Effects of Growth Promoters on Sheep Metabolism and Growth

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وَمَا بِكُم مِن نِعْمَةٍ فَمِنَ اللَّهِ
(سورة النحل الآية 53)
And whatever of blessings and good things you have, it is from Allah (God)
(surah Alnahil, the Bees 53)

Abstract

The aim of this thesis was to investigate the mechanisms that mediate the effects of beta-adrenergic agonists (BA) and Growth Hormone (GH) in sheep, by examining the changes in skeletal muscle transcriptome and blood metabolome in order to identify the predominant metabolic mechanisms by which muscle hypertrophy was mediated.

Male lambs (120 days old) were all fed a high protein/energy feed *ad-libitum,* with the GH group (n=10) receiving a single subcutaneous injection of bovine GH (3.75mg/kg body weight, POSILAC, Monsanto) on day 1; the BA group (n=10) receiving BA (cimaterol) at 10mg/kg feed, whereas the control group (CO, n=11) only had the *ad-libitum* feed*.* After 6 days sheep were slaughtered, plasma and samples of the *Longissimus dorsi* (LD), *Supraspinatus* (SS) muscles and liver were collected. The effect of treatments on the LD transcriptome was assessed on a subset of samples (n=3 from each treatment) via a cross-species approach using the Affymetrix Human U133+2 GeneChip array (47K human microarray). Verification of identified differentially expressed genes and proteins was by quantitative RT-PCR or western blotting, respectively, on all animals. Metabolomics analysis of plasma samples was carried out by Metabolon Inc. (USA) using GC/MS and LC/MS/MS platforms.

BA, but not GH, significantly ($P<0.05$) increased muscle weights and this was associated transition to large fast-glycolytic muscle fibre types. In GH, but not BA treated animals, there was an increase in liver weights ($P<0.001$). This was associated with an increase in the whole liver content of glycogen ($P<0.001$), protein ($P<0.01$), and lipid ($P<0.05$) content.

Analysis of the LD transcriptome of the treated sheep identified 477 and 316 transcripts were significantly altered ($P<0.05$ and 1.5 fold change) by BA and GH respectively, relative to controls. This muscle was selected as it is a commercial valuable muscle and is commonly used for muscle biochemical studies therefore this would allow us to make comparisons to other studies, including our own. In addition it is a fast glycolytic muscle fibre type there could be compared against SS muscle (oxidative muscle fibre type). BA decreased the expression of genes involved with oxidative phosphorylation and upregulated those serine biosynthetic pathways. Subsequent qRT-PCR analysis showed a BA induced increase in expression of phosphoglycerate dehydrogenase (PHGDH) ($P<0.05$) and phosphoserine-aminotransferase (PSAT) ($P<0.05$) mRNA in both LD and SS but not liver. In LD there was also an increase ($P<0.001$) in PHGDH protein in muscle from BA treated sheep relative to GH treated sheep. Up-regulation of this pathway has been previously reported in cancer cells which has a tendency to be associated with an increase in gene expression of a specific isoform of the glycolysis enzyme pyruvate kinase (PKM2) which has reduced activity. Total PKM and PKM1 and PKM2 isoforms were increased in the SS and LD of BA treated sheep ($P<0.05$). Previous studies in cancer cells have suggested that increases in serine synthesis are mediated by changes in PKM2 expression and associated enzyme activity. The lack of a differential increase in PKM2 suggested that the regulation of muscle PK in BA treated animals was not critical to the potential increase in serine synthesis capacity. No clear change in PKM gene expression suggested this was not the mechanism by which the serine synthesis pathway was stimulated. There was an increase ($P<0.05$) in the expression the mitochondrial form of phosphoenolpyruvate carboxykinase (PCK2) in the LD of BA treated sheep, which might be expected to increase gluconeogenic

potential thereby increasing intermediates that could be used for serine synthesis. There was no effect of this gene on sheep treated with GH. An increase in the gene expression of asparagine synthetase (ASNS) was also seen in the muscles of BA but not GH treated sheep ($P<0.001$) and there was no effect on their livers, which further suggested that BA was influencing the production of nonessential amino acids.

