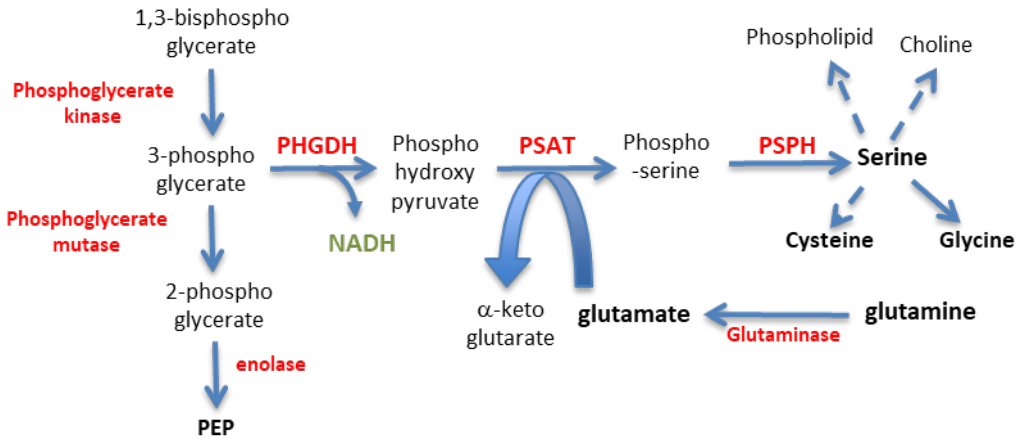


Figure 6.1: Schematic representation of the *de novo* biosynthesis of serine. The first step is the synthesis of Phosphohydroxypyruvate (PPHP) from 3-Phosphoglycerate catalysed by PHGDH. PPHP is then converted to Phosphoserine and then finally to serine catalysed by PSAT and PSPH respectively (Source Parr *et al* unpublished).

6.2.4 Gene expression of Asparagine synthetase (ASNS) in divergent growing muscles of Ross 308 and Hy-Line chicken genotypes

The observation in this trial where a higher expression of ASNS in the faster growing muscle was seen suggests that requirements for Asparagine are increased in these chickens and more specifically in the muscles that of growing the fastest. The nature of ASNS regulation suggests that asparagine may play a role in cellular amino acid homeostasis as ASNS expression is

upregulated in response to amino acid shortfalls. Therefore, the upregulated expression of ASNS seen in the faster growing chicken skeletal muscle tissues in this study, which are majorly composed of post mitotic differentiated muscle cells suggests that a similar metabolic mechanism seen in highly proliferating tumour cells having an abnormally very high demand for amino acids, characterised by increased protein synthesis, also occurs in genetically selected chickens that have the phenotype of differentiated fast growing skeletal muscle cells. Thus, the postulation is that the upregulated ASNS mRNA expression seen in the faster growing chicken muscles may be due to the activation of the amino acid response (AAR) pathway in faster growing muscles as a result of the high metabolic demand for amino acids for protein synthesis. As described above similar activating mechanism exists for co-ordinately activating the serine synthesis pathway genes via an AAR pathway, which has been predominantly described associated with the unfolded protein response, modulated by the feedback circuit of asparagine biosynthesis and the activation of ATF4 transcriptional target through the GCN2/eIF2α axis (figure 6.3).

6.2.5. Interaction between ASNS expression and serine biosynthesis

Although Asparagine is required for protein synthesis, it is thought to be a metabolic dead end, the mechanistic way it acts to regulate protein synthesis may be by serving as an amino acid exchange factor to enable cell acquisition of amino acids in limiting conditions. Cells transferred to in asparagine deficient media will export asparagine into the media (Krall *et al*., 2016) suggesting that the cells may be using asparagine as an amino exchange factor. Notably among the amino acids exchanged into the cell is serine. Krall *et al*., (2016) also noted that increased ASNS expression strongly correlated with the expression of genes encoding enzymes in the serine/glycine biosynthetic pathway. The authors reported that a knockdown of ASNS, resulting in reduced intracellular asparagine synthesis, stimulated an upregulated gene and protein expression of serine synthesis pathway enzymes as well as an upregulated ATF4 mRNA expression which were reversed by the incorporation of extracellular asparagine into the culture medium. Suggesting that cells may compensate for decreased intracellular asparagine levels and consequent decreased ability to exchange intracellular asparagine for extracellular serine by transcriptionally upregulating the serine synthesis pathway through ATF4 activation. ASNS, PHDH, PSAT and PSPH are among the seven ATF4-depedent transcripts involved in amino acid and protein anabolism. Furthermore, repression of ATF4 expression has been reported to reduce intracellular levels of serine during insulin stimulated

protein synthesis. While increased the expression of ATF4 resulted in the upregulated expression of ATF4 dependent amino acid synthesising enzymes ASNS and PSAT which in turn the increased the capacity to synthesise asparagine and serine respectively (Adams 2007).

Cells can acquire serine by *de novo* biosynthesis or importing extracellular serine from the environment (Mattiani *et al*., 2016). Xenografts of HCT116 colon cancer cells grow roughly half as fast when mice are fed a serine and glycine free diet as opposed to a normal diet (Maddocks *et al*., 2013). In human colon cancer cell lines, withdrawal of serine from growth medium reduces proliferation even in the presence of increased concentrations of glycine suggesting that cells preferentially take up serine and excrete glycine when serine is available and consume glycine only when serine is depleted (Labuschange *et al*., 2014; Jain *et al*., 2012). The seen upregulated ASNS expression seen in the faster growing Ross 308 muscles in this study, may also influence intracellular serine uptake in exchange for asparagine. Thus, the uptake of serine may be highly dependent on the biosynthesis of asparagine irrespective of the observed increased expression of enzymes in the serine biosynthetic pathway in the faster growing chicken muscles in this study. This phenomenon has also been established in some cancer cells where *de novo* serine biosynthesis from the glycolytic pathway has been observed even in the presence of abundant extracellular serine (Mattaini *et al*., 2016). Thus, this may be the situation in the faster growing chicken muscles where serine biosynthesis and extracellular serine exchange with asparagine are simultaneously upregulated. It also suggests that despite the upregulated asparagine synthesis that may be taking place in the fast-growing chicken muscles, the concentration of the amino acid may still be insufficient thus the cells may need to increase serine synthesis and these processes may be mediated by the increased expression of ATF4 (figure 6.3).

Asparagine is derived from aspartic acid which is synthesised from oxaloacetate and the transamination of glutamate which yields ketoglutarate (a-KG). Furthermore, in the serine biosynthesis pathway, the transaminase PSAT regulates the second step and produces a-KG by the transamination of glutamate (Brown *et al*., 2016). a-KG can then be used as a precursor for resynthesizing glutamate and in turn glutamine (Wu, 2014) (see figure 6.2 and 6.3), however a-KG has also been linked to muscle growth and protein synthesis, muscle hypertrophy and growth. Administration of 2% (w/v) a-KG in drinking water of pigs resulted in enhanced gastrocnemius muscle weight and fibre size (Cai *et al*., 2016). Thus a-KG produced from the synthesis of the serine and asparagine may acting mechanistically in conjunction with these amino acids to accentuate muscle growth seen in the faster growing muscles in this study.

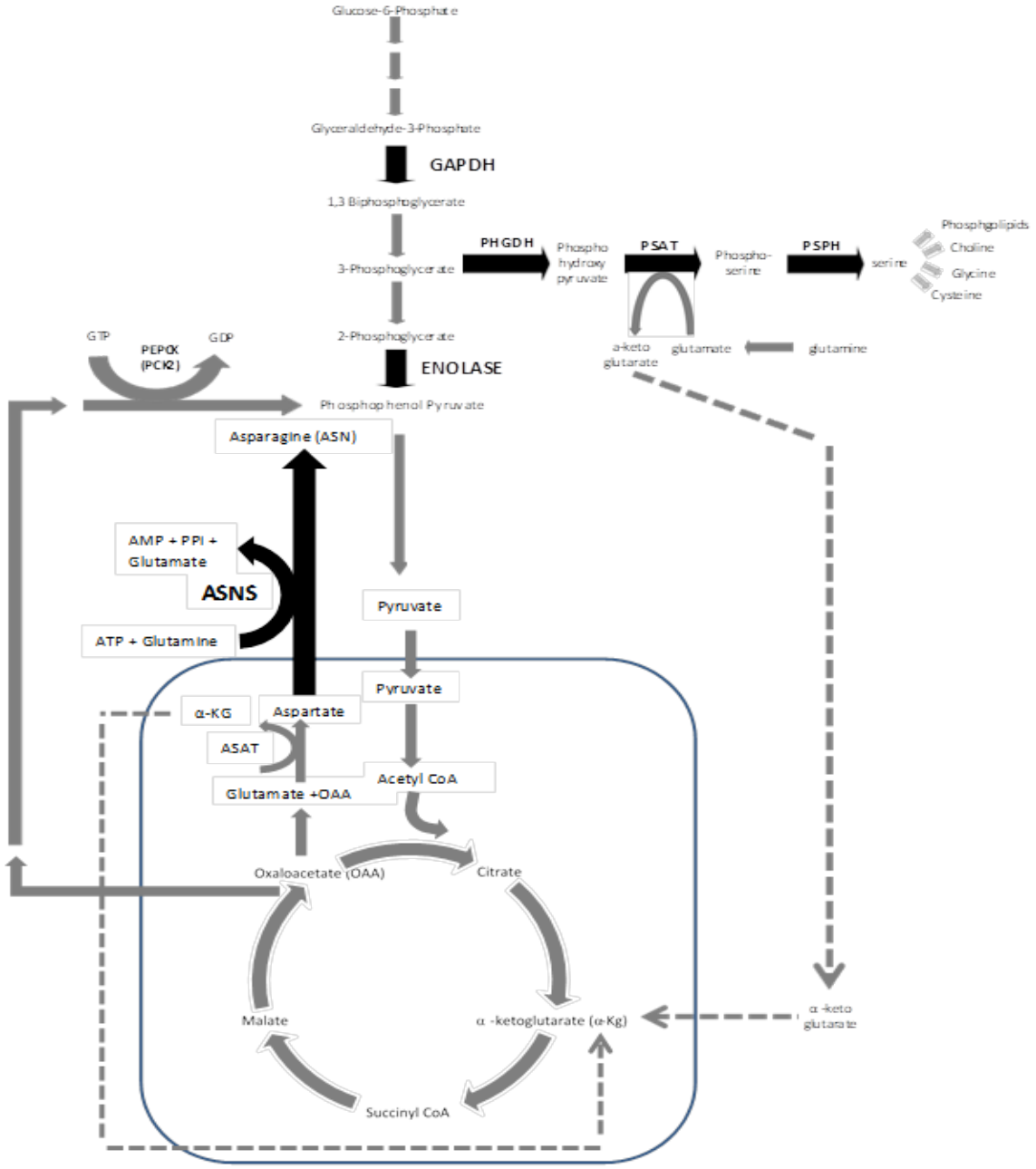


Figure 6.2: A schematic illustrating the relationship between glycolysis, the TCA cycle and pathways associated with serine biosynthesis and asparagine synthesis. The model shows the upregulated expression of *de novo* serine biosynthetic protein enzyme genes from 3-phosphoglcerate as well as pathway synthesis of ASNS catalysed asparagine biosynthesis and PCK2 catalysed phosphophenol pyruvate from the TCA cycle as well as α-ketoglutarate biosynthesis from the serine and asparagine biosynthetic pathway. Block arrows show an upregulation in the expression of GAPDH, enolase, PHGDH, PSAT, PSPH and ASNS in faster growing muscles. PCK2 was not upregulated in faster growing chicken skeletal muscles.

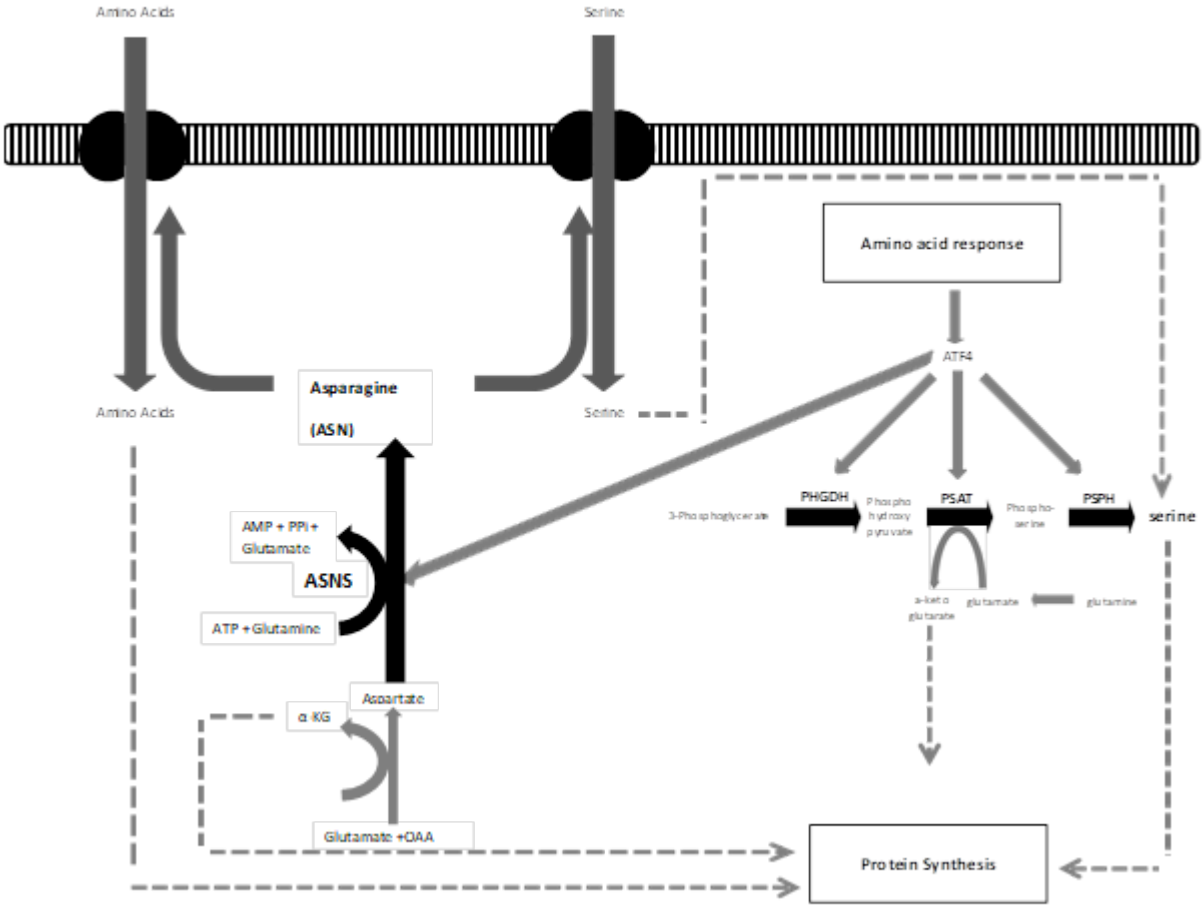


Figure 6.3: Model showing the proposed interaction between intracellular serine and asparagine biosynthesis and extracellular serine/amino acid exchange with asparagine in fast growing chicken skeletal muscle. Block arrows indicate an upregulated expression of enzymes catalysing the synthesis of asparagine and serine. Model depicts the simultaneous upregulated serine biosynthesis and intracellular asparagine/extracellular serine amino acid exchange.

6.3. STUDIES ON PROTEIN SYNTHESIS SYSTEMS IN CHICKEN SKELETAL MUSCLE

6.3.1 Differential expression of downstream AKT/mTOR (P70S6K, and rps6) intermediates and muscle growth in divergent growing chicken muscles and genotypes

In Trail 1 and 2 there was a significantly P70S6K mRNA expression PM than PT whilst in trial 2 it appeared that faster growing Ross 308 had higher expression than Hy-Line. Although only the protein expression of ribosomal protein S6 (rpS6) in the RPM and HPM was measured at day 35, which corresponded to a time point with the highest RPM mRNA expression in both trial 1 and 2, there was higher quantities of protein in RPM We were unable to monitor whether the difference in phosphorylated levels of P70S6K and rps6 proteins due to a lack of availability of reliable antibodies. However, the observations seen in total protein expression of P70S6k and rps6 suggest that there may be a higher rate of protein synthesis in the faster growing RPM muscles when compared to the other slower growing muscle types. This agrees with earlier reports associating the upregulated synthesis and activation of P70S6K and RPS6 with an increased skeletal muscle protein synthesis in chicken (Deng *et al*., 2014; Wang *et al*., 2013; Boussaid Om-Ezzine *et al*., 2010; Tesseraud 2000). P70S6K and rps6 are activated through the AKT/mTOR signalling pathway. The mTOR pathway is reported to regulate protein synthesis through translational capacity and efficiency which leads to an upregulated mRNA translation resulting in increased fibre size (Zanchi and Lancha 2008).

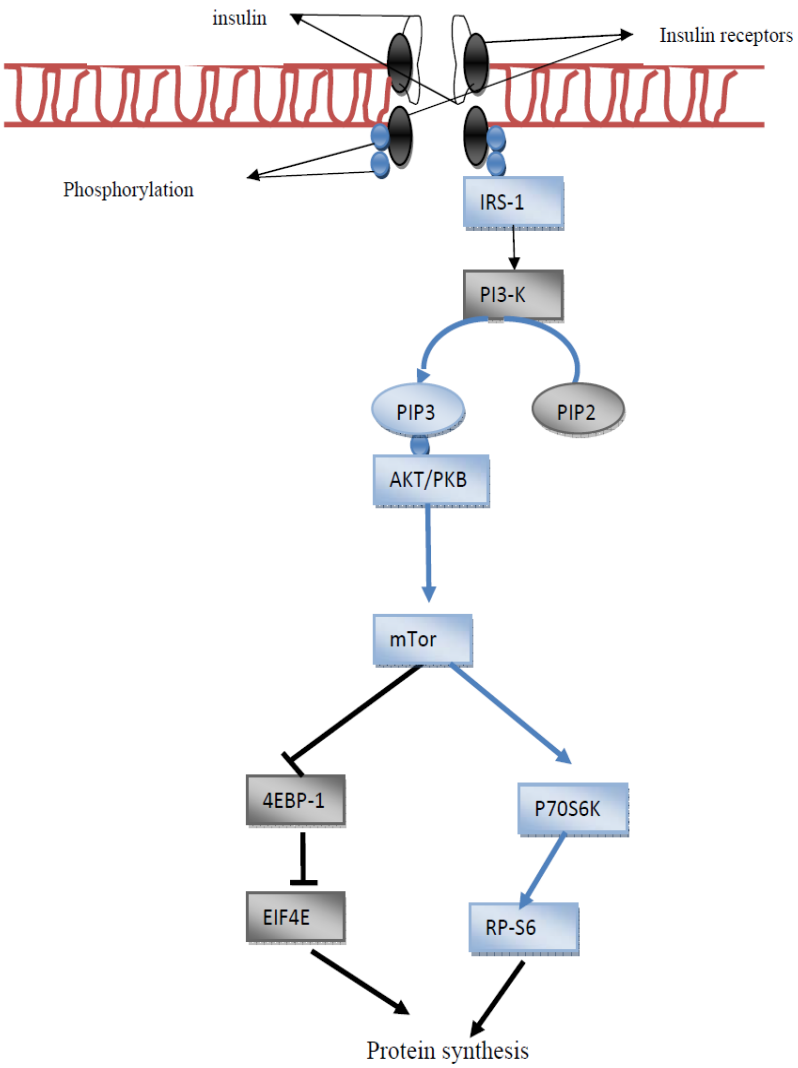


Figure 6.4: proposed pathway for AKT/mTOR skeletal muscle protein synthesis resulting in increased muscle growth in chicken. There was an upregulated mRNA expression of P70S6K in faster growing chicken muscles as well as an upregulated total protein expression of P70S6K and RPS6 in faster growing chicken muscles.

6.4 STUDIES ON PROTEOLYTIC SYSTEMS IN CHICKEN MUSCLE

6.4.1 Calpain proteolytic system in chicken muscles

The calpain proteolytic systems are believed to be a gateway to facilitate skeletal muscle protein proteasome degradation process. They selectively cleave protein myofibre substrates rather than completely degrading them, thus releasing myofibrillar polypeptide substrates which the proteasome further degrades (Goll *et al*., 2003; Powers *et al*., 2005). Thereby contributing to protein ubiquitination and proteasome enzyme activity (Kramerova *et al*., 2005; Fareed *et al*., 2006). Calapastatin is the specific endogenous inhibitor of calpain and thereby is a mechanism by which calpain activity can be regulated. However, in in Trial 1 there was no significant difference in calpastatin mRNA expression between the PM and PT at all time points in trial 1.

In Trial 2, of the three-time points measured (day 14, 35 or 42 post hatch), there was no significant difference in the activity of both calpain isoforms in all muscles between and within genotype at day 14 and day 35. These observations are in agreement with Ballard *et al*., (1988) where the activities of both calpains and calpastatin were not significantly altered in chickens with muscle diverse growth rates accomplished by feeding them diets with varying protein levels to attain differing growth. It also agrees with reports by Nakashima *et al*., (2009) where there was no difference in the expression of milli-calpain larger sub unit, between divergent growing broiler and layer *Pectoralis major* muscles. In contrast to observations broiler type chicken with a high fractional growth rate were reported to have a lower low m-calpain and higher calpastatin activities when compared to layers with a low fractional growth rate (Johari *et al*., 1993; Johari *et al*., 1996). In mammalian skeletal muscle, μ-calpain was reported to have higher activity values in fast fibre type Longissimus dorsi than slow fibre type trapezius muscle respectively, whilst for m-calpain the activity values were reversed (Jones *et al*., 1998). Taken together, these reports tend to support the observation made in this current study where there was a time point difference in the comparative μ and m calpain activities between broiler and layer muscle types. We observed here that at day 14 and 42 there was no difference in the activities of μ and m calpain in all muscle types between and within the two genotypes but there was a significant genotype effect with a higher activity of μ and m calpain seen in the HPM and HPT when compared to the RPM and RPT at day 35.

Myofibrils are composed of a mixture of sacromeric and cytoskeletal proteins which have all been reported to be substrates of calpains *in vivo* or *in vitro* (Huang and Zhu 2015). The calpain

proteolytic system also plays a limited role in muscle protein turnover as they are believed to be responsible for the removal of proteins from the myofibrillar structure without disrupting the myofibril, these proteins include troponin T, desmin, vinculin, talin, spectrin, nebulin, and titin. The removed myofilaments could either reassemble back to the surface of the myofibril or be degraded to small peptides and amino acids by cytoplasmic proteinases in the proteasome (Yang and Mcelligot 1989) (see Figure 6.4). Therefore, the calpain system is probably involved in the initiation events of protein degradation rather than gross degradation. The relatively low level of calpain activity in the fast-growing Ross 308 chickens compared to the slow growing Hy-Line at day 35 is rather interesting as it indicates a lower level of calpain proteolytic activity at this time point. This is the at the same point where relatively high protein synthesis capacity and non-essential amino acid synthesis (serine and asparagine) was observed (see above). This could reflect a metabolic adaptation to accentuate protein deposition by increasing synthesis capacity whilst reducing myofibril degradation.

6.4.2 Proteasome enzyme activities and E3 ubiquitin ligases MAFbx and MuRF1 mRNA expression and chicken skeletal muscle growth

There is no previous information available on genetic differences in differential growing chicken with regard ubiquitin proteasome activity, this will be the first reported case. In this study, we observed a higher activity of the three-catalytic proteasome sub-units (trypsin-like, chymotrypsin-like and caspase-like activities) measured in the muscles of the faster growing Ross 308 genotype when compared to Hy-Line genotype at all time points. In contrast, there was an upregulated mRNA expression of E3 ubiquitin ligases MuRF1 and MAFbx in the slower growing Hy-Line muscles when compared to the Ross 308 counterparts. These observations are similar to reports by other researches. Baehr *et al*., (2014) reported that 20S and 26S 5β chymotrypsin-like proteasome activity was elevated in mouse plantaris muscle where hypertrophy and protein synthesis was induced by subjugating the mice to bilateral functional overload. The proteasome activities also remained elevated for a much longer period than did the induction of MuRF1 and MAFbx expression post functional overload. In another study, Baehr *et al*., (2011) reported that despite the significantly upregulated expression of MAFbx and MuRF1 in the triceps surae muscles of mice following muscle atrophy induced dexamethasone treatment, there was no change in caspase-like, trypsin-like and chymotrypsin-like activities. Also, under denervation conditions, the three catalytic subunits of the proteasome have also been shown to have a greater activation in MuRF1 null mice in

comparison to their wild type counterparts (Gomes *et al*., 2012). Lastly, during the recovery of mice hindlimb muscle post 7-day atrophy induced hindlimb immobilisation, MAFbx and MuRF1 expression were not increased at any time point studied but chymotrypsin-like protease activity increased significantly at day 1 of recovery along with a significant increase in protein synthesis from day 1 to 6 in comparison to the control group.

Further insight into how the proteasome works to simulate muscle growth can be seen in resistance training and aerobic exercise. Acute bouts of exercise have been shown to stimulate both skeletal muscle protein synthesis and degradation (Rennie and White 2000). This surge in muscle protein breakdown during exercise is hypothesised to be adaptive, getting rid of damaged muscle tissues and facilitates myofilament restructuring and muscle growth (Bell *et al*., 2016). Cunha *et al*., (2011) reported an increase in 26S chymotrypsin-like proteasome activity as well as an upregulation in the mRNA and protein expression of all other proteolytic component in the *plantaris* skeletal muscle of mice after 8 weeks of moderate intensity aerobic exercise training which was accompanied by hypertrophy. However, the authors also reported that 24 hours after the exercise session, the only component that remained above resting level was the proteasome activity.

The hypothesis based on observations from the study of this chicken model is that the higher rate of proteasome activity seen in the fast-growing muscles of Ross 308 in contrast to the slower growing Hy-Line skeletal muscles may be because the proteasome serves a basic housekeeping function as an amino acid recycling machine, rather than being just a waste disposer. Thus, for the fast-growing Ross 308 chickens to meet their genetic and metabolic demand for skeletal muscle protein synthesis and growth, the proteasome may be acting to replenish the pool of intracellular amino acids available for muscle uptake due to amino acid limitation to maintain adequate amino acid levels for sustaining muscle protein synthesis. See figure 6.4. Furthermore, the upregulated proteasome activity, ASNS expression as well as the mRNA expression of enzymes in the serine biosynthetic pathway seen in this current study taken together with reported interactions with UPR, proteasome activity and ATF4 signalling suggests that this process may be mediated by the eif2a/ATF4 signalling pathway. This speculation is echoed in an earlier publication by Suraweera *et al*., (2012). The researchers reported that treatment of mammalian cells with proteasome inhibitors MG-132 or Bortezomib under conditions of nutrient sufficiency decreased the intracellular levels of the amino acids asparagine/aspartate and cysteine with lethal consequences to the cells. They also demonstrated that supplementation of the proteasome deficient cells with these amino acid dramatically increased cell survival and markedly reduced apoptosis in a dose dependent manner.

Furthermore, they reported that the integrated stress response is induced by the amino acid shortage resulting from proteasome inhibition manifested by the phosphorylation of eIF2a, expression of ATF4 and the pro-death proteins CHOP and GADD34.

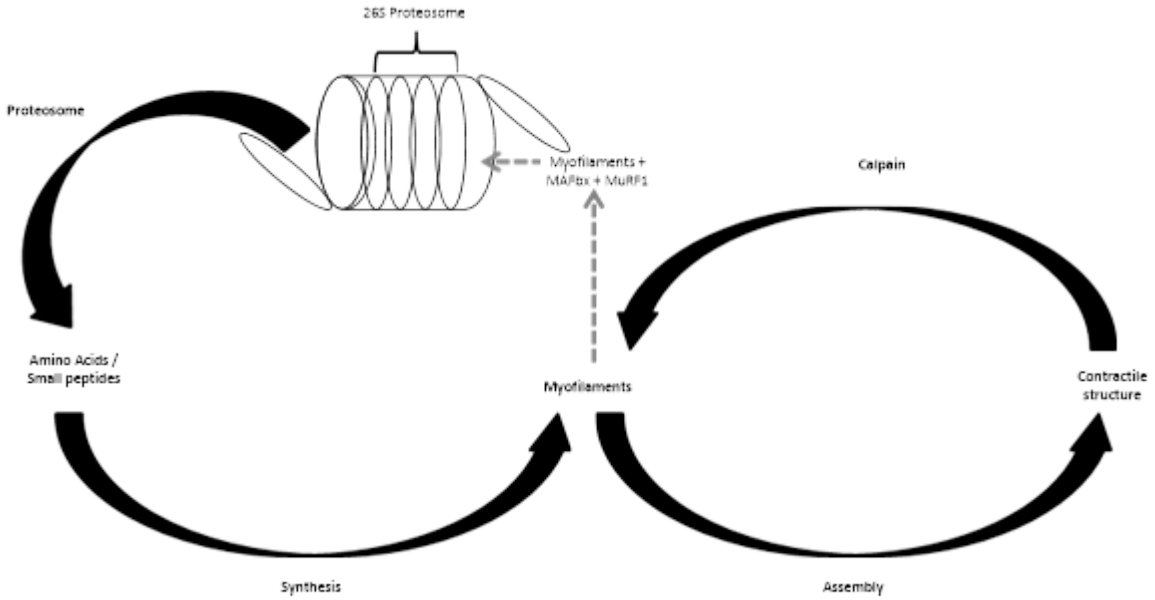


Figure 6.5: Model showing the proposed relationship between proteolytic system and skeletal muscle assembly in fast growing skeletal muscle of chicken. Model shows the breakdown of contractile structures by calpains which are then reassembled back to whole muscles or further broken down to amino acids and small peptide in the proteasome which are recycled and synthesized back to myofilament and re-assembled back to contractile proteins

6.5 MUSCLE GROWTH AND CELL CYCLE, DIFFERENTIATION, FUSION AND REGENERATION

6.5.1. Expression of genes modulating the muscle cell differentiation and regeneration. LIM domain proteins (CSRP3 and FHL2) and MUSTN1.

There was significantly higher CSRP3 mRNA expression in the slower growing PT muscle when compared to the PM observed in Trial 1, which was also observed in both genotypes in trial 2. This agrees with earlier reports where a lower in expression of CSRP3 transcripts was seen in faster growing breast muscles of broilers compared to slower growing layer breast muscles. (Zheng *et al*., 2009). CSRP3 is a known positive regulator of myogenesis promoting myogenesis through differentiation by enhancing the activity of MyoD. CSRP3 has also been reported to have an upregulated expression in broiler chickens in a muscle wasting myopathic condition known as 'wooden breast' (Mutryn *et al.,* 2015). Decreased CSRP3 expression has also been reported in humans undergoing pathogenic cardiac hypertrophy (Ecarnot-Laubriet *et al*., 2000). CSRP3 knockout mice displayed an array of skeletal muscle defects such as shorter sarcomere length and muscle atrophy suggesting CSRP3 may play a role in the maintenance of skeletal muscle and also support skeletal muscle regeneration following injury through structural repair and gene regulation (Barash *et al*., 2005). CSRP3 has also been reported to be significantly increased in expression after periods of exercise in humans (Kostek *et al*., 2007). This may occur in order to promote muscle growth as CSRP3 has also been linked to involvement in muscle hypertrophy and regeneration through the calcineurin/NFAT signalling pathway and myogenic differentiation (MYOD) expression (Arber *et al*., 1994) However, it has been observed that CSRP3 is mainly expressed in slow skeletal muscle, compared to its low expression in fast skeletal muscle as reported by (Schnider *et al*., 999) where CSRP3 expression was upregulated during fast-to-slow fiber type switch in rats. This supports the findings of this study as chicken leg muscles are generally characterised with slow growing muscle fibres whereas the breast is mainly composed of fast twitch fibres.

The significantly higher levels of FHL2 expression in the slower growing PT when compared to the faster PM muscle in Trial 1 as well as the higher rate of expression in the slower growing HPM and HPT muscles when compared to the RPM at days 23, 35 and 42 in Trial 2 is also consistent with reports from Zheng *et al*., (2009) where the expression level was lower in broiler breast muscles when compared to layers. These observations have also been seen in growth promoter induced muscle growth in pigs (Parr *et al*., unpublished). A study by Shi *et*

al., (2010) demonstrated that FHL2 is involved in cell cycle, where FHL2 knockdown in mice were reported to induce cell cycle arrest, reduced myogenic progenitor cell population, perturbed muscle regeneration and interacted with Foxk1 (a factor expressed in adult myogenic cell population positively upregulating muscle regeneration) to bring about the repression of Foxo4 and P21 which both serve as negative muscle regenerative factors. FHL2 has also been shown to play a role in the regulation of myogenic proliferative capacity in adult cell by upregulating the expression of interleukin 6 (IL-6). Impaired satellite cell proliferation and myonuclei incorporation have been attributed to IL-6 deficiency (Serano *et al*., 2008; Kurek *et al*., 1997).

In this study, we also found that the level of expression of MUSTN1 increased significantly with age and its expression was highly correlated with muscle weight in the slower growing PT of broilers in trial 1. MUSTN1 has been reported to play a role in muscle development and hypertrophy in different species (Lombardo *et al*., 2005; Han 2010). Zheng *et al*., (2009) reported higher expression of MUSTN1 in the slower growing breast muscles of layers when compared to broilers. a Silencing of rat MUSTN1 leads to the inhibition of myogenic fusion and differentiation, indicating its role in muscle development (Liu et al., 2010). MUSTN1 expression has also been reported to be upregulated in exercise induced muscle damage supporting its possible involvement in skeletal muscle regeneration (Kostec *et al*., 2007). An elevated expression of MUSTN1 has also been seen in chicken myopathic disorder known as wooden breast where it's upregulation in affected birds was attributed to facilitate induced compensatory growth (Mutryn *et al*., 2010).

Chickens are ground-dwelling bird that rarely fly. They use their leg muscles for unhurried, long-duration movements such as roaming around and searching for food. In contrast, a chicken hardly uses its wings except for balance. When it flaps them energetically, it's usually to make a quick escape from a threat. Because the muscle requirements of the chicken leg and breast are different, the two sets of muscles evolve differently. The legs consist predominantly of slow-contraction muscle fibres, while the breast is composed chiefly of fast-contraction muscle fibres to help flap the wings. The slow-contraction muscle fibre is for the long-duration jobs and the fast-contraction muscle fibre for the quick-energy spurts. Thus, the legs of the chicken are more susceptible to muscle damage and repair possibly resulting in the higher expression of CSRP3 and FHL2 as well age dependent upregulation of MUSTN1 in the leg PT muscles when compared to the breast PM as observed in this study. Muscle damage and regeneration have been shown to upregulate the expression of CSRP3 (Rashid *et al*., 2015; Sannoudou *et*

al., 2006; Jensen *et al*., 2012), FHL2 (Shi *et al*., 2010) and MUSTN1 (Kostec *et al*., 2007). Thus, the upregulation of these three genes in the PT muscle in chickens likely indicates a response to compensatory hypertrophy or muscle repair secondary to muscle damage rather than physiologic hypertrophy during bird growth.

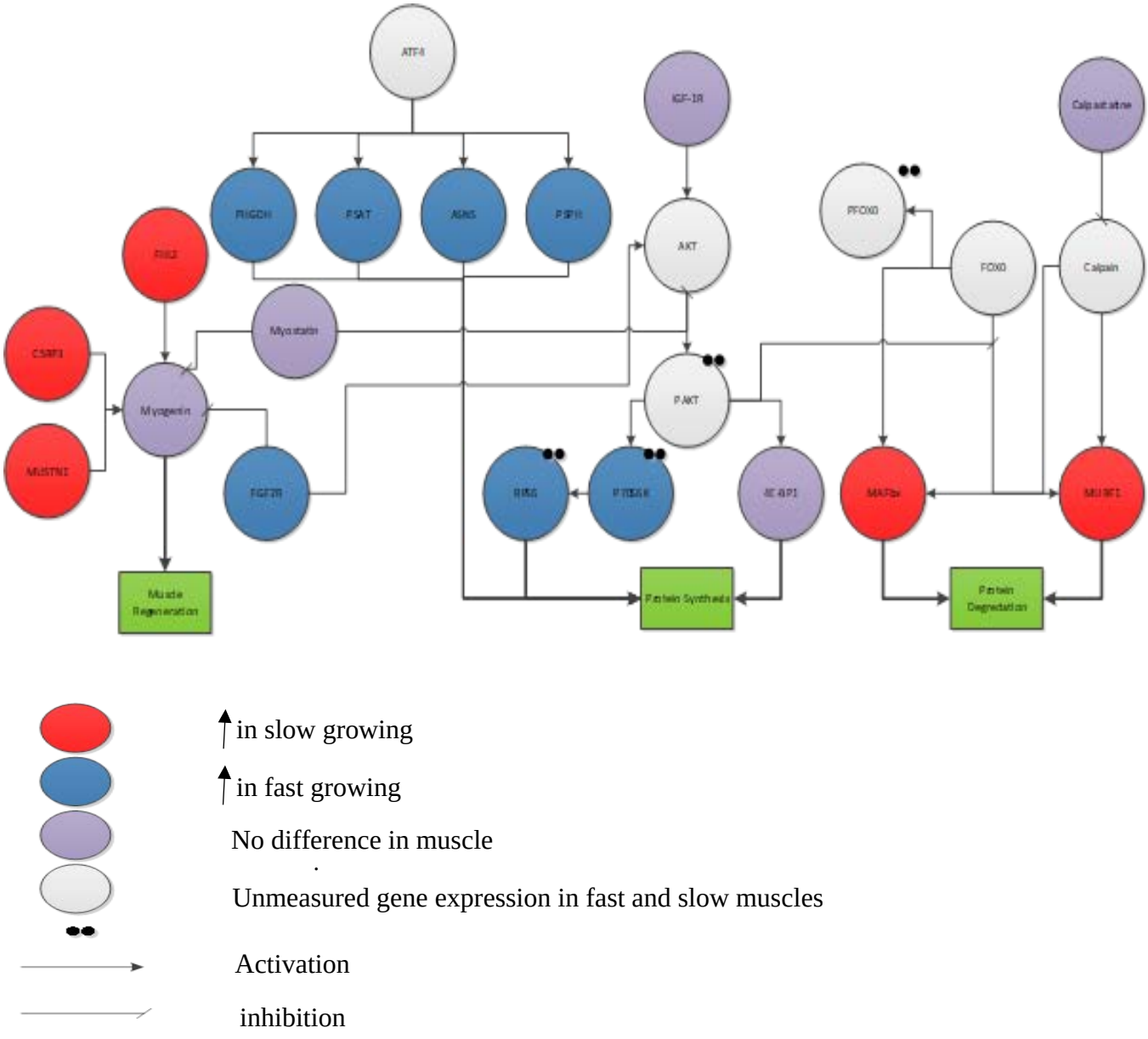


Figure 6.6: expression analysis showing the relationship and interaction of some measured genes on chicken skeletal muscle protein synthesis, degradation, and regeneration. FHL2, CSRP3 an MUSTN1 activate muscle regeneration by upregulating the expression of myogenenin and muscle regenerating promoting genes, FGF2 and myostatin inhibit the process while simultaneously activating and inhibiting respectively the phosphorylation of AKT. Phosphorylated AKT activates the phosphorylation of P70S6K and RPS6 proteins which upregulates protein synthesis. FOX0 and calpains activate the expression of E3 ubiquitin ligases MAFbx and MURF1 which lead to protein degradation while FoX01 and calpain expression are inhibited by phosphor-AKT and calpastatin respectively. ATF4 leads to the upregulation of PHGDH, PSAT, PSPH and ASNS expression attenuating protein synthesis.