Metabolomics analysis showed that products of triacylglycerol breakdown, glycerol and free fatty acids, were all elevated in the plasma of both BA and GH treatments, indicating lipolytic effects but the increase in the free fatty acid profile were more pronounced with GH treatment ($P<0.05$). Likewise GH rather than BA had a greater impact on elevating plasma glucose and associated metabolites such as pyruvate ($P<0.05$). There was no effect of either treatment on plasma serine or asparagine concentrations. However there was a decrease in glycine ($P<0.05$) and glutamine ($P<0.05$) in GH relative to control, with BA decreasing histidine ($P<0.05$) and methionine ($P<0.01$) relative to control.

Cell culture experiments were carried out in the myogenic C2C12 cell line to determine if the genes associated with the GH and BA response in sheep were affected during myogenesis and whether there was an effect of des (1-3) IGF-I and dibutyryl cyclic adenosine monophosphate (dbcAMP) that stimulates GH and BA signalling pathways respectively. During differentiation, without treatment, gene expression of PHGDH and PSAT enzymes declined ($P<0.05$), which might be expected as cells move from a proliferative to a terminally differentiate state. There was no clear effect of treatment on genes associated with the serine synthesis pathway suggesting that the effects of BA, in particular, are on muscle fibres rather than differentiating cells.

Of the two growth promoters examined in this thesis BA appears to be the most potent in skeletal muscle. A clear effect of this agent was an increase in the gene expression of the serine biosynthetic pathway, which has been shown to be upregulated in various cancers and, in this pathology, is thought to be a novel mechanism for hyperplastic growth. The associated changes in the expression of genes such as ASNS and PCK2 indicate that their co-ordinated upregulation could be mediated via endoplasmic reticulum stress response mechanisms. Unlike GH, BA does not appear to have a major effect upon the systemic mobilisation of nutrients, but instead seems to targets muscle fibres, activating muscle biosynthetic pathways that potentially provide the substrates required for growth.

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Abbreviations

%	Percentage
µl	Microliter
3’	Three prime
5’	Five prime
ADP	Adenosine diphosphate
AR	Adrenergic receptor
ATP	Adenosine triphosphate
BA	Beta-adrenergic agonist
Bp	Base pair
BSA	Bovine serum albumin
BW	Body weight
cDNA	Complementary deoxyribonucleic acid
dbcAMP	Dibutyryl-Cyclic adenosine monophosphate
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
g	Gram
g/kg	Gram per kilogram
GH	Growth hormone
Hr	Hour
IGF-I	Insulin-like growth factor 1
IGFs	Insulin-like growth factors

Kb	Kilo base
kDa	Kilodalton
LD	*Longissimus dorsi*
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mM	Mill molar
mm	Millimeters
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
ng	Nanogram
nm	Nanometers
^{o}C	Degrees Celsius
PCA	Perchloric acid
PCR	Polymerase chain reaction
Pi	Inorganic phosphate
r.t.	Room temperature
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Rotation per minute
RT-PCR	Reverse transcriptase polymerase chain reaction

SDS	Sodium dodecyl sulphate
sec	Second
SED	Standard error of difference
SEM	Standard error of mean
SS	*Supraspinatus*
Tris	2 amino-2-(hydroxymethyl) propane-1,3-diol
U	Units
v/v	Volume per volume
w/v	Weight per volume

1 Introduction

1.1 General Introduction

An increase in urbanisation of developing countries will lead to a change in consumer demands with a likely elevated desire for meat within their diet and associated pressure of animal feed (Bruinsma, 2009, UNPD, 2007), United Nations Population Division .Therefore there is a requirement to ensure Global Food Security. Animal production systems seek to increase growth by partitioning nutrients into lean tissue and increasing nutrient utilisation efficiency. Growth promoters such as beta-adrenergic agonists (BA) and Growth Hormone (GH) achieve this to varying degrees. Growth promoters may be defined as any natural or synthetic substances that are administered at low doses in order to maximize animal production and improve the quality, especially in the meat, resulting in higher protein and lower fat contents (Hughes and Heritage, 2004). However, growth promoters include an extensive range of substances that clearly have a variety of ways of action (Unruh, 1986). Many methods have been used and developed to improve animal production, and there have been many metabolic modifiers used to improve meat quality on their own or combined, including growth hormone (GH), BA, anabolic implants and conjugated linoleic acid (CLA) (Dunshea et al., 2005). The fatty acid CLA has been described as decreasing body fat and increasing lean tissue, as well as enhanced weight gain and improved feed efficiency when fed to laboratory animals (Chin et al., 1994). In livestock species such as pigs CLA it has been reported to increase lean tissue and decrease fat deposition (Ostrowska et al., 1999).