6.6 SUMMARY

This study sought to investigate how changes in skeletal muscle growth pattern in differential growing genotypes and muscle types of chickens interact with proteolytic, synthesis and metabolic systems as well as the expression of some key genes involved in the modulation of these processes to regulate skeletal muscle growth. Some of these molecular factors that modulate skeletal muscle growth rates were comparatively studied in this research work and differences in the way they interact and associate with muscle growth were observed. This would be the first time the expression of genes involved in some metabolic pathways as well as proteins in some physiological signalling pathways would be reported. The main findings in this work were the differential expression of metabolic genes which include protein enzyme genes involved in the glycolytic pathway, *de novo* serine biosynthesis and differential PHGDH protein expression and asparagine synthesis from the TCA cycle. Genes modulating these metabolic pathways were found to be generally upregulated in faster growing muscles between and within the of the two differential chicken genotypes studied. Generally, observations in this study suggest an increased rate of protein synthesis in the fast-growing muscles. This is evidenced in the seen upregulation of the mRNA expression of genes that stimulate the AKT/mTOR pathway namely P70S6K and FGF2R as well as protein expression of P70S6K and end target RPS6 downstream the same signalling pathway in the faster growing muscles. Proteolytic systems were also explored where the activities of two isoforms of calpains as well as activities of 26S UPS proteasome enzyme like activities were comparatively measured. Results indicated that proteolytic systems in chicken may be contributing to muscle growth by acting to recycle amino acids to support protein synthesis. Suggesting that protein synthesis and degradation are co-ordinately regulated in fast growing chicken muscles. The expression of genes encoding two LIM domain proteins CSRP3 and FHL2 that may play a role in muscle regeneration were seen to have a higher expression in slower growing PT muscles in both genotypes when compared to the faster growing PM. MUSTN1 which has been reported to play a role in muscle regeneration and repair had a higher expression with increasing age in the slower growing PT muscle. Suggesting these genes likely play a role in response to compensatory hypertrophy or muscle repair secondary to muscle damage rather than physiologic hypertrophy during bird growth.

The potential benefits of these observations are that it could be an added benefit to animal meat production potentially identifying molecular targets for manipulation in a bid to improve feed

efficiency and could also be beneficial to health and medical research where conditions of muscle wasting such as muscle atrophy and dystrophy are concerned.

6.7 CONCLUSIONS AND FUTURE WORK

6.7.1 Final conclusions and directions for future work

Traditionally, research aimed at improving animal production in meat animals have mostly been aimed at improving and manipulating animal genetics through breeding programmes and improving feedstuffs to enhance their utilisation efficiency. The body of work presented herein aimed to utilise molecular techniques to improve our understanding of how these genetic and nutrition researched based programmes have impacted on chicken growth physiologically and metabolically. In Trial 1, two muscle types within the same fast-growing Ross 308 genotype were studied. While in Trial 2, the same muscles were studied in 2 genotypes having divergent skeletal muscle growth phenotype namely Ross 308 and Hy-Line. This study also investigated how potential molecular targets express themselves differently between divergent growing muscles within and across the two different genotypes. The main observations seen were a difference in growth rate in muscle types within and between genotypes. There was a clear association in gene expression and protein quantity associated with differences in enzyme activity. This present study also highlighted that there is an increase in protein synthesis which leads to clear increase in protein accretion in faster growing chicken muscles supporting the wealth of literature detailing protein turnover in chicken skeletal muscles. There also appears to be an interaction between protein synthesis and degradation, however in most cases protein synthesis seems to be more dynamic and these changes seem to appear around day 35.

The novel findings of this study were the observed increase in the expression genes that attenuate the synthetic capacity of non-essential amino acid (non-EEA) in fast growing muscles. Thus, the proposed model is that to increase protein deposition, the muscles require an increase in its endogenous non-essential amino acid synthetic capacity. This need to increase this capacity may be induced by an increase in the synthesis of asparagine, serine and other non-essential amino acids as well as the upregulation in the expression of enzymes that catalyse their synthesis. This situation in chicken muscle cells is analogous to cancer cells where massive proliferation rates requires an increase in biosynthetic capacity. The question therefore is how dependent are chicken muscles on this capacity and how is it regulated? In cancer cells, proliferation rates are very high and in order for the cells to satisfy this condition, there is an

increased biosynthesis of endogenous amino acids and their metabolites for example the biosynthesis of serine as a precursor to choline. There is also an increased biosynthesis of nucleic acids. The expectation in muscle would be that there would be a differential regulation of the nucleus and how it influences growth in differentiated muscle cells. Which leads to the questions of what mechanisms regulate theses non-EAAs synthesis and are these non-EAAs geared towards protein synthesis in differentiated muscle cells or towards the synthesis of other metabolites such as choline and nucleic acids as seen in cancer cells?

Future studies would be geared towards the investigation signalling pathways that modulate non-EEA synthesis. One of such is the ATF4 signalling pathway. A proposed study would be

- To investigate the effect of blocking ATF4 expression on the synthetic capacity of non EAA such as serine and asparagine and how this mechanism generally influences growth in chicken muscle cells.
- To investigate the effect of blocking ASNS and enzymes in the SOG pathway expression on growth in chicken muscle growth in chicken muscle cells.
- To elucidate the mechanism of action of serine biosynthesis on muscle growth and determine if serine exerts its muscle growth effect by solely upregulating the muscle capacity for protein synthesis or solely upregulating the biosynthesis of other serine metabolites or a combination of both processes.
- To elucidate the mechanism of action of ASNS and SOG enzymes on protein synthesis by determining the extent to which their expression is attenuated by the AKT/mTOR and ATF4 signalling pathways and determine if a co-ordinated mechanism of these pathways influences muscle growth.

An investigation of these mechanisms would give more insight to the question as to whether the capacity for accelerate growth is limited by endogenous non-EAA availability. It could also potentially lead to the research into the modification of chicken diets by increasing the amount of non-EEA's and checking its effect on growth.

6.7.2 Characterisation of expression of Pyruvate kinase (PKM 1 and PKM 2) genes in differential growing muscles and genotypes of chicken.

A low level of intracellular serine leads to a reduction in aerobic glycolysis, and the low pyruvate kinase activity of PKM2 facilitates accumulation of glycolytic intermediates that serve as substrates for endogenous serine synthesis. In turn a low level of serine leads to

activation of GCN2 and the enhanced translation of ATF4. The ATF4 induction increases the transcription of the genes necessary for serine biosynthesis from the accumulating glycolytic intermediates upstream of PKM2. The combined functions of GCN2-ATF4 and PKM2 are necessary for cells to maintain cell proliferation when deprived of extracellular serine Ye *et al*., (2012). Therefore, as the difference in growth rate between fast and slow growing muscles in chicken are inherent, it is likely the mechanism causing this is an internal difference in key metabolic steps between the two muscles one of which may be the activity of PKM2.

Pyruvate kinase muscle is a glycolytic enzyme that catalyses the conversion of phosphoenolpyruvate to pyruvate. In mammals, the M1 isoform (PKM1) is expressed in most adult tissues The M2 isoform (PKM2) is an alternatively spliced variant of M1 that is expressed during embryonic development (Christofk 2008). Cancer cells have been reported to exclusively express PKM2 (Dombrauckas *et al*., 2005). Results in our lab has shown an increased expression of both PKM 1 and 2 in ovine *Longissimus dorsi* and *supraspinatus* muscles of the back (Al-Doski *et al*., 2015). The Pkm2 splice variant has been implicated as a regulatory component controlling metabolic flux through to mitochondrial ATP production or re-directing glucose carbons into biosynthetic pathways stemming from glycolysis. The splice variant Pkm2 is less effective than Pkm1 at facilitating the conversion of PEP to pyruvate and its activity can be regulated by various metabolic inputs. Therefore, the ratio of Pkm2:Pkm1 is likely critical in controlling the metabolic fate of glucose carbons. A high ratio of Pkm2:Pkm1 is theorised to cause a “back-log” effect, re-directing glucose carbons to biosynthetic pathways (Lyssiotis et al. 2012).

PKM2 is generally thought to interact with the biosynthesis of serine and has an association with muscle growth as described in section 2.13.2. Two transcripts of chicken PKM was identified in this study by online bioinformatic tools (see appendix 5). Due to the similarity in sequence base pairs, specific primers for each transcript could not be designed. However, it may be possible to measure the expression of the proteins by Western blotting. There appears to be antibodies developed specifically for the two isoforms. See (www.cellsignal.com).

6.7.3 Investigating the relationship between integrated stress response signalling and muscle growth in chicken.

The hypothesis is that a high demand for amino acids required for protein synthesis as a result of the phenotype of fast muscle growth in chicken has resulted to the upregulation of intermediates of ISR signalling for the purpose of upregulating protein synthesis and muscle growth. The goal would be to characterise ISR signalling in chicken skeletal muscle by measuring the phosphorylation of eIf2α and the expression of ATF4 and GCN2 and establish a relationship between the expression these ISR intermediates and expression of ASNS, serine biosynthesis genes as well as proteasome activity in relation to muscle growth in chicken.

The integrated stress response (ISR) is an elaborate signalling pathway present in eukaryotic cells, which is activated in response to a range of physiological changes and different pathological conditions (Pakos-Zebrucka *et al*.., 2016). The ISR consists of four kinases that converge on the phosphorylation of eukaryotic translation initiation factor (eIF2α) in response to a diverse array of stimuli sensed by individual mechanisms. Among these stimuli are amino acid deprivation and unfolded protein accumulation resulting in ER stress (Lehman *et al*., 2015). The stress-induced signalling pathway in human and mammalian cells that involves eIF2α phosphorylation and downstream ATF4 induction is referred to as the integrated stress response (Pakos-Zebrucka *et al*.., 2016). Conventionally, the ISR has been associated with a reduction in general protein synthesis by the phosphorylation of eIF2α at the serine 51 interphase consequently supressing translation initiation (Yano and Yano 2017). However, this may not always be the case. For example, the initiation of translation of ATF4 (activating transcription factor 4) mRNA which could be upregulated by unfolded protein accumulation induced ER stress (Harding *et al*., 2003; Vattem and Rek 2004) encodes a transcription factor that induces gene expression for adaptive responses. It has been reported that the post transcriptional repression of ATF4 leads to a downregulation of mRNAs encoding amino acid transporters and metabolic enzymes suggesting that the function of ATF4 under non-stress conditions is to maintain the homeostatic balance between translation supply and demand by tuning the expression of the many enzymes and transporters required for delivering amino acids to the translation machinery (Park *et al*., 2017).

6.7.4 Investigation of the insulin signalling pathways and its intermediates modulating protein synthesis in chicken skeletal muscle.

The characterization of the AKT/mTOR pathway in relation to the insulin cascade regulating protein synthesis may not give an accurate reflection of the anabolic effect of insulin in protein synthesis as well as its sensitivity in chickens. Thus, it would be necessary to investigate the GCN2/ATF4 pathway in addition to the AKT/mTOR pathway to elucidate the anabolic effects of insulin and muscle growth in faster growing chicken muscles. The hypothesis is that insulin signalling attenuates muscle protein synthesis through a combined regulation of the GCN2/ATF4 and AKT/mTOR pathways.

Insulin has an anabolic effect on the metabolism of carbohydrates, lipids and proteins promoting the uptake of amino acids, glucose and the synthesis of protein and glycogen while inhibiting catabolic processes such as protein breakdown, gluconeogenesis, glycogenolysis, lipolysis, fatty acid oxidation, and ketogenesis (Foster and McGarry 1996). Insulin and mTOR activation has been reported to increase protein synthesis and ATF4 mRNA prior to the induction of ATF4-dependent mRNAs. By increasing ATF4 expression, mTORC1 sets in motion a series of events that presumably allow cells to acquire and generate amino acids and aminoacyl-tRNAs needed for continued protein synthesis and cell growth (Adams 2007).

In both mammals and chickens, various post-receptor steps regulate cell sensitivity to insulin and alterations of insulin sensitivity are not necessarily associated with changes in insulin receptor number or kinase activity (Dupont *et al*., 2009). Chickens have been shown to be hyperglycaemic and insulin intolerant mimicking the early stages of type two diabetes in humans (ji et al., 2012). However, insulin has also been shown to be involved in protein synthesis in chicken by the downstream activation of the AKT/mTOR and ATF4/GCN2 signalling pathways (Duchene et al 2008; Adams 2007).

CHAPTER 7 (REFERENCES)

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CHAPTER 8 (APPENDICES)

APPENDIX 1: Muscle and body weights (Trial 1)

Day 14

Ross 308	Animal no	Body wt (g)	P.major wt (g)	P.tertius wt (g)	% PM to body wt	% PT to body wt
	1	523.26	25.12	1.3	4.8	0.2
	2	-	20.21	1.28		
	3	339.33	18.17	0.91	5.3	0.2
	4	424.52	21.77	1.15	5.1	0.3
	5	457.48	22.18	1.02	4.8	0.2
	6	392.91	19.86	1.02	5.1	0.3
Mean		428	21.0	1.0	4.9	0.3
STD		69.0	2.4	0.2		
SEM		30.9	0.9	0.1		

Day 36

Ross 308	Animal no	Body wt (g)	P.major wt (g)	P.tertius wt (g)	% PM to body wt	% PT to body wt
	1	2105	142.8	7.3	6.8	0.3
	2	2164	134.3	13.1	6.2	0.6
	3	2266	148.4	12.2	6.5	0.5
	4	2460	187.0	17.3	7.6	0.7
	5	2070	138.3	21	6.7	1.0
	6	1880	145.0		7.7	
Mean		2158	149.0	14.0	7.0	0.7
STD		195.3	19.1	5.2		
SEM		79.7	7.8	2.3		

Day 43

Ross 308	Animal no	Body wt (g)	P.major wt (g)	P.tertius wt (g)	% PM to body wt	% PT to body wt
	1	2686	178.3	13	6.6	0.5
	2	2666	145.53	15.6	5.4	0.6
	3	3030	166.7	10.6	5.5	0.3
	4	2880	177.1	11.12	6.2	0.4
	5	3230	206.5	10	6.4	0.3
	6	2880	142.9	11.5	5.0	0.4
Mean		2895	169.5	12.0	5.9	0.4
STD		213.1	23.6	2.0		
SEM		87	9.7	0.8		

APPENDIX 2: Muscle and body weights (Trial 2)

Day 4 02.05.14

Ross 308	Animal no	Body wt (g)	P.major wt (g)	P.tertius wt (g)
	1	62.7	0.7	0.5
	2	65.7	0.9	0.6
	3	75.2	0.8	0.4
	4	69.9	0.6	0.2
	5	77.3	0.8	0.4
	6	66.5	0.6	0.4
Mean		70.0	0.73	0.42
STD		5.7		
SEM		2.3		
Hy-line	1	44.7	0.6	0.4
	2	55.8	0.5	0.2
	3	53.3	0.7	0.3
	4	44	0.3	0.1
	5	53	0.7	0.2
	6	53	0.4	0.2
Mean		51	0.53	0.23
STDV		5.0		
SEM		2.0		

Day 14 12.05.14

Ross 308	Animal no	Body wt (g)	P.major wt (g)	P.tertius wt (g)
	1	259.5	7.2	2.2
	2	191.9	5.3	1.2
	3	247.6	6.3	1.2
	4	231.9	6	1.7
	5	271.6	9.9	1.9
	6	238.3	7.5	2.1
Mean		240.1	7.03	1.72
STD		27.6		
SEM		11.3		
Hy-line	1	136.9	3.5	0.8
	2	138.6	3.6	0.9
	3	131.1	3.2	0.7
	4	139.9	3.4	0.9
	5	130.7	3.6	0.8
	6	141.8	4.1	1
Mean		136.50	3.57	0.85
STDEV		4.60		
SEM		1.90		

23 21.05.14

Ross 308	Animal no	Body wt (g)	P.major wt (g)	P.tertius wt (g)
	1	442.1	15.1	3.3
	2	477.3	13.9	3.7
	3	547.2	20.4	4.3
	4	576.4	19.2	4.1
	5	653.8	23	4.6
	6	503.8	17.6	3.5
Mean		533.43	18.20	3.92
STDV		76.0		
SEM		31.0		
Hy-line	1	306	8.8	2.2
	2	259.1	8.1	1.5
	3	289.2	9.1	1.8
	4	264	8.2	1.5
	5	230	6.5	1.3
	6	262.5	7.7	1.9
Mean		268.5	8.07	1.70
STDV		26.3		
SEM		10.7		

Day 35 02.06.14

Ross 308	Animal no	Body wt (g)	P.major wt (g)	P.tertius wt (g)
	1	1286	53.9	11
	2	1226.6	52.5	11
	3	1078.8	50.6	7.9
	4	1208.7	41.2	9.6
	5	1207.3	55.2	10.2
	6	1291.3	52.3	9.3
Mean		1216.4	50.95	9.83
	stdv	77.0		
	sem	31.4		
Hy-line	1	523.5	15.9	4.1
	2	542.4	19.3	3.8
	3	532.3	15.9	3.3
	4	556.6	18.9	4.3
	5	472.2	15.4	3.6
	6	523.2	15.8	4.2
Mean		525.0	16.87	3.88
STD		28.8		
SEM		11.8		