Efficiency of growth promoters range between 0 up to 20% depending on the type of substance and the experimental conditions (Meyer, 2001). Administration of growth promoter implants in cattle and sheep have been shown to improve growth rates (10 - 30%), feed conversion (5 – 15%) and carcass leanness (5 – 8%) (Preston, 1999). The overall aim of this project was to investigate the mechanisms by which these agents (BA and GH) affect sheep, as they show the greatest differential response to these agents within farm species (highest response to BA and a low level response to GH). In this study although a microarray approach was used to examine the effects of these growth promoters on LD muscle the research sought to determine whether there was any indication of a relationships between muscle and the liver. The liver has a central role in the metabolism of the nutrients being utilised or released by the muscle and fat depots, respectively, and thereby has nutrient partitioning role which may be critical in the response to growth promoters. The aim of the studies described in this thesis was to identify the key metabolic processes associated with the most effective response to growth promoters and thereby potentially identify some of the targets for manipulation to enhance growth.

1.2 Global Food Security and Demand for Food

1.2.1 Introduction

Food security is the ability of all people living in the world to have access to sufficient and healthy food (World Food Summit, 1996). There are many reasons for an increasing demand for food, such as the growth in population and the increased consumption per person due to increased personal wealth (Kearney, 2010).

Two of the most important factors that could advance in sustainable food production and availability are the application of agriculture production technologies and increasing research to investigate the known and unknown challenges that effect food systems in the coming future (Godfray et al., 2010).

Increasing population, especially in low income countries, will combine with other transformational changes leading to the movement of people from rural areas to cities that will need to be supplied with food, water and energy. Fifty per cent of the world's population now live in urban area but this will increase to 60% by 2030 (UNPD, 2007). Projected population increases in the world are shown in Figure 1-1.

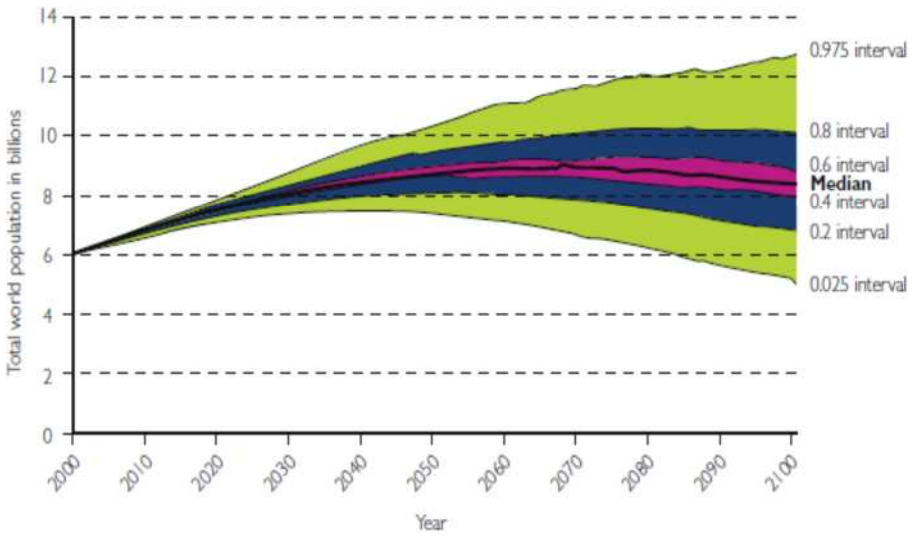


Figure 1-1: Total world population in billions.

Probabilistic estimate up until 2100 (green 95% interval; blue 60%; pink 20%). Reprinted from (Lutz et al., 2008).

Many interaction factors such as cultural, social, religious and economic effect the pattern of dietary changes, therefore predicting will be complex, but a particular issue which has a significant impact on food security is increase demand for meat in the diet. The expected increases in meat consumption per *capita*

are from the current 37 to about 52kg/person/year by year 2050, with the increase in the underdeveloped countries being from 26 to 44kg/person/year (Bruinsma, 2009). This increased demand will be associated with an expected increase in global farm animal population; from 1.5 billion in 2000 to around 2.6 billion in 2050, a 70% increase for cattle and approximately 60% for sheep and goat, from 1.7 billion to about 2.7 billion over the time period (Group, 2009).

In developing countries the quantity of meat consumed has increased three fold over the past 20 years, whilst in developed countries the changes have been relatively small. This has been associated with a rise in income above the poverty level across the world which leads to consumption of more animal products (Delgado, 2003). The nutrition and income of many rural people in the world is improving, but there are still many problems associated with these dietary changes which are related to environmental and health issues. These that need to be actively addressed in order to prevent these populations being undernourished (Delgado, 2003). In the long term, consumption of livestock products will be expected to continue to increase, but the extent of the change will differ among countries and livestock sectors (Table 1-1).

Table 1-1: World production by livestock sector. Adapted from (Alexandratos and Bruinsma, 2012).

World	1961/1963	2005/2007	2050	1961-2007	1987-2007	1997-2007	2005/2007-2050
	Million tonnes			Annual growth (% p.a.)			
Total meat	72	258	455	2.9	2.5	2.2	1.3
Beef	30	64	106	1.6	0.9	1.2	1.2
Mutton	6	13	25	1.7	1.8	2.1	1.5
Pig meat	26	100	143	3.1	2.3	1.7	0.8
Poultry	9	82	181	5.2	4.7	3.9	1.8
Milk	344	664	1077	1.4	1.3	2.2	1.1
Eggs	14	62	102	3.5	3.3	2.3	1.1

Therefore if the population has an increased demand for meat the efficiency of its production has to be increased in order to ensure that the demand for animal feed does not become a factor in limiting the availability of nutrients to an increasing global population.

1.3 Skeletal Muscle Development

Skeletal muscle is a unique tissue basically consisting of myofibers with various contractile and metabolic properties. The number and size of the myofibers are the main determinants of postnatal muscle growth (Oksbjerg et al., 2004). During prenatal and postnatal events, myofiber number and size are formed, respectively, following a complex sequence. The number of muscle fibre is very important to postnatal growth as an animal with muscles containing a high fibre number tends to grow faster and more efficiently than animals with a lower fibre number (Dwyer et al., 1993).

Myogenesis (skeletal muscle development) covers the period of development where there is proliferation of myoblast cells followed by differentiation and fusion, resulting in three populations of muscle cells. These include primary and secondary muscle fibres, as well as a satellite cell population (Oksbjerg et al., 2004). Myogenesis is regulated by the myogenic regulatory factors (MRFs), consisting of MyoD, Myf-5, myogenin, and MRF4 (Rehfeldt et al., 2011). The MRFs regulate muscle making process, and appear at different times during development of muscle. During myogenesis, myoblasts develop from mesenchymal precursor cells by proliferation and myogenic commitment. Myoblasts then differentiate and fuse to form myotubes which then continue to form muscle fibres (Figure 1-2).