Day 42 09.06.14

Ross 308	Animal no	Body wt (g)	P.major wt (g)	P.tertius wt (g)
	1	1997.8	83.5	19.8
	2	2185.8	112.9	20.5
	3	2128.3	104.2	21.9
	4	2061	93.1	18.5
	5	2042	104.7	19.5
	6	1915	92.5	16.6
Mean		2055	98.48	19.47
STDV		95.5		
SEM		39.0		
Hy-line	1	660.3	23.6	6.8
	2	740.3	25	7
	3	785.5	28.2	6.4
	4	569.9	19.6	4.8
	5	704.5	23	6.6
	6	725	25.1	7.2
Mean		698	24.08	6.47
STDV		74.9		
SEM		30.6		

APPENDIX 3: Example of some standard and melt curves charts of measured genes from Trial 1 and 2.

a **b**

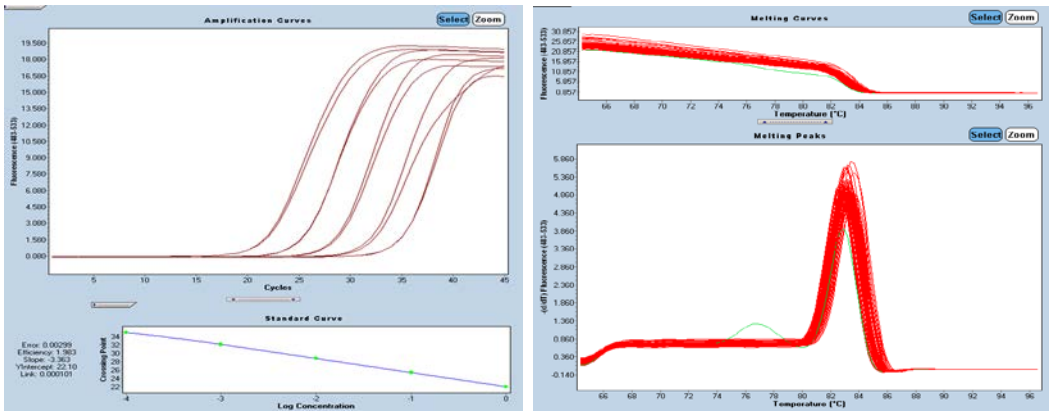

Figure: a). Standard curve of cyclophilin gene expression. 1:8 neat cDNA to RNase free water dilution ratio. b). Melting curve of cyclophilin gene expression. 1:8 neat cDNA to RNase free water dilution ratio.

a **b**

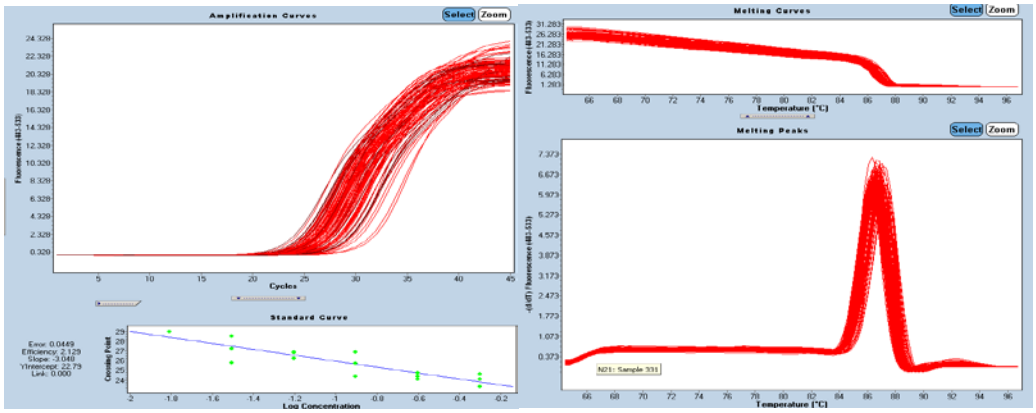

Figure: a). Standard curve of GAPDH gene expression. 1:8 neat cDNA to RNase free water dilution ratio. b). Melt curve of GAPDH gene expression. 1:8 neat cDNA to RNase free water dilution ratio.

a **b**

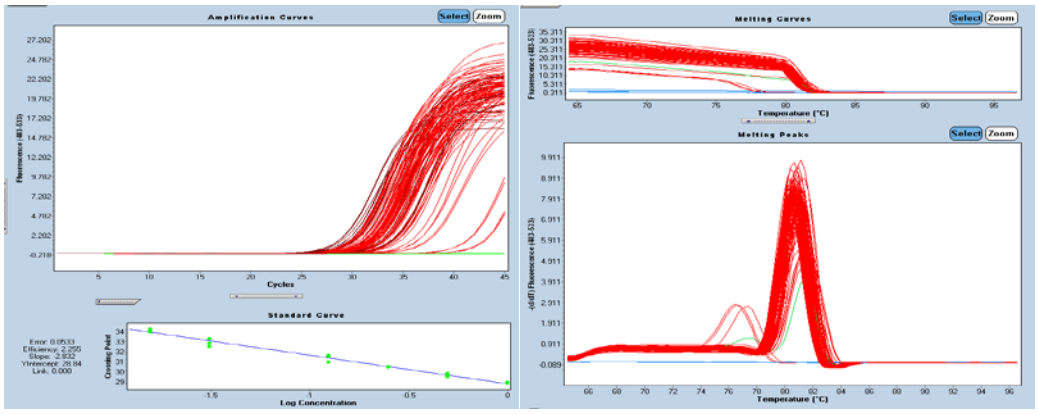

Figure: a). Standard curve of calpstatin gene expression. 1:8 neat cDNA to RNase free water dilution ratio. b). Melt curve of calpstatin gene expression. 1:8 neat cDNA to RNase free water dilution ratio.

a **b**

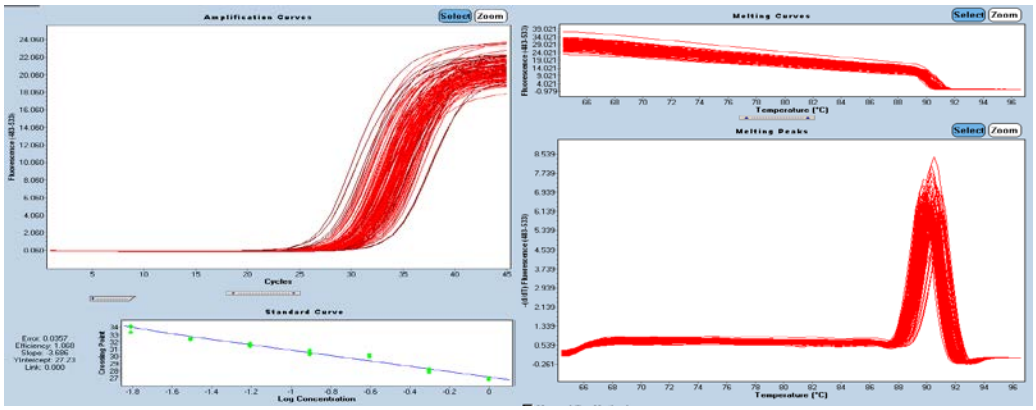

Figure: a). Standard curve of myostatin gene expression. 1:8 neat cDNA to RNase free water dilution ratio. b). Melt curve of myostatin gene expression. 1:8 neat cDNA to RNase free water dilution ratio.

a **b**

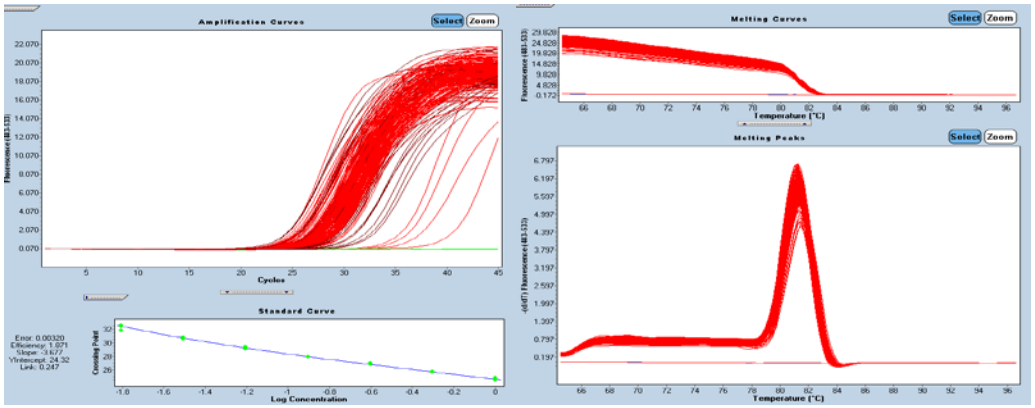

Figure: a). Standard curve of α-enolase gene expression. 1:8 neat cDNA to RNase free water dilution ratio. b). Melt curve of α-enolase gene expression. 1:8 neat cDNA to RNase free water dilution ratio.

a **b**

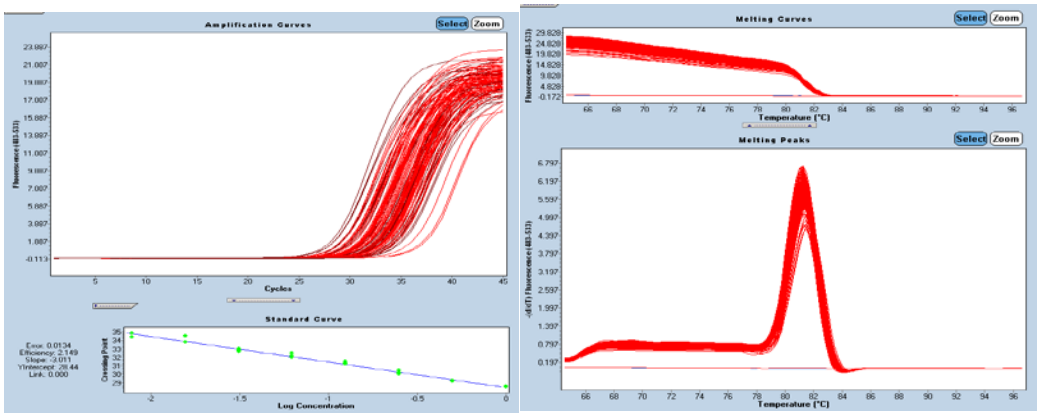

Figure: a). Standard curve of PSAT gene expression. 1:8 neat cDNA to RNase free water dilution ratio. b). Melt curve of PSAT gene expression. 1:8 neat cDNA to RNase free water dilution ratio.

a **b**

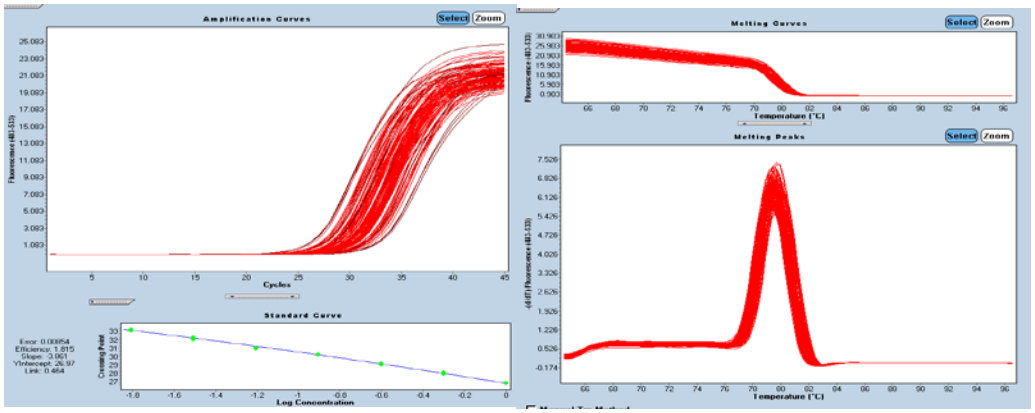

Figure: a). Standard curve of PSPH gene expression. 1:8 neat cDNA to RNase free water dilution ratio. b). Melt curve of PSPH gene expression. 1:8 neat cDNA to RNase free water dilution ratio.

a **b**

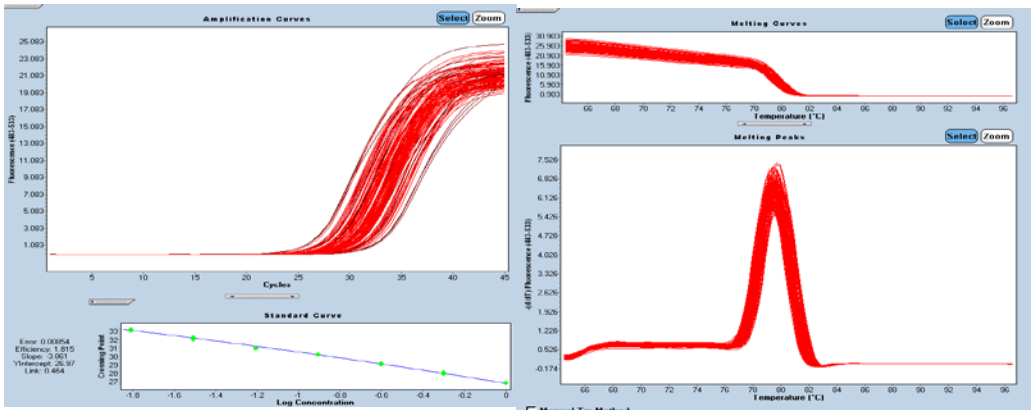

Figure: a). Standard curve of PSPH gene expression. 1:8 neat cDNA to RNase free water dilution ratio. b). Melt curve of PSPH gene expression. 1:8 neat cDNA to RNase free water dilution ratio.

a **b**

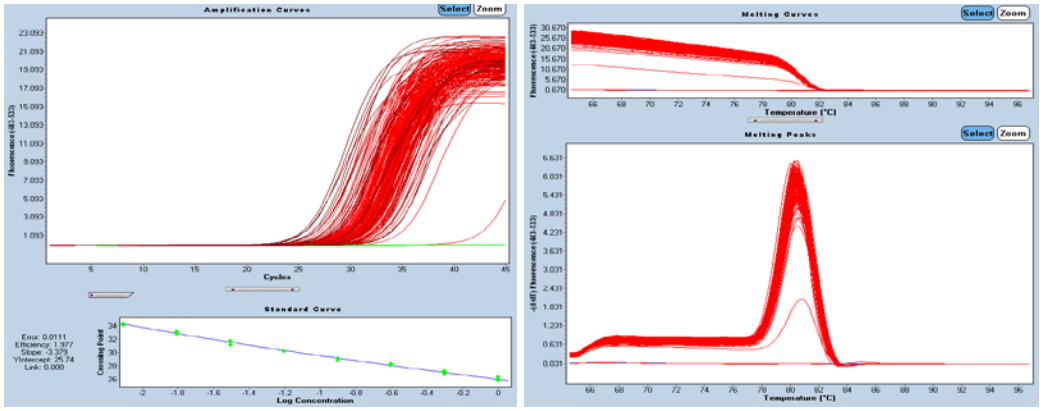

Figure: a). Standard curve of P70S6K RPM, HPM, RPT and HPT (trial 2) gene expression 1:8 neat cDNA to RNase free water dilution ratio. b). Melt curve of P70S6K RPM, HPM, RPT and HPT (trial 2) gene expression 1:8 neat cDNA to RNase free water dilution ratio. b).

a **b**

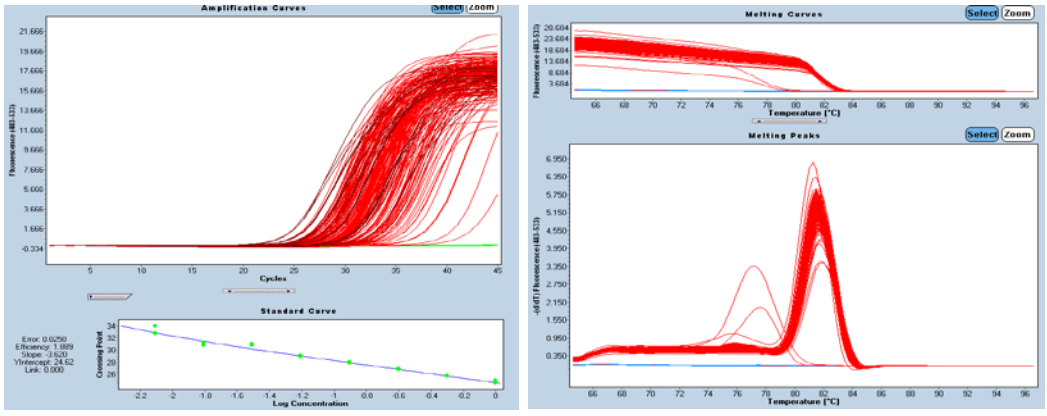

Figure: a). Standard curve of CSRP3 RPM, HPM, RPT and HPT (trial 2) gene expression 1:8 neat cDNA to RNase free water dilution ratio. b). Melt curve of CSRP3 RPM, HPM, RPT and HPT (trial 2) gene expression 1:8 neat cDNA to RNase free water dilution ratio. b).

a **b**

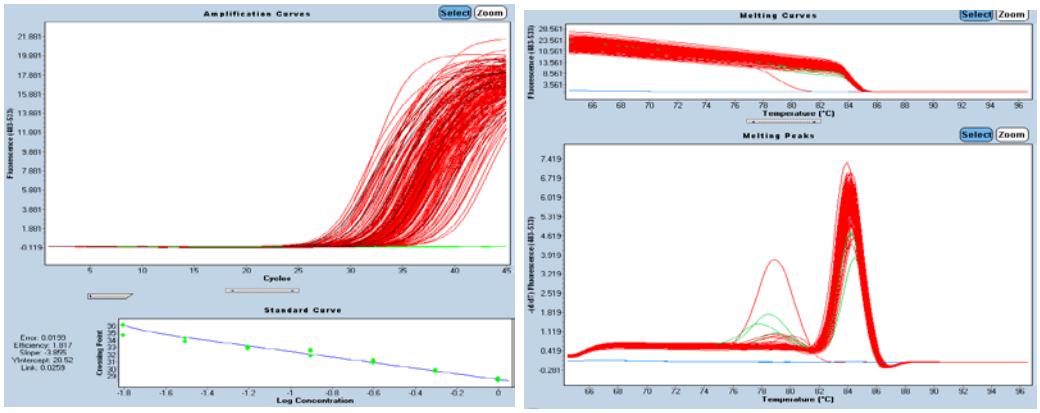

Figure: a). Standard curve of FHL2 RPM, HPM, RPT and HPT (trial 2) gene expression 1:8 neat cDNA to RNase free water dilution ratio. b). Melt curve of FHL2 RPM, HPM, RPT and HPT (trial 2) gene expression 1:8 neat cDNA to RNase free water dilution ratio. b).