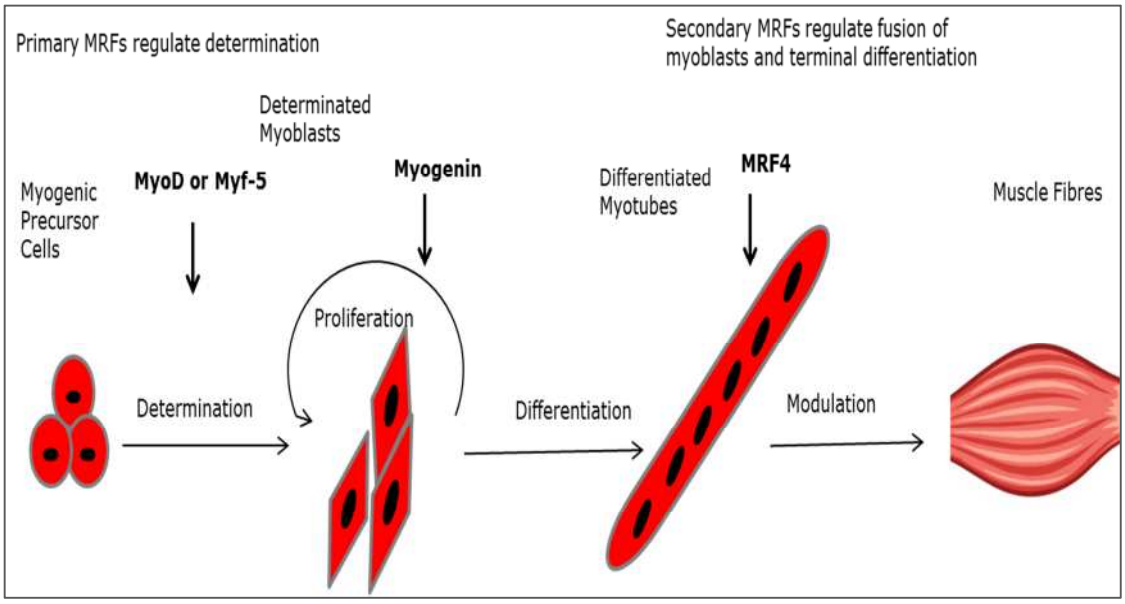


Figure 1-2: The basic stages of myogenesis

Myofibre development is a biphasic phenomenon with the formation of primary fibres first and then secondary fibres in lambs (Ashmore et al., 1972) and in pigs (Berard et al., 2011). However

at birth the total number of fibres is set, there being no postnatal hyperplasic growth of fibres, fibre number does not change.

Postnatal growth is associated with myofibre hypertrophy and involves satellite cell proliferation and differentiation, and associated protein turnover (Oksbjerg et al., 2004). In mammals and birds the increase in skeletal muscle mass during postnatal growth is due to an increase in muscle fibre size (hypertrophy) without a change in fibre number (Rehfeldt et al., 2000). This process is accompanied by proliferation of satellite cells, as these cells provide new nuclei which are incorporated into the muscle fibres.

In the pigs postnatal muscle hypertrophy is associated with an increased DNA content due to division and fusion of satellite cells residing between the basement membrane and the sarcolemma. A high percentage of satellite cells remain proliferative during rapid postnatal muscle growth (Mesires and Doumit, 2002). In mammals and poultry postnatal growth and function of muscles depends on the hypertrophy of the fibres formed during myogenesis and the composition of fibre type, in terms of metabolic and contractile properties, whereas in fish, hyperplasia still plays a major role (Rehfeldt et al., 2011). However, both muscle mass and meat quality have been affected by selection (genetic) for both muscle fibre number and muscle fibre size (Rehfeldt et al., 2000).

1.4 Muscle Fibres

The objective of increasing animal growth efficiency is to enhance the proportion of the feed consumed which gets deposited into the carcass and ideally this deposition needs to be into lean tissue (muscle), as this is the component of the carcass which has the greatest commercial value. The feed conversion efficiency in sheep

is about 6-7kg feed/kg gain (Mahgoub et al., 2000, Demirel et al., 2006), and in pigs ~ 3kg feed/kg gain (Dugan et al., 1997, Andersson et al., 1997), while in broilers about 2kg feed/kg gain (Waldroup et al., 2003). This range of feed conversion efficiencies indicates that there is potential for species to improve their efficiency. Therefore an understanding of muscle is essential in order to gain an insight into the processes by which enhanced efficiency of high quality meat production in livestock can be achieved.

1.4.1 Muscle Fibre Type

Muscle reacts strongly to changes in functional demands (Goldspink, 1999). Skeletal muscle fibres are made up of multinucleate, membrane-bound cells that vary in both diameter and length, the typical diameter of muscle fibres range between 10 to 100μm, and their lengths can vary from several millimetres to more than 30 cm (Bechtel, 2012). The diversity of the individual skeletal muscle fibres and the mosaic composition of the multiple fibre types can be attributed to the heterogeneous characteristics of muscle fibres (Schiaffino and Reggiani, 1996). Depending on muscle function, fibre type composition can vary significantly in various species and muscle types (Klont et al., 1998). Characteristics of different muscles may change in living animals as a result of environmental conditions and genetic selection (Klont et al., 1998). However, there are many different factors affecting fibre type and meat quality, such as age, weight at slaughter (Candek-Potokar et al., 1998), sex (Ozawa et al., 2000), breed (Ryu et al., 2008), hormones (Florini et al., 1996), and the impact of physical exercise (Pette and Staron, 2000).

Many different histochemical staining techniques have been used to determine the muscle characteristics and to identify different muscle fibre types (Ryu et al., 2008, Morita et al., 2000). These fibre type variations vary depending on their molecular, structural, metabolic, and contractile characteristics (Jurie et al., 1999, Schiaffino et al., 1989). Therefore knowledge of muscle fibre properties will allow an understanding of the overall muscle characteristics and quality of meat.

1.4.2 Muscle Fibre Type Classification

Many methods of muscle fibre classification have been used and all classifications can be applied to animal muscle. The original classification was based on contractile activity (slow or fast twitch) which became further defined based on metabolic performance (Bottinelli and Reggiani, 2000). Utilising metabolic based screening approaches, three main fibre types have been identified; red, intermediate, and white fibres. Red fibres (slow oxidative) contain higher concentrations of enzymes that are involved in oxidative metabolism and lower concentrations of glycolytic enzymes, and have greater myoglobin contents than white fibres (fast glycolytic). While intermediate fibres (fast oxidative glycolytic) are intermediate between red and white fibres having both oxidative and glycolytic capacities (Dubowitz and Pearse, 1960, Jurie et al., 1999).

The main descriptive classification of muscle fibres is based on differences in the acid and alkaline stability of the myosin ATPase reaction. These distinguish three fibre types that included fibre types I, IIA, and IIB (Brooke and Kaiser, 1970, Hintz et al., 1984). This is the predominant nomenclature which is used to histologically identify muscle fibres. Although type I fibres show

low ATPase activity after pre-incubation in alkaline at pH 10.4, it has a stable and high ATPase activity after acid pre-incubation at either pH 4.3 or 4.6. Type IIB fibres lose ATPase activity after acid pre-incubation at pH 4.3. Whilst type IIA have a strong activity after alkaline pre-incubation, but has no reaction after acid pre-incubation at pH 4.3 or 4.6 (Brooke and Kaiser, 1970). Fibres can also be classified according to histochemical reactions of muscle fibre type based on oxidative capacity, using the enzyme succinate dehydrogenase (SDH), or glycolytic capacity using the enzyme NADH-tetrazolium reductase (NADH-TR) (Rahelic and Puac, 1981, Baker and Santer, 1990). In general terms fibres defined as Type I by ATPase staining have a high oxidative capacity, whilst Type IIA and Type IIB have increasing glycolytic capacity. Type IIA having fast-oxidative metabolic capacity (a significant glycolytic capacity) whilst Type IIB is fast-glycolytic, having high dependence on glycolysis.

1.4.3 Characteristics of Muscle Fibres

In adult animals, the main muscle fibre types differ in the function, structure, and metabolic characteristics (Bottinelli and Reggiani, 2000). Fibre type I, or slow-twitch fibres, produce energy for ATP resynthesizes primarily by aerobic energy transfer. The activity of myosin ATPase is lower in type I fibres than in fast-twitch fibres and they have less glycolytic capacity than fast-twitch fibres (Sjostrom and Squire, 1977). Type I fibres contain relatively larger numbers of mitochondria, myoglobin, and iron than type II. High levels of mitochondrial enzymes and myoglobin may also support to increased aerobic metabolic capacity (Nemeth and Lowry, 1984). Furthermore, type I fibres have a higher levels of lipid, some of which may be used as a source of aerobic metabolic energy, but they contain lower amounts of glycogen and glucose

than type IIB fibres (Peter et al., 1972, Hintz et al., 1984). Therefore, type IIB fibres predominantly use glucose as source of energy.

Type II fibres, especially type IIB fibres have more rapid contraction speeds than type I fibres that give ability to rapid transfer energy for fast, powerful muscle actions. The ATP hydrolysis rate for example is three to four times faster in type IIB fibres than in type I fibres. Between the metabolic and physical extreme characteristics of type I and IIB are type IIA and IIX fibres. These are intermediate in their metabolic and contractile characteristics with IIA being most oxidative (Bottinelli et al., 1994, Stienen et al., 1996). The ratio between ATPase and tension which is called the tension cost, is several times lower in type I fibres than in types IIA, IIX, and IIB fibres (Bottinelli et al., 1994, Stienen et al., 1996).

1.4.4 Muscle Fibre Type and Meat Quality

The size and number of muscle fibres are the most important factors that influence muscle mass and meat quality (Rehfeldt et al., 2004, Rehfeldt and Kuhn, 2006). It has been suggested that muscles with high numbers of lower or medium size fibres tend to display good quality of meat, without great differences in muscle mass. Conversely, production of large muscle fibres in farm animals, especially type IIB fibres, seems to result in poorer meat quality (Gentry et al., 2002, Ryu and Kim, 2006).