APPENDIX 4: Non cyclophilin corrected mRNA expression of measured genes bar charts

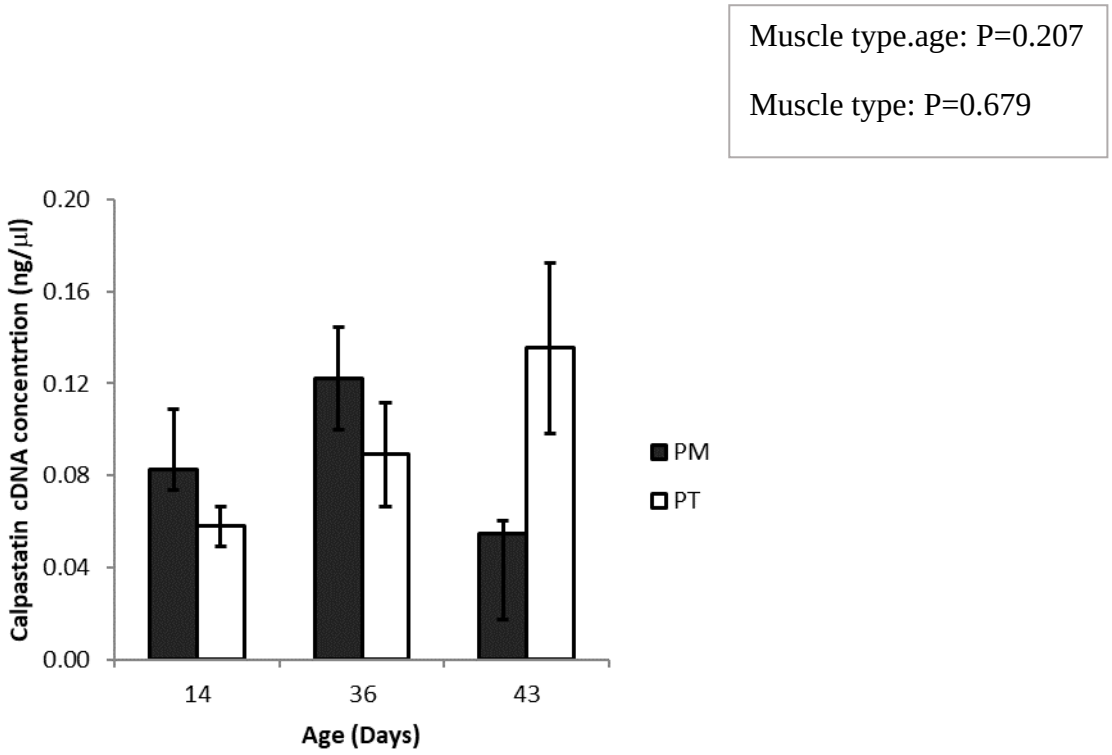

Figure: Calpastatin non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

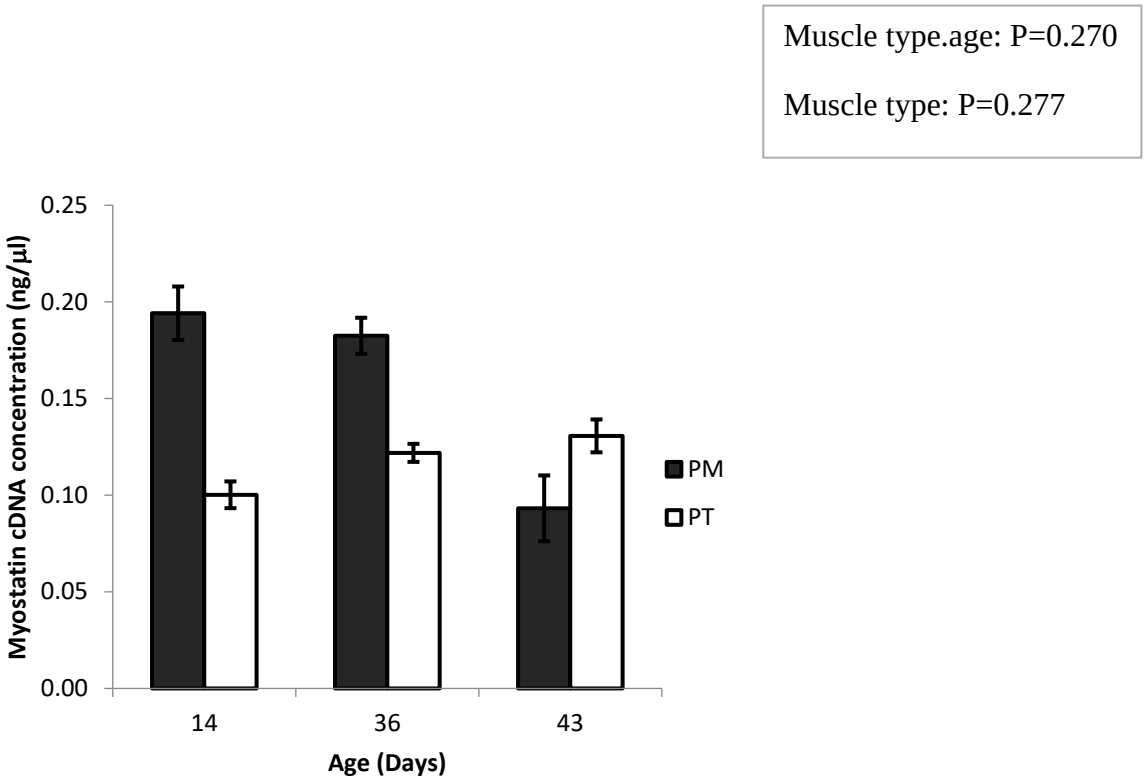

Figure: Myostattin non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

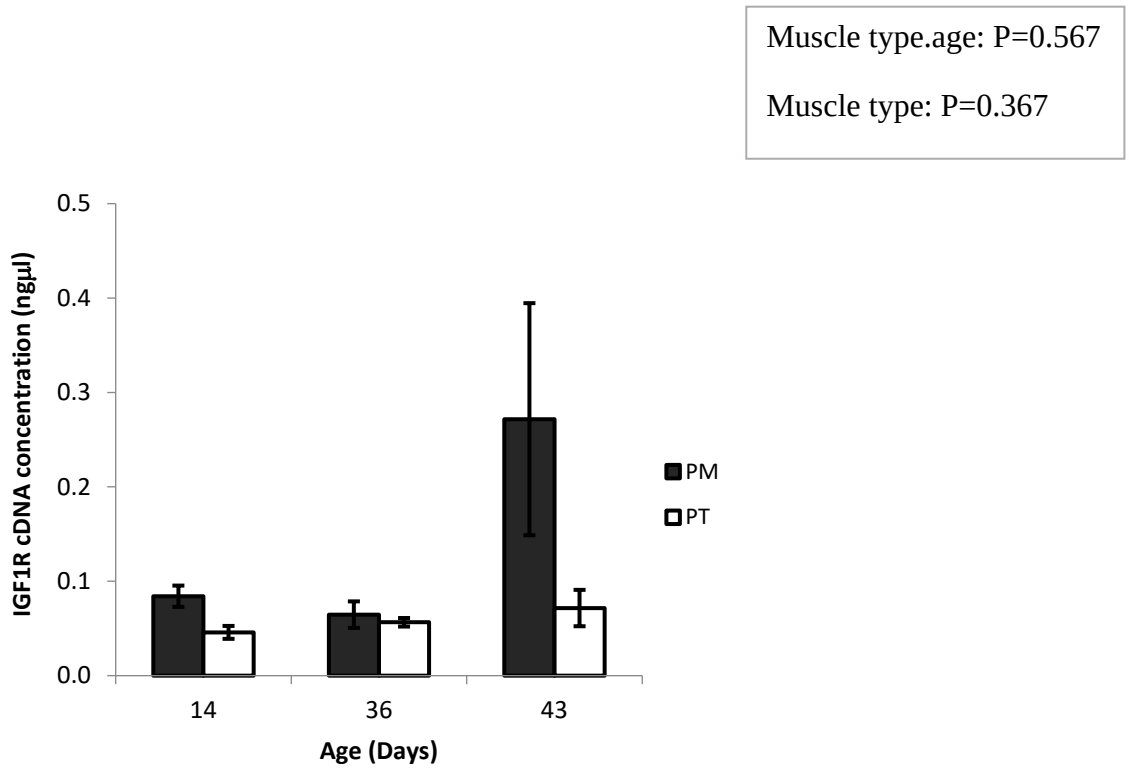

Figure: IGF1R non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

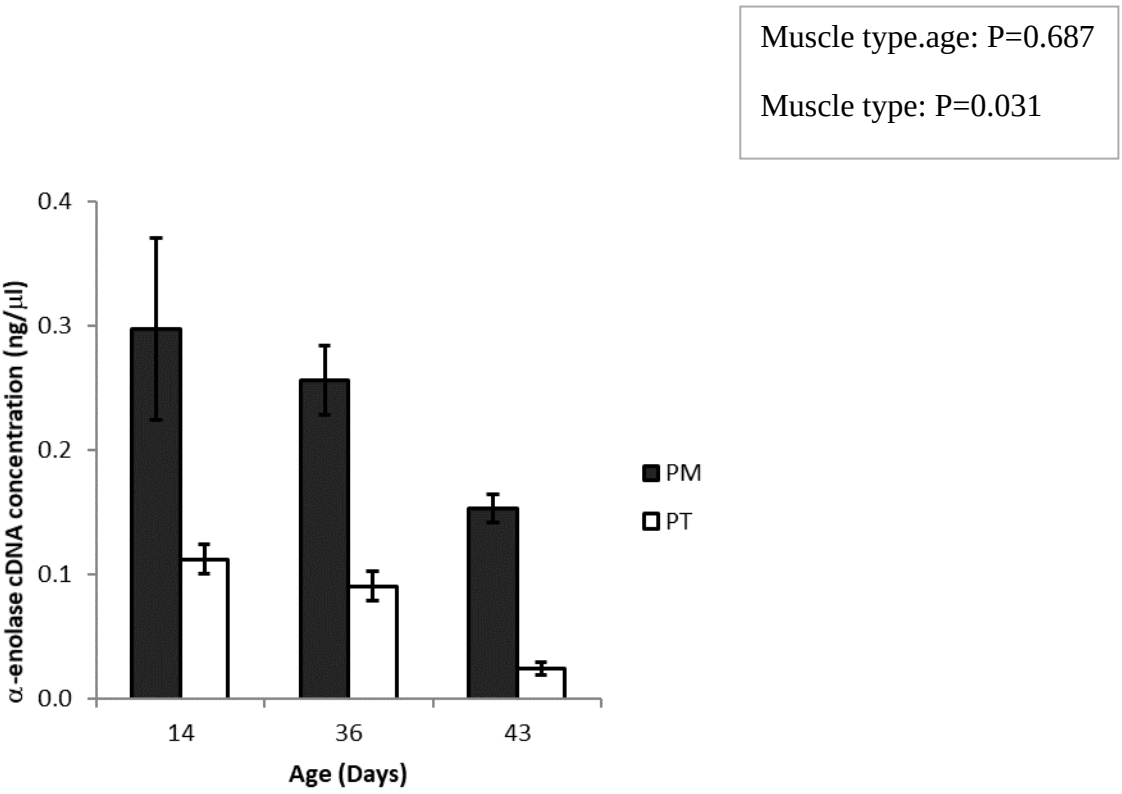

Figure: α-Enolase non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/µl from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

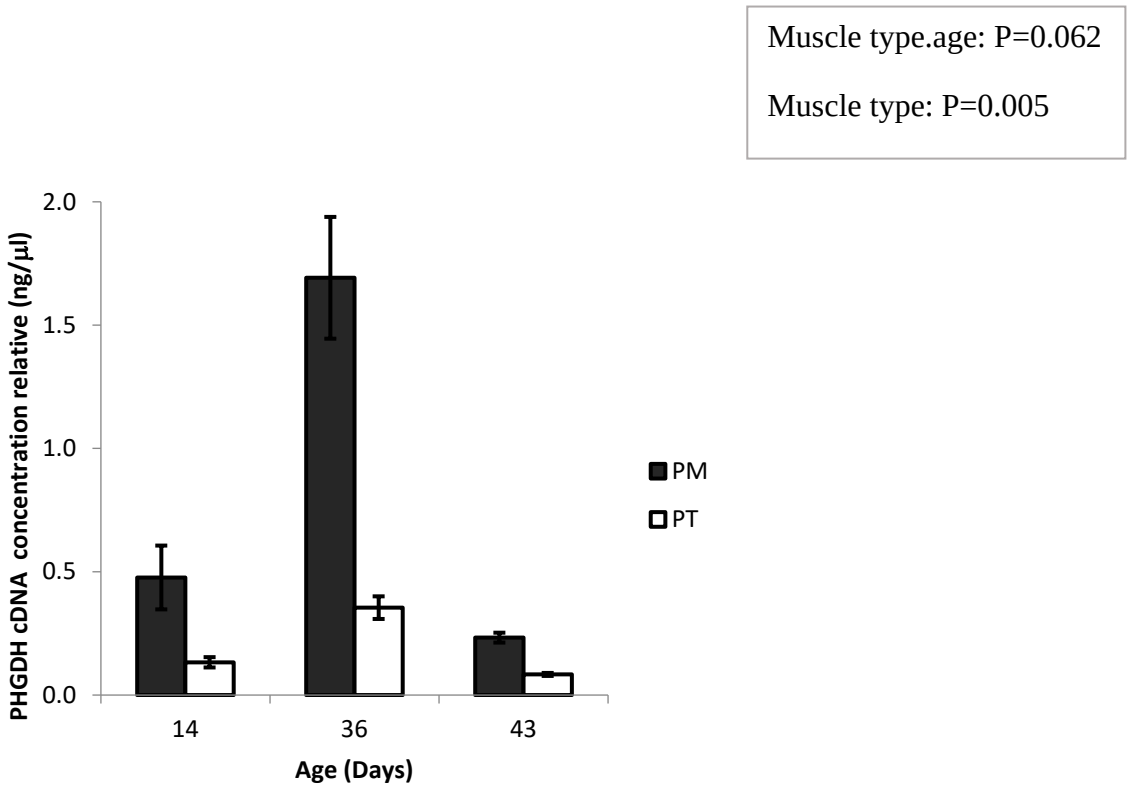

Figure: PHGDH non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

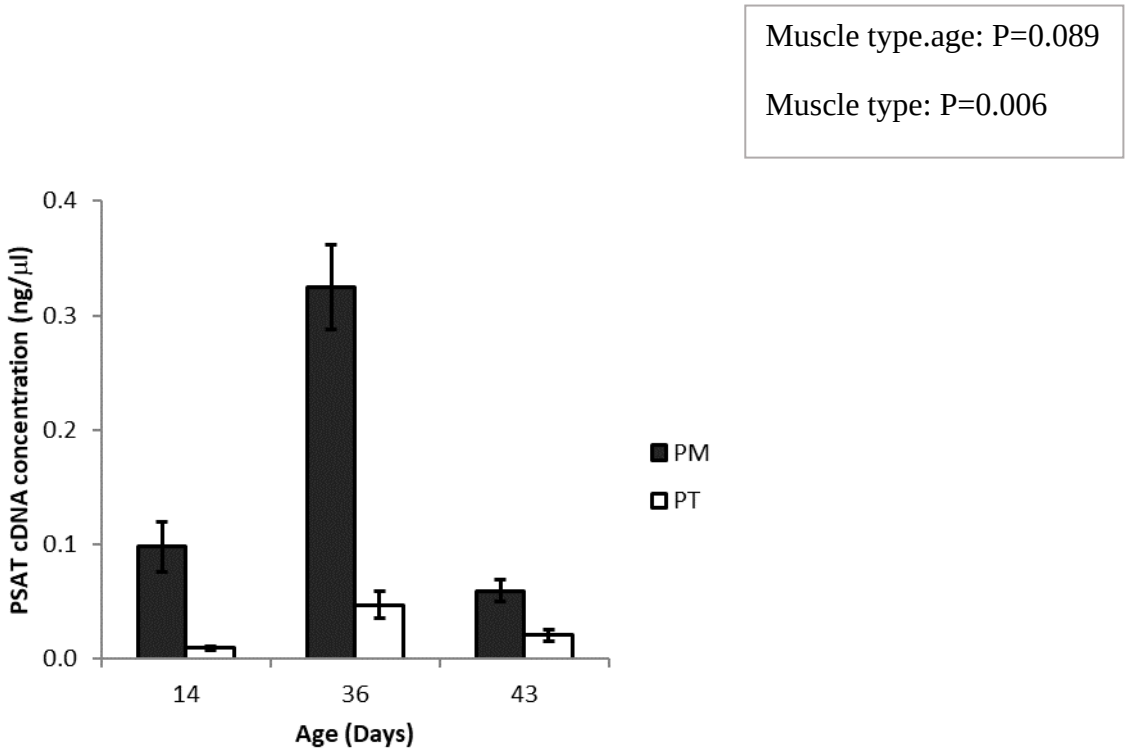

Figure: PSAT non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

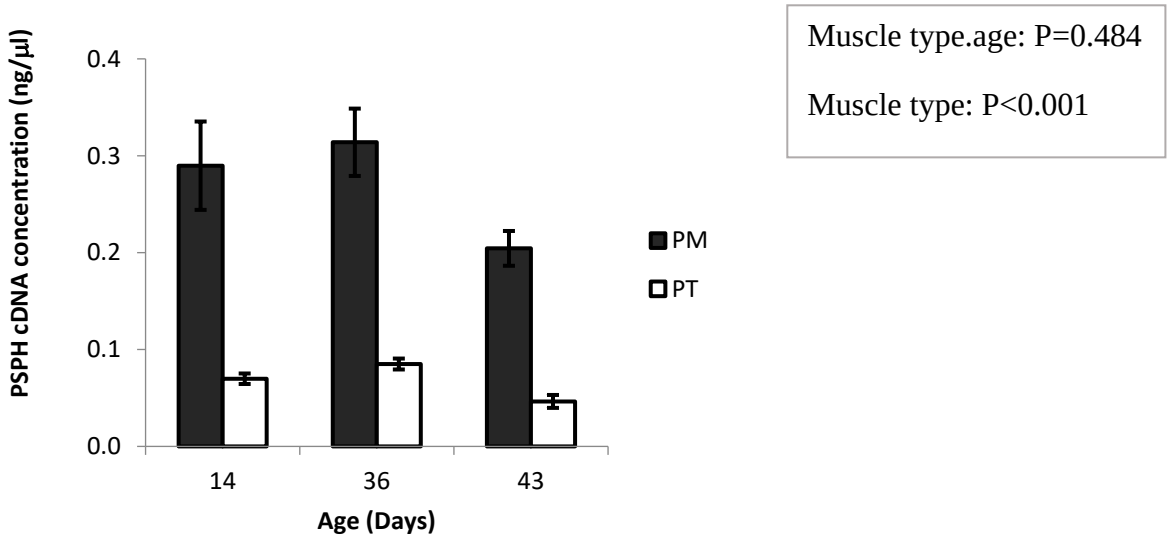

Figure: PSPH non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

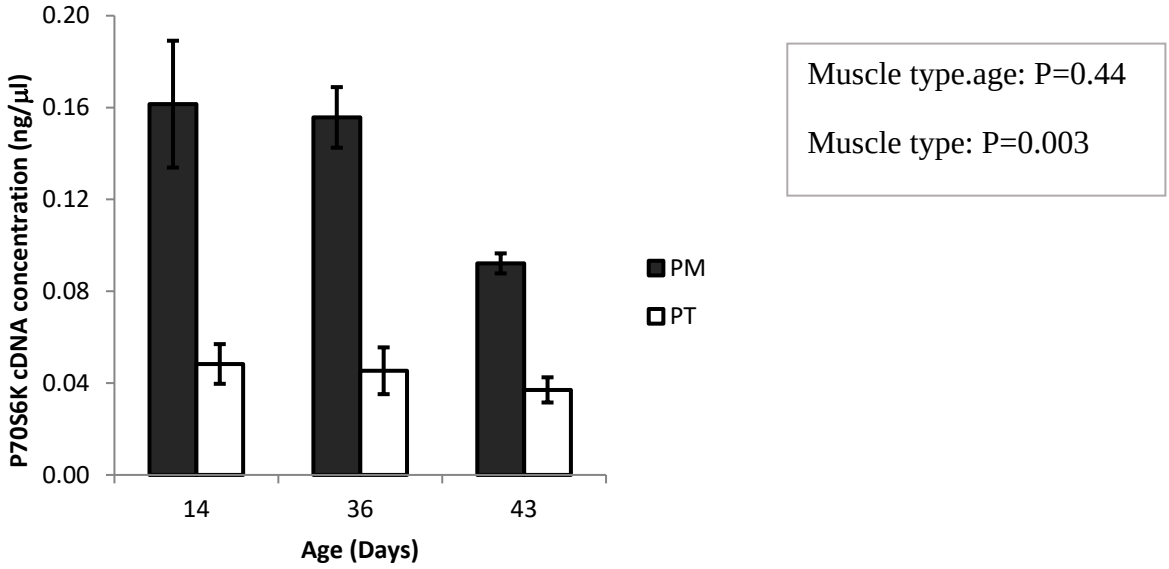

Figure: P70S6K non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

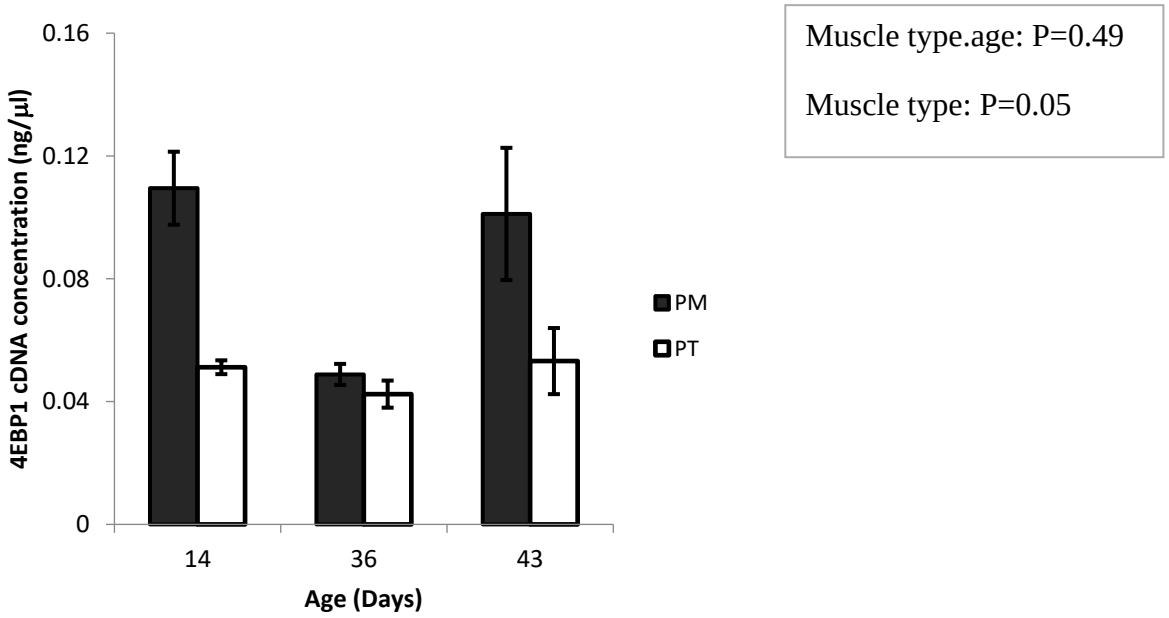

Figure: 4E-BP1 non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

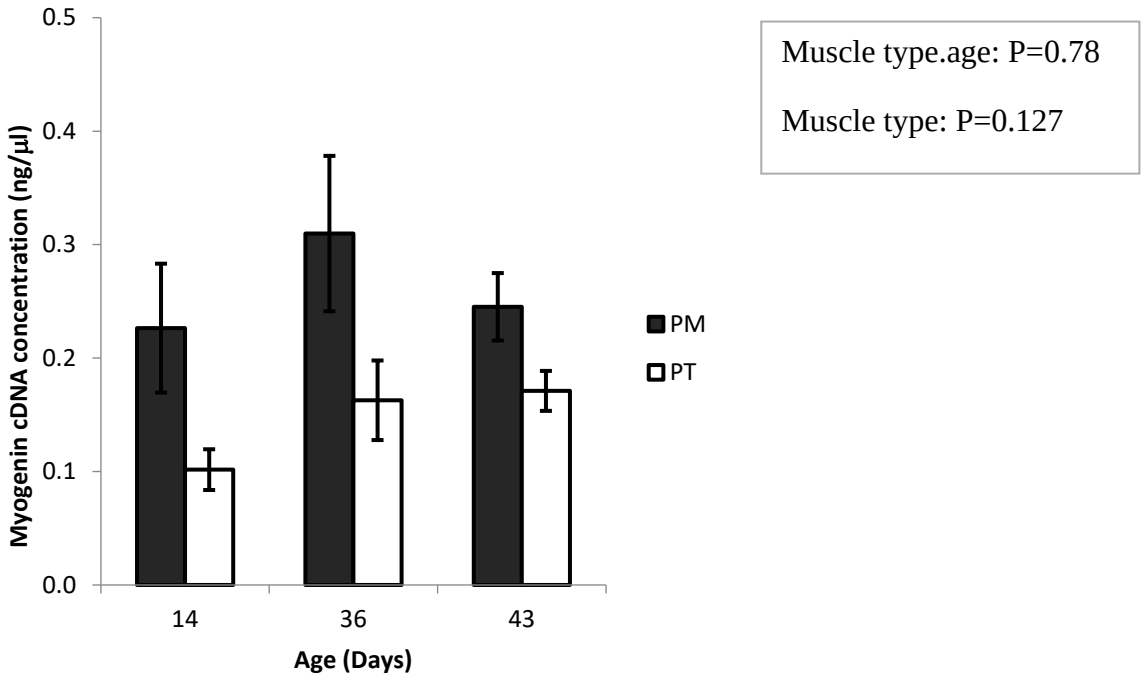

Figure: Myogenin non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

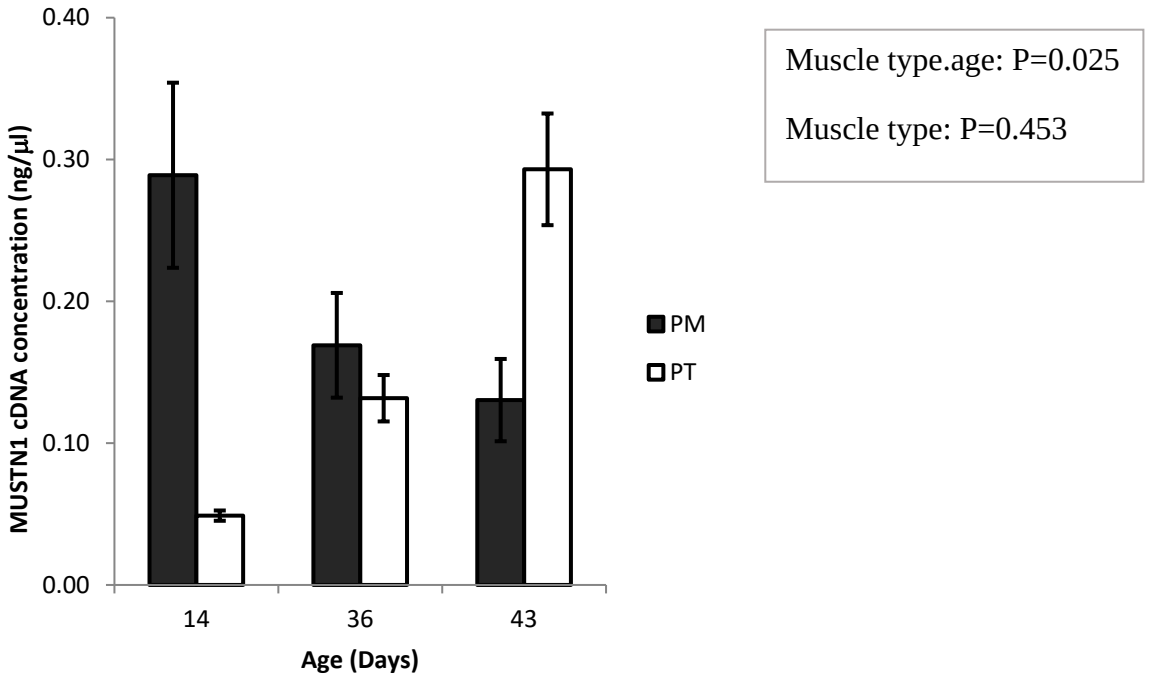

Figure: MUSTN1 non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

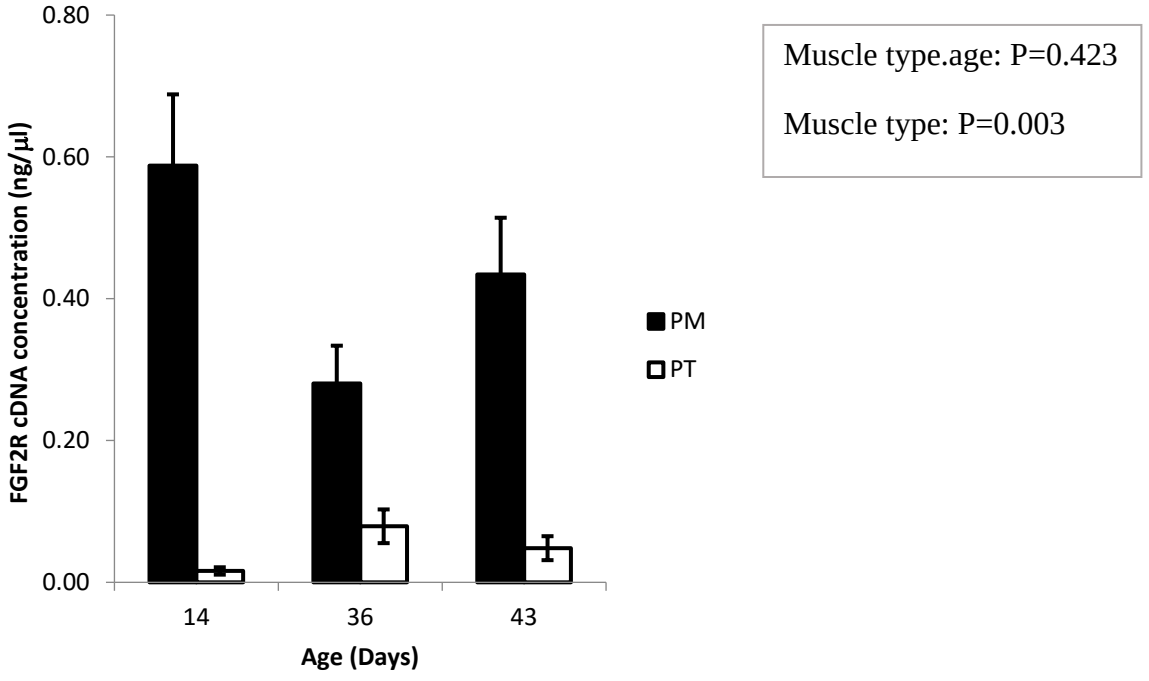

Figure: FGF2R non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

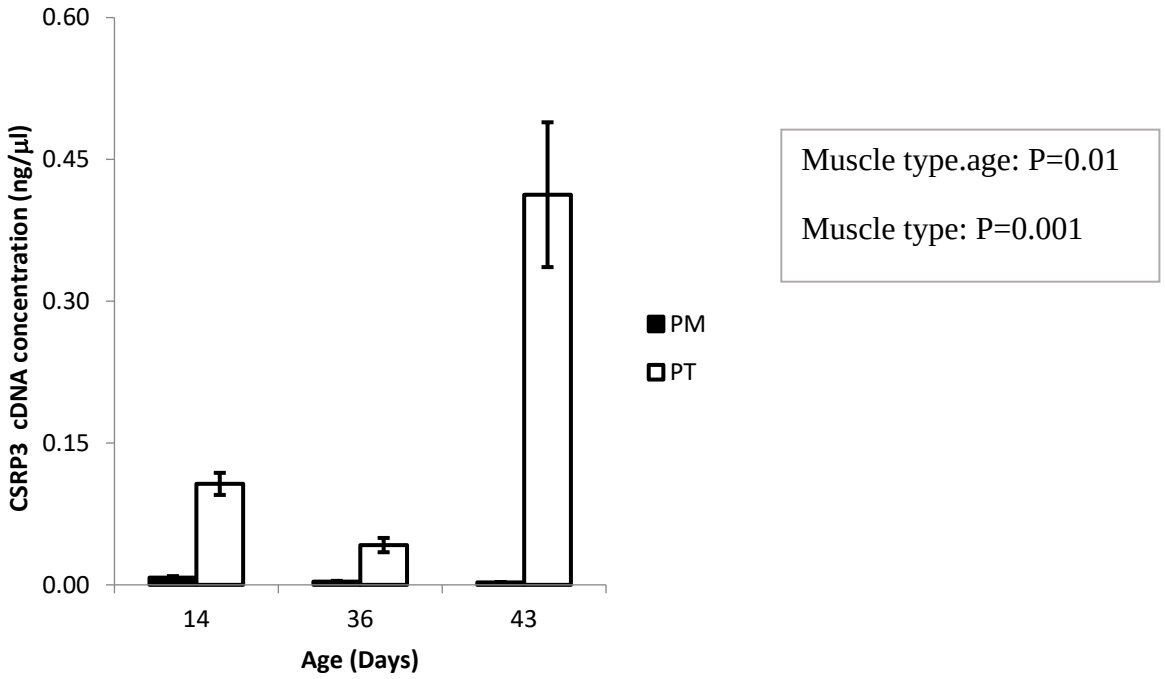

Figure: CSRP3 non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

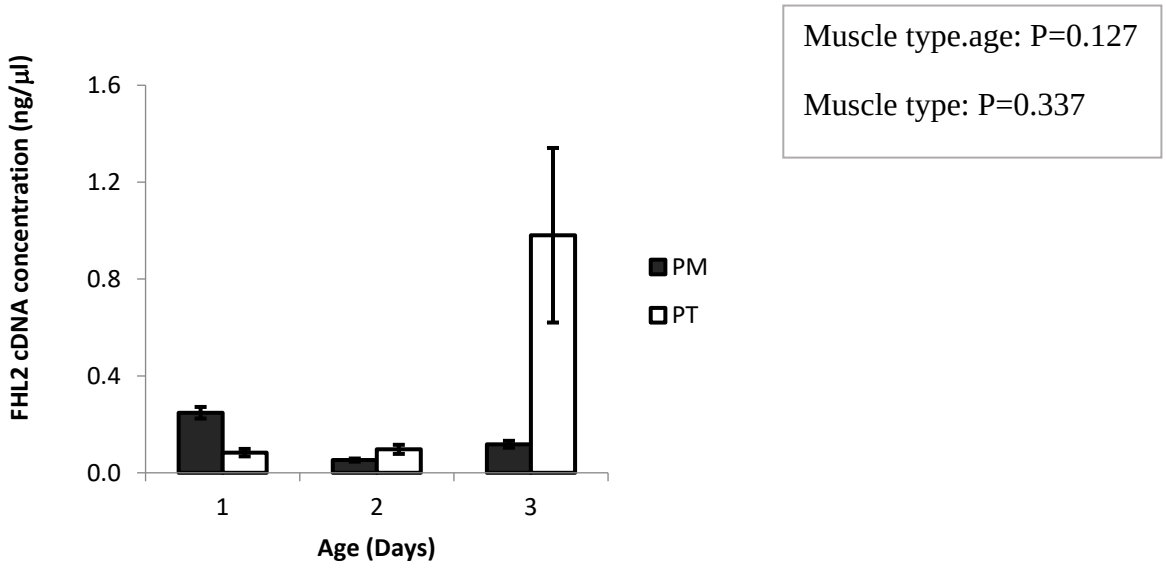

Figure: FHL2 non cyclophilin corrected mRNA expression in the PM and PT muscles of Ross 308 broiler chickens at days 14, 36 and 43 post hatch n=6, quantified concentrations in ng/ml from light cycler crossing point cycle number, expressed as means ± Standard Error of the mean.

APPENDIX 5: PKM 1 gallus gallus nucleotide sequence (Ascension number NM_205469.1)

AGCGGCGGCGGCGCTCGGGCAGGACAGGCTTTGGGCACGGCGGCGGAAGCAGCAGCAGGAGACACCGAACTCCAGTAAC
CATGTCGAAGCACCACGATGCAGGGACCGCTTTCATCCAGACCCAGCAGCTGCACGCTGCCATGGCAGACACCTTTCTG
GAGCACATGTGCCGCCTGGACATCGACTCCGAGCCAACCATTGCCAGAAACACCGGCATCATCTGCACCATCGGCCCAG
CCTCCCGCTCTGTGGACAAGCTGAAGGAAATGATTAAATCTGGAATGAATGTTGCCCGCCTCAACTTCTCGCACGGCAC
CCACGAGTATCATGAGGGCACAATTAAGAACGTGCGAGAGGCCACAGAGAGCTTTGCCTCTGACCCGATCACCTACAGA
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CAGGTGCTGCGGTCGACCTGCCTGCAGTCTCAGAGAAGGACATTCAGGACCTGAAATTTGGCGTGGAGCAGAATGTGGA
CATGGTGTTCGCTTCCTTCATCCGCAAAGCTGCTGATGTCCATGCTGTCAGGAAGGTGCTAGGGGAAAAGGGAAAGCAC
ATCAAGATTATCAGCAAGATTGAGAATCACGAGGGTGTGCGCAGGTTTGATGAGATCATGGAGGCCAGCGATGGCATTA
TGGTGGCCCGTGGTGACCTGGGTATTGAGATCCCTGCTGAAAAAGTCTTCCTCGCACAGAAGATGATGATTGGGCGCTG
CAACAGGGCTGGCAAACCCATCATTTGTGCCACTCAGATGTTGGAAAGCATGATCAAGAAACCTCGCCCGACCCGCGCT
GAGGGCAGTGATGTTGCCAATGCAGTTCTGGATGGAGCAGACTGCATCATGCTGTCTGGGGAGACCGCCAAGGGAGACT
ACCCACTGGAGGCTGTGCGCATGCAGCACGCTATTGCTCGTGAGGCTGAGGCCGCAATGTTCCATCGTCAGCAGTTTGA
AGAAATCTTACGCCACAGTGTACACCACAGGGAGCCTGCTGATGCCATGGCAGCAGGCGCGGTGGAGGCCTCCTTTAAG
TGCTTAGCAGCAGCTCTGATAGTTATGACCGAGTCTGGCAGGTCTGCACACCTGGTGTCCCGGTACCGCCCGCGGGCTC
CCATCATCGCCGTCACCCGCAATGACCAAACAGCACGCCAGGCACACCTGTACCGCGGCGTCTTCCCCGTGCTGTGCAA
GCAGCCGGCCCACGATGCCTGGGCAGAGGATGTGGATCTCCGTGTGAACCTGGGCATGAATGTCGGCAAAGCCCGTGGA
TTCTTCAAGACCGGGGACCTGGTGATCGTGCTGACGGGCTGGCGCCCCGGCTCCGGCTACACCAACACCATGCGGGTGG
TGCCCGTGCCATGAGCTGCCGGCGCCCCCGCCTCTTCCACCCCCGCGCCCCTTCCCCATGCATTAGGCCAGCAGTCGCT
TGCGATGTTCTCCTCGTCCCTAGCGTGGATTTAGGTTGCTCAACACCACCGAGTGCAGCAGCGGGGGAAGAGACCTTAA
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GCTCCCCACGTCCCCCTAGCTTAGCACTGCCCTCAGGGTGTGGCTGTGCCTCTCCTTGCCCCGGCTGTGGGGTGATGGG
GAAGGAGTGGGCGCTTATGGTAACGGTTCTGCTGCTGCACACCGCAGCTCCCCCAGGTTGGTGCCCTCTGTCCATGAGG
GGTTATAGGTTGTCCCCTCTGGGCTTACACTCCAAAGGTGTGCTCCACGGTTCCTACAAGAATCCCAAACCAGGGTGCT
GCTCGTTGTCCCCCTGGGTGCAGGTGGAGTCCTGCGGGTTGTTGGGGTGCAGGATGGGTGGGGGACGCACGCTTCATCT
CCATGCAGCCACCTGCCCTTCTATGGTGTCTGTCTGTGTTGTCGCCTTTCCTGAGAGCGTTCCTTTTGTCTGGGCGCCC
TGTTTGCTCCGGTGGGTGTATTGGGTTGTGGAGCACCACTGTGCACGTCAATAAAGAGCTGAG

APPENDIX 6: PKM 2 gallus gallus nucleotide sequence(Ascension number XM_015278795.1)

CTTGCGCGTCCGCCGGAAAAAAAGAGTCCCGGCCGTTCTCCCCGCTGCGCAGTGGGCGGAGGGATCCGAGGGGCGGTGC
TTCGGCAGGGATCACCTTGGCGGCGCGGCCGAGAGGCGTGCAGAGCGGCGGCGGCGCTCGGGCAGGACAGGCTTTGGGC
ACGGCGGCGGAAGCAGCAGCAGGAGACACCGAACTCCAGTAACCATGTCGAAGCACCACGATGCAGGGACCGCTTTCAT
CCAGACCCAGCAGCTGCACGCTGCCATGGCAGACACCTTTCTGGAGCACATGTGCCGCCTGGACATCGACTCCGAGCCA
ACCATTGCCAGAAACACCGGCATCATCTGCACCATCGGCCCAGCCTCCCGCTCTGTGGACAAGCTGAAGGAAATGATTA
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GCCAGGCACACCTGTACCGCGGCGTCTTCCCCGTGCTGTGCAAGCAGCCGGCCCACGATGCCTGGGCAGAGGATGTGGA
TCTCCGTGTGAACCTGGGCATGAATGTCGGCAAAGCCCGTGGATTCTTCAAGACCGGGGACCTGGTGATCGTGCTGACG
GGCTGGCGCCCCGGCTCCGGCTACACCAACACCATGCGGGTGGTGCCCGTGCCATGAGCTGCCGGCGCCCCCGCCTCTT
CCACCCCCGCGCCCCTTCCCCATGCATTAGGCCAGCAGTCGCTTGCGATGTTCTCCTCGTCCCTAGCGTGGATTTAGGT
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GTGGGGGTGG

APPENDIX 7: multiple sequence alignments of chicken protein kinase muscle 1 (PKM1) vs protein kinase muscle 2 (PKM2) and chicken (*Gallus gallus*) genomic DNA nucleotide sequences. A=Adenine, T=Thymine, G=Guanine, C=Cytosine

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PKM202          ------------------------------------------------------------
PKM201          CTTGCGCGTCCGCCGGAAAAAAAGAGTCCCGGCCGTTCTCCCCGCTGCGCAGTGGGCGGA 60

PKM202          ------------------------------------------------------------
PKM201          GGGATCCGAGGGGCGGTGCTTCGGCAGGGATCACCTTGGCGGCGCGGCCGAGAGGCGTGC 120

PKM202          --AGCGGCGGCGGCGCTCGGGCAGGACAGGCTTTGGGCACGGCGGCGGAAGCAGCAGCAG 58
PKM201          AGAGCGGCGGCGGCGCTCGGGCAGGACAGGCTTTGGGCACGGCGGCGGAAGCAGCAGCAG 180
                  ************************************************************

PKM202          GAGACACCGAACTCCAGTAACCATGTCGAAGCACCACGATGCAGGGACCGCTTTCATCCA 118
PKM201          GAGACACCGAACTCCAGTAACCATGTCGAAGCACCACGATGCAGGGACCGCTTTCATCCA 240
                ************************************************************

PKM202          GACCCAGCAGCTGCACGCTGCCATGGCAGACACCTTTCTGGAGCACATGTGCCGCCTGGA 178
PKM201          GACCCAGCAGCTGCACGCTGCCATGGCAGACACCTTTCTGGAGCACATGTGCCGCCTGGA 300
                ************************************************************

PKM202          CATCGACTCCGAGCCAACCATTGCCAGAAACACCGGCATCATCTGCACCATCGGCCCAGC 238
PKM201          CATCGACTCCGAGCCAACCATTGCCAGAAACACCGGCATCATCTGCACCATCGGCCCAGC 360
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PKM202          CTCCCGCTCTGTGGACAAGCTGAAGGAAATGATTAAATCTGGAATGAATGTTGCCCGCCT 298
PKM201          CTCCCGCTCTGTGGACAAGCTGAAGGAAATGATTAAATCTGGAATGAATGTTGCCCGCCT 420
                ************************************************************
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PKM202          CAACTTCTCGCACGGCACCCACGAGTATCATGAGGGCACAATTAAGAACGTGCGAGAGGC 358
PKM201          CAACTTCTCGCACGGCACCCACGAGTATCATGAGGGCACAATTAAGAACGTGCGAGAGGC 480
                ************************************************************

PKM202          CACAGAGAGCTTTGCCTCTGACCCGATCACCTACAGACCTGTGGCTATTGCACTGGACAC 418
PKM201          CACAGAGAGCTTTGCCTCTGACCCGATCACCTACAGACCTGTGGCTATTGCACTGGACAC 540
                ************************************************************

PKM202          CAAGGGACCTGAAATCCGAACTGGACTCATCAAGGGAAGTGGCACAGCAGAGGTGGAGCT 478
PKM201          CAAGGGACCTGAAATCCGAACTGGACTCATCAAGGGAAGTGGCACAGCAGAGGTGGAGCT 600
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PKM202          CAAGAAGGGCGCAGCTCTCAAAGTGACGCTGGACAATGCCTTCATGGAGAACTGCGATGA 538
PKM201          CAAGAAGGGCGCAGCTCTCAAAGTGACGCTGGACAATGCCTTCATGGAGAACTGCGATGA 660
                ************************************************************

PKM202          GAATGTGCTGTGGGTGGACTACAAGAACCTCATCAAAGTTATAGATGTGGGCAGCAAAAT 598
PKM201          GAATGTGCTGTGGGTGGACTACAAGAACCTCATCAAAGTTATAGATGTGGGCAGCAAAAT 720
                ************************************************************

PKM202          CTATGTGGATGACGGTCTCATTTCCTTGCTGGTTAAGGAGAAAGGCAAGGACTTTGTCAT 658
PKM201          CTATGTGGATGACGGTCTCATTTCCTTGCTGGTTAAGGAGAAAGGCAAGGACTTTGTCAT 780
                ************************************************************

PKM202          GACTGAGGTTGAGAACGGTGGCATGCTTGGTAGTAAGAAGGGAGTGAACCTCCCAGGTGC 718
PKM201          GACTGAGGTTGAGAACGGTGGCATGCTTGGTAGTAAGAAGGGAGTGAACCTCCCAGGTGC 840
                ************************************************************

PKM202          TGCGGTCGACCTGCCTGCAGTCTCAGAGAAGGACATTCAGGACCTGAAATTTGGCGTGGA 778
PKM201          TGCGGTCGACCTGCCTGCAGTCTCAGAGAAGGACATTCAGGACCTGAAATTTGGCGTGGA 900
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PKM202          GCAGAATGTGGACATGGTGTTCGCTTCCTTCATCCGCAAAGCTGCTGATGTCCATGCTGT 838
PKM201          GCAGAATGTGGACATGGTGTTCGCTTCCTTCATCCGCAAAGCTGCTGATGTCCATGCTGT 960
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                ************************************************************

PKM202          CAGGAAGGTGCTAGGGGAAAAGGGAAAGCACATCAAGATTATCAGCAAGATTGAGAATCA 898
PKM201          CAGGAAGGTGCTAGGGGAAAAGGGAAAGCACATCAAGATTATCAGCAAGATTGAGAATCA 1020
                ************************************************************

PKM202          CGAGGGTGTGCGCAGGTTTGATGAGATCATGGAGGCCAGCGATGGCATTATGGTGGCCCG 958
PKM201          CGAGGGTGTGCGCAGGTTTGATGAGATCATGGAGGCCAGCGATGGCATTATGGTGGCCCG 1080
                ************************************************************

PKM202          TGGTGACCTGGGTATTGAGATCCCTGCTGAAAAAGTCTTCCTCGCACAGAAGATGATGAT 1018
PKM201          TGGTGACCTGGGTATTGAGATCCCTGCTGAAAAAGTCTTCCTCGCACAGAAGATGATGAT 1140
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PKM202          TGGGCGCTGCAACAGGGCTGGCAAACCCATCATTTGTGCCACTCAGATGTTGGAAAGCAT 1078
PKM201          TGGGCGCTGCAACAGGGCTGGCAAACCCATCATTTGTGCCACTCAGATGTTGGAAAGCAT 1200
                ************************************************************

PKM202          GATCAAGAAACCTCGCCCGACCCGCGCTGAGGGCAGTGATGTTGCCAATGCAGTTCTGGA 1138
PKM201          GATCAAGAAACCTCGCCCGACCCGCGCTGAGGGCAGTGATGTTGCCAATGCAGTTCTGGA 1260
                ************************************************************

PKM202          TGGAGCAGACTGCATCATGCTGTCTGGGGAGACCGCCAAGGGAGACTACCCACTGGAGGC 1198
PKM201          TGGAGCAGACTGCATCATGCTGTCTGGGGAGACCGCCAAGGGAGACTACCCACTGGAGGC 1320
                ************************************************************

PKM202          TGTGCGCATGCAGCACGCTATTGCTCGTGAGGCTGAGGCCGCAATGTTCCATCGTCAGCA 1258
PKM201          TGTGCGCATGCAGCACGCTATTGCCCGTGAGGCGGAGGCCGCCATCTTTCACAGGCAGCT 1380
                ********************** ******** ********.** ** ** .* ****:

PKM202          GTTTGAAGAAATCTTACGCCACAGTGTACACCACAGGGAGCCTGCTGATGCCATGGCAGC 1318
PKM201          GTTTGAGGAGCTGCGTCGCCTGACCTCCCTGAACTGTGATCCCACCGAGGCCGCTGCTGT 1440
                ******.**..*   :****: *    .*: .**:* ** ** .* ** ***.  **:*
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PKM202          AGGCGCGGTGGAGGCCTCCTTTAAGTGCTTAGCAGCAGCTCTGATAGTTATGACCGAGTC 1378
PKM201          TGGCGCTGTGGAAGCGTCCTTCAAGTGCTGCAGCGGGGCCATTATTGTCCTCACCAAGTC 1500
                :***** *****.** ***** ******* .. .* .** .* **:** .* ***.****

PKM202          TGGCAGGTCTGCACACCTGGTGTCCCGGTACCGCCCGCGGGCTCCCATCATCGCCGTCAC 1438
PKM201          TGGAAGGTCTGCACACCTGGTGTCCCGGTACCGCCCGCGGGCTCCCATCATCGCCGTCAC 1560
                ***.************************************************************

PKM202          CCGCAATGACCAAACAGCACGCCAGGCACACCTGTACCGCGGCGTCTTCCCCGTGCTGTG 1498
PKM201          CCGCAATGACCAAACAGCACGCCAGGCACACCTGTACCGCGGCGTCTTCCCCGTGCTGTG 1620
                ************************************************************

PKM202          CAAGCAGCCGGCCCACGATGCCTGGGCAGAGGATGTGGATCTCCGTGTGAACCTGGGCAT 1558
PKM201          CAAGCAGCCGGCCCACGATGCCTGGGCAGAGGATGTGGATCTCCGTGTGAACCTGGGCAT 1680
                ************************************************************

PKM202          GAATGTCGGCAAAGCCCGTGGATTCTTCAAGACCGGGGACCTGGTGATCGTGCTGACGGG 1618
PKM201          GAATGTCGGCAAAGCCCGTGGATTCTTCAAGACCGGGGACCTGGTGATCGTGCTGACGGG 1740
                ************************************************************

PKM202          CTGGCGCCCCGGCTCCGGCTACACCAACACCATGCGGGTGGTGCCCGTGCCATGAGCTGC 1678
PKM201          CTGGCGCCCCGGCTCCGGCTACACCAACACCATGCGGGTGGTGCCCGTGCCATGAGCTGC 1800
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PKM202          CGGCGCCCCCGCCTCTTCCACCCCCGCGCCCCTTCCCCATGCATTAGGCCAGCAGTCGCT 1738
PKM201          CGGCGCCCCCGCCTCTTCCACCCCCGCGCCCCTTCCCCATGCATTAGGCCAGCAGTCGCT 1860
                ************************************************************

PKM202          TGCGATGTTCTCCTCGTCCCTAGCGTGGATTTAGGTTGCTCAACACCACCGAGTGCAGCA 1798
PKM201          TGCGATGTTCTCCTCGTCCCTAGCGTGGATTTAGGTTGCTCAACACCACCGAGTGCAGCA 1920
                ************************************************************

PKM202          GCGGGGGAAGAGACCTTAAATCACTCAGAATACTTGAAGTGTGTAGTTGTTTCCAGACCT 1858
PKM201          GCGGGGGAAGAGACCTTAAATCACTCAGAATACTTGAAGTGTGTAGTTGTTTCCAGACCT 1980
```

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                ************************************************************

PKM202          CTCTCCCTGTAGTTAGCCATGTCAGAGCGGGGCCCTGTGCTCCCCACGTCCCCCTAGCTT 1918

PKM201          CTCTCCCTGTAGTTAGCCATGTCAGAGCGGGGCCCTGTGCTCCCCACGTCCCCCTAGCTT 2040

                ************************************************************

PKM202          AGCACTGCCCTCAGGGTGTGGCTGTGCCTCTCCTTGCCCCGGCTGTGGGGTGATGGGGAA 1978

PKM201          AGCACTGCCCTCAGGGTGTGGCTGTGCCTCTCCTTGCCCCGGCTGTGGGGTGATGGGGAA 2100

                ************************************************************

PKM202          GGAGTGGGCGCTTATGGTAACGGTTCTGCTGCTGCACACCGCAGCTCCCCCAGGTTGGTG 2038

PKM201          GGAGTGGGCGCTTATGGTAACGGTTCTGCTGCTGCACACCGCAGCTCCCCCAGGTTGGTG 2160

                ************************************************************

PKM202          CCCTCTGTCCATGAGGGGTTATAGGTTGTCCCCTCTGGGCTTACACTCCAAAGGTGTGCT 2098

PKM201          CCCTCTGTCCATGAGGGGTTATAGGTTGTCCCCTCTGGGCTTACACTCCAAAGGTGTGCT 2220

                ************************************************************

PKM202          CCACGGTTCCTACAAGAATCCCAAACCAGGGTGCTGCTCGTTGTCCCCCTGGGTGCAGGT 2158

PKM201          CCACGGTTCCTACAAGAATCCCAAACCAGGGTGCTGCTCGTTGTCCCCCTGGGTGCAGGT 2280

                ************************************************************

PKM202          GGAGTCCTGCGGGTTGTTGGGGTGCAGGATGGGTGGGGGACGCACGCTTCATCTCCATGC 2218

PKM201          GGAGTCCTGCGGGTTGTTGGGGTGCAGGATGGGTGGGGGACGCACGCTTCATCTCCATGC 2340

                ************************************************************

PKM202          AGCCACCTGCCCTTCTATGGTGTCTGTCTGTGTTGTCGCCTTTCCTGAGAGCGTTCCTTT 2278

PKM201          AGCCACCTGCCCTTCTATGGTGTCTGTCTGTGTTGTCGCCTTTCCTGAGAGCGTTCCTTT 2400

                ************************************************************

PKM202          TGTCTGGGCGCCCTGTTTGCTCCGGTGGGTGTATTGGGTTGTGGAGCACCACTGTGCACG 2338

PKM201          TGTCTGGGCGCCCTGTTTGCTCCGGTGGGTGTATTGGGTTGTGGAGCACCACTGTGCACG 2460

                ************************************************************
```

```
PKM202          TCAATAAAGAGCTGAG---------------------------------------- 2354
PKM201          TCAATAAAGAGCTGAGCACCCCTGCCCGCGGCTTCGTGTGCTGATGCTGCGCTACGGCGG 2520
                ****************


PKM202          ------------------
PKM201          GATCACGCGTGGGGGTGG 2538
```

APPENDIX 8: AKT multiple sequence amino acid alignments

```
CLUSTAL 2.1 Multiple Sequence Alignments


Sequence type explicitly set to Protein

Sequence format is Pearson

Sequence 1: chicken      480 aa

Sequence 5: MOUSE        480 aa

Start of Pairwise alignments

Aligning...



Sequences (1:5) Aligned. Score: 95.8333


CLUSTAL-Alignment file created  [clustalw.aln]
```

---

clustalw.aln

```
CLUSTAL 2.1 multiple sequence alignment


MOUSE           MNDVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYKERPQDVDQRESPLNNFSVAQC

chicken         MNEVAIVKEGWLHKRGEYIKTWRPRYFLLKNDGTFIGYKERPQDVDQRESPLNNFSVAQC

                **:**:************************************::**************
```

```
MOUSE           QLMKTERPRPNTFIIRCLQWTTVIERTFHVETPEEREEWATAIQTVADGLKRQEEETMDF
chicken         QLMKTERPKPNTFIIRCLQWTTVIERTFHVETPEEREEWTKAIQTVADSLKKQEEEMMDF
                :*******:*****************************:.*******.**:***  ***

MOUSE           RSGSPSDNSGAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKI
chicken         RSGSPSDNSGAEEMEVSMTKPKHKVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKI
                *****.:**********:::***:************************************

MOUSE           LKKEVIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLS
chicken         LKKEVIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLS
                ************************************************************

MOUSE           RERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEGI
chicken         RERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEGI
                *****.******************************************************

MOUSE           KDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFEL
chicken         KDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFEL
                ****************************************:*****************

MOUSE           ILMEEIRFPRTLGPEAKSLLSGLLKKDPTQRLGGGSEDAKEIMQHRFFANIVWQDVYEKK
chicken         ILMEEIRFPRTLSPEAKSLLSGLLKKDPKQRLGGGPDDAKEIMQHKFFAGIVWQDVYGKK
                ************.***************.******.:********:**:.******* **

MOUSE           LSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDSERRPHFPQFSYSASGTA
chicken         LVPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMDCVDNERRPHFPQFSYSASGTA
                * ********************************** *:  *.*****************
```

Figure: Attempted AKT protein expression in C2C12 mouse muscle cell lines and chicken muscle protein extracts. There was no detection of bands indicating that primary antibodies were incompatible with extracted proteins

APPENDIX 9: MAFbx multiple sequence amino acid alignments

```
CLUSTAL 2.1 Multiple Sequence Alignments


Sequence type explicitly set to Protein

Sequence format is Pearson

Sequence 1: CHICKEN      355 aa

Sequence 2: MOUSE        355 aa

Start of Pairwise alignments

Aligning...



Sequences (1:2) Aligned. Score: 90.1408


CLUSTAL-Alignment file created  [clustalw.aln]
```

---

clustalw.aln

```
CLUSTAL 2.1 multiple sequence alignment


MOUSE           MPFLGQDWRSPGQSWVKTADGWKRFLDEKSGSFVSDLSSYCNKEVYSKENLFSSLNYDVA
CHICKEN         MPFLGQDWRSPGQSWVKTADGWTRFLDEKSGGFVGDLSSFCKKEDHNKENLFNSLNYDVA
                **********************.******** **.**.*:*.     *****.*******


MOUSE           AKKRKKDIQNSKTKTQYFHQEKWIYVHKGSTKERHGYCTLGEAFNRLDFSTAILDSRRFN
```

```
CHICKEN         AKKRKKDLLNNKAKIQYFHQEKWIYVHKGSTKERHGYCTLGEAFNRLDFSSAILDSRRFN
                *******: *.*:* ***********************************:*********

MOUSE           YVVRLLELIAKSQLTSLSGIAQKNFMNILEKVVLKVLEDQQNIRLIRELLQTLYTSLCTL

CHICKEN         YVVRLLELIAKSQLTSLSGIAQKNYMNILEKVVQKVLEDQQNIRLIRELLQTLYTSLCTL
                ************************:******** ************************

MOUSE           VQRVGKSVLVGNINMWVYRMETILHWQQQLNSIQISRPAFKGLTITDLPVCLQLNIMQRL

CHICKEN         VQRVGKSVLVGNINMWVHRMETILHWQQQLNNIQITRPAFKGTTFTDLPLCLQLNIMQRL
                *****************:**** ********.***:***::* *:****:**********

MOUSE           SDGRDLVSLGQAAPDLHVLSEDRLLWKRLCQYHFSERQIRKRLILSDKGQLDWKKMYFKL

CHICKEN         SDGRDLVSLGQVAPDLQVLSEDRLLWKKLCQYHFTDRQIRKRLILSDKGQLDWKKMYFKL
                **********.****:**********:******::*

MOUSE           VRCYPRREQYGVTLQLCKHCHILSWKGTDHPCTANNPESCSVSLSPQDFINLFKF

CHICKEN         IRCYPRKEQYGDTLQLCRHCHILSWKGTDHPCTANNPETCSTSLSPQDFINLFRF
                                  :************:** ***********:*
```

Figure: Attempted MAFbx/Atrogen-1 protein expression in C2C12 mouse muscle cell lines and chicken muscle protein extracts. There was no detection of bands indicating that primary antibodies were incompatible with extracted proteins

APPENDIX 10: P70S6K multiple sequence amino acid alignments

```
CLUSTAL 2.1 Multiple Sequence Alignments


Sequence type explicitly set to Protein

Sequence format is Pearson

Sequence 1: CHICKEN      502 aa

Sequence 5: MOUSE        472 aa

Start of Pairwise alignments

Aligning...




MOUSE           ------------------------------------------------------MDHGGVG

CHICKEN         ----------------------MAGVFDIDLDQPEDAGSDEELEEGGQLSESMDHGGVG

                                                                     *******


MOUSE           PYELGMEHCEKFEISETSVNRGPEKIRPECFELLRVLGKGGYGKVFQVRKVTGANTGKIF

CHICKEN         QYDLGMEHCEKFEISETSVNRGPEKIRPECFELLRVLGKGGYGKVFQVRKVTGANTGKIF

                 *:**************************************************


MOUSE           AMKVLKKAMIVRNAKDTAHTKAERNILEEVKHPFIVDLIYAFQTGGKLYLILEYLSGGEL

CHICKEN         AMKVLKKAMIVRSAKDTAHTKAERNIVEEVKHPFIVDLIYAFQTGGKLYLILEYLSGGEL

                ************.************:********************************


MOUSE           FMQLEREGIFMEDTACFYLAEISMALGHLHQKGIIYRDLKPENIMLNHQGHVKLTDFGLC

CHICKEN         FMQLEREGIFMEDTACFYLAEISMALGHLHQKGIIYRDLKPENIMLNHQGHVKLTDFGLC

                ************************************************************
```

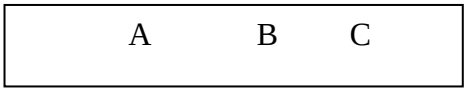
```
MOUSE           KESIHDGTVTHTFCGTIEYMAPEILMRSGHNRAVDWWSLGALMYDMLTGAPPFTGENRKK
CHICKEN         KESIHDGTVTHTFCGTIEYMAPEILMRSGHNRAVDWWSLGALMYDMLTGAPPFTGENRKK
                ************************************************************

MOUSE           TIDKILKCKLNLPPYLTQEARDLLKKLLKRNAASRLGAGPGDAGEVQAHPFFRHINWEEL
CHICKEN         TIDKILKCKLNLPPYLTQEARDLLKKLLKRNAASRLGAGPGDAGEVQAHPFFRHINWDEL
                ********************************************.*********:**

MOUSE           LARKVEPPFKPLLQSEEDVSQFDSKFTRQTPVDSPDDSTLSESANQVFLGFTYVAPSVLE
CHICKEN         LARKVEPPFKPLLQSEEDVSQFDSKFTRQTPVDSPDDSTLSESANQVFLGFTYVAPSVLE
                ****** ******************************:*********************

MOUSE           SVKEKFSFEPKIRSPRRFIGSPRTPVSPVKFSPGDFWGRGASASTANPQTPVEYPMETSG
CHICKEN         SVKEKFSFEPKIRSPRRFIGSPRTPVSPVKFSPGEFWGRGASASASNTQTPVEYPMETSG
                *******************************.*:*********::*.************

MOUSE           IEQMDVTVSGEASAPLPIRQPNSGPYKKQAFPMISKRPEHLRMNL
CHICKEN         IEQMDVTVCGEASAPLPIRQPNSGPYKKQAFPMISKRPEHLRMNL
                ******* .***********************************
```



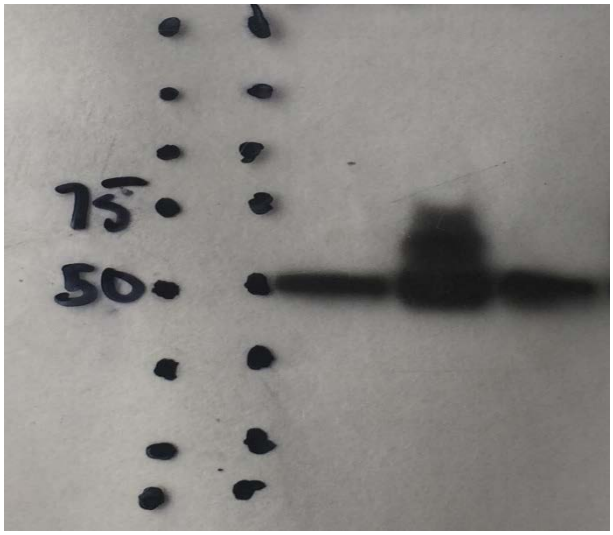


Figure: P70S6K protein expression in C2C12 mouse muscle cell lines, chicken muscle protein extracts. Blots from well A on the flim are C2C12 bands used as positive control, blots from well B and are chicken muscle extracts with decreasing concentrations of protein of, 100 and 80 mg of protein loaded per well. Blots were transferred from 7.5% mini-protean $^{(R)}$ TGX Stain-Free^{TM} precast gels.

APPENDIX 11: 4E-BP1 multiple amino acid alignments

```
CLUSTAL 2.1 Multiple Sequence Alignments


Sequence type explicitly set to Protein

Sequence format is Pearson

Sequence 1: MOUSE        117 aa

Sequence 2: CHICKEN      116 aa

Start of Pairwise alignments

Aligning...



Sequences (1:2) Aligned. Score: 61.2069
```

---

clustalw.aln

```
CLUSTAL 2.1 multiple sequence alignment


MOUSE           MSAGSSCSQTPSRAIP--TRRVALGDGVQLPPGDYSTTPGGTLFSTTPGGTRIIYDRKFL
CHICKEN         MSGRCCQGQTPSRDIPGPGKRLALPDGAPLPPGDYSTTPGGTVFGTTPGGTRIIYDRKFL
                **. .. .***** **   :*:** **. *************:*.***************

MOUSE           MECRNSPVAKTPPKDLPAIPGVTSPTSDEPPMQASQSQLPSSPEDKRAGGEESQFEMDI
CHICKEN         MECRNSPVAKTPPSDLPDIPGVTSPIVEELKIENNHVQN---YDEKANVGEEEQFDMDI
                *************.*** *******  :*  :: .: *     ::*   ***.**:***
```

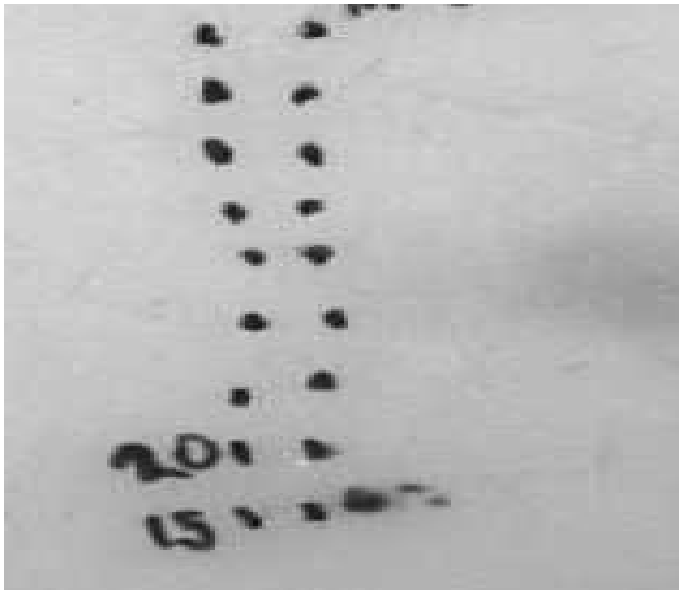
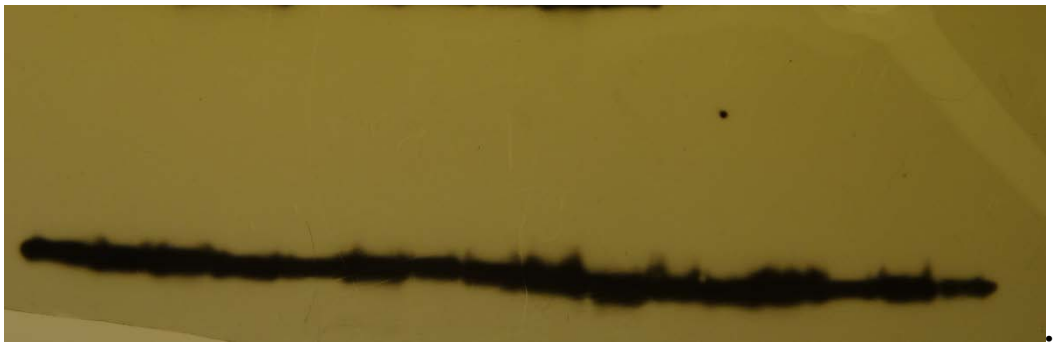


Figure 3.12: Attempted 4E-BP1 protein expression in C2C12 mouse muscle cell lines and chicken muscle protein extracts. There was no detection of bands indicating that primary antibodies were incompatible with extracted proteins

APPENDIX 12: Optimisation of western blotting procedure

During the course of measuring the specific proteins with their corresponding antibodies, a series of adjustments in techniques had to be made in order to get adequate quantifiable blots on the film. The adjustments in technique optimisation were specific to antibody used and specific protein measured. Specific adjustments made were primary and secondary antibody used, concentration in blocking agent, duration of membrane washing, duration of time left in the cassette, and duration of time films were immersed in developing solution. Below are examples of potential bands that were generated during the optimiation procedure.

1. Signal saturation: This occurred either as a result of a high concentration of antibodies used to incubate membranes or where the film and membranes were left in contact for too long. The bands were therefore too thick and saturated rendering individual bands quantifiable.
2. No bands detected on film: This occurred either as a result of a low concentration of antibodies used to incubate membranes or where the film and membranes were left in contact for a short periond of time. The bands were therefore undetectable.
3. High background overexposure: This occurred as a result of non specific binding but was also minimised by washing membranes for a longer perion of time.

a  **.**

b.

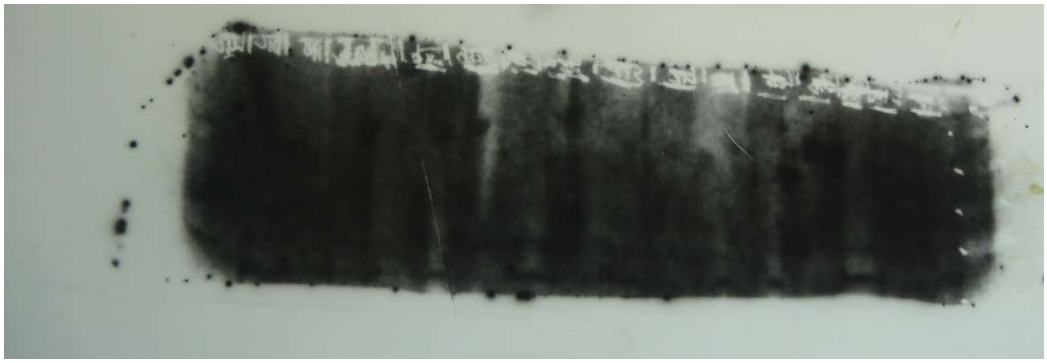


Figure: Variation in detecting proteins using western blotting and immunoprobing of blots. (a) Saturated unquantifiable bands using Ribosomal protein S6 (rps6) antibody developed by cell signalling technology (b) High background exposure due to non-specific binding using Calpastatin antibody developed in our laboratory.

APPENDIX 13: Optimal procedures adopted for specific antibody

Table: List of antibodies and optimisation conditions

Antibody	Antibody conc μl/ml	Exposure time with film (secs)	Exposure time in developer (Secs)
PHGDH	0.2	60	30
RPS6	0.1	30	30
Phospho RPS6	1.0	60	60
AKT	0.2	60	60
Phospho AKT	0.1	60	60

APPENDIX 14: ANOVA Table of multilinear regressions (Trial 1)

Gene	Multiple R	R squared	Significance (F)	Intercept coefficient	Age coefficient	Muscle type coefficient	P value intercept	P value Age	P value Muscle type
GAPDH	0.5898	0.3479	0.0039	0.9509	0.0019	0.0148	0.2066	0.9457	0.0055
ENOLASE	0.3914	0.1532	0.1251	0.2876	-0.0064	0.0010	0.0022	0.0539	0.1031
PHGDH	0.4270	0.1823	0.0808	7.1501	-0.1267	0.0791	0.1882	0.5436	0.0389
PSAT	0.4447	0.1977	0.0637	1.3008	-0.0261	0.0222	0.3958	0.6391	0.0307
PSPH	0.6501	0.4227	0.0008	3.0469	-0.1014	0.0392	0.0341	0.0562	0.0002
P7S6K	0.5265	0.2773	0.0147	1.9652	-0.0385	0.0130	0.0039	0.1079	0.0040
MUSTN1	0.2546	0.0648	1.5690	1.5690	0.0627	-0.0073	0.2239	0.1933	0.3898
CSRP3	0.6688	0.4473	-0.9193	-0.9193	0.1476	-0.0296	0.3856	0.0008	0.0002
FHL2	0.4800	0.2304	0.0332	-1.4879	0.2869	-0.0491	0.6303	0.0182	0.0230

APPENDIX 15: ANOVA Table of multilinear regressions (Trial 2)

Gene	Muscle type	Multiple R	R squared	Significance (F)	Intercept coefficient	Age coefficient	Muscle type coefficient	P value intercept	P value Age	P value Muscle type
PSAT	P. Major	0.23081207	0.053274	0.234391298	0.0336488	0.00056556	0.00027837	0.0585297	0.5378	0.520018
	P. Tertius	0.23420923	0.054854	0.305828031	0.01687673	-0.0002645	0.00084872	0.0001617	0.24178	0.125974
PSPH	P. Major	0.23081207	0.053274	0.234391298	0.0336488	0.00056556	0.00027837	0.0585297	0.5378	0.520018
	P. Tertius	0.43242951	0.186995	0.015917004	0.02608113	-0.000212	0.00325991	0.0203587	0.72437	0.032086