Muscle fibre morphology is represented by their total number of fibres (TNF), cross-sectional area of muscle fibre (CSAF), and length of muscle fibre. Muscle growth potential is related to TNF and CSAF (Rehfeldt et al., 2000, Ruusunen and Puolanne, 1997). TNF and CSAF are inversely correlated to each other, but both are

positively correlated with muscle growth (Fiedler et al., 1999, Rehfeldt et al., 2000, Henckel et al., 1997).

In previous studies, it has been suggested that increasing muscle fibre size negatively influenced meat tenderness (Rehfeldt et al., 2000, Seideman and Crouse, 1986). Muscles with smaller fibres showed less tough meat than muscles with bigger fibres (Karlsson et al., 1993, Maltin et al., 1997). It has been reported there is a positive correlation between intramuscular fat contents (IMF) and cross-sectional area of muscle fibre (CSAF) (Larzul et al., 1997). Intramuscular fat (IMF) content is a major factor that impacts on meat quality, including, juiciness, tenderness, and flavour, and is affected by genetic factors and environmental conditions. However, intramuscular fat (IMF) content is positively correlated to tenderness (Renand et al., 2001), and negatively correlated with fibre area (Karlsson et al., 2000). Muscle fibre type composition and morphology of muscle are associated with colour stability, tenderness and water holding capacity in beef (Klont et al., 1998).

Double muscled (DM) syndrome cattle possess 40% more fibres than normal cattle at birth due to an increase in muscle cell number (hyperplasia) prior to differentiation (Gerrard and Grant, 1994). Myoblasts from DM cattle proliferate more rapidly than normal and differentiation is delayed which leads to more fibres being produced (Gerrard and Grant, 1994). Variations in the myostatin gene which result in it not being expressed have been identified as the cause of the double-muscled phenotype in cattle (Grobet et al., 1997).

The meat from double muscled animals contains less saturated fatty acids and collagen compared to non-double muscled animals. Therefore the meat from DM animals is generally more tender (Fiems, 2012).

1.5 Molecular Mechanisms Controlling Muscle Fibres

In adult skeletal muscles, the slow and fast-twitch myofibers express single sets of muscle specific genes, and these special programmes of gene expression are, in part, controlled by differences in motor neuron activity. It has been identified that a various models of motor nerve activity stimulate selective variations in gene expression to contribute to the establishment of the specialized properties of slow and fast myofibres (Chin et al., 1998). For example, it is quite well established that slow fibres have greater levels of intracellular free calcium than fast fibres, as a results of more frequent neural stimulation which has an effect on the gene expression profile (Chin et al., 1998). A number of transcription factors are associated with different fibre types. For example, the expression of the transcription factors peroxisome proliferator-activated receptor delta (PPARdelta) is affected by muscle activity and induces slow muscle properties in adult rat muscles (Lunde et al., 2007), whilst peroxisome proliferator-activated receptor gamma (PPARgamma) tends to increase the oxidative capacity of muscle. Transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha) is a major factor regulating muscle fibre type determination and its over expression increases muscles oxidative capacity switching it to a type I fibres (Lin et al., 2002). In addition the activation of enzymes such as calcineurin, (a calmodulin dependent phosphatase) has also been found to induce the slow fibre gene regulatory program *in vivo* by its capacity to activate downstream transcription factors (Naya et al., 2000). It appears that molecular mechanisms regulating the slow twitch muscle fibre phenotype have received a great deal of attention which has resulted in the identification of these regulating factors. The reason

for this is that oxidative type I are effective at metabolising fatty acids which is a problem when individuals develop insulin resistance.

In contrast the molecular mechanisms that regulate the expression of the fast-twitch muscle fibre phenotype are not as well established. However, a study by Grifone et al. (2004) identified that the transcription factors Six1 and Eya1 appear to be involved in controlling the expression of the fast-twitch glycolytic phenotype, and is able to reprogram adult slow-twitch oxidative fibres toward fast-twitch glycolytic fibres. In their study, the proteins of Six1 and Eya1 accumulate preferentially in the nuclei of fast-twitch muscles. The expression of both Six1 and Eya1 in the slow-twitch soleus muscle induced a fibre type transition described by the replacement of myosin heavy chain I and IIA isoforms by the faster IIB and/or IIX isoforms. This activation of specific genes associated with fast-twitch fibre, lead to a switch toward glycolytic metabolism (Grifone et al., 2004), a similar response to that noticed with administration of BA (Figure 1-3).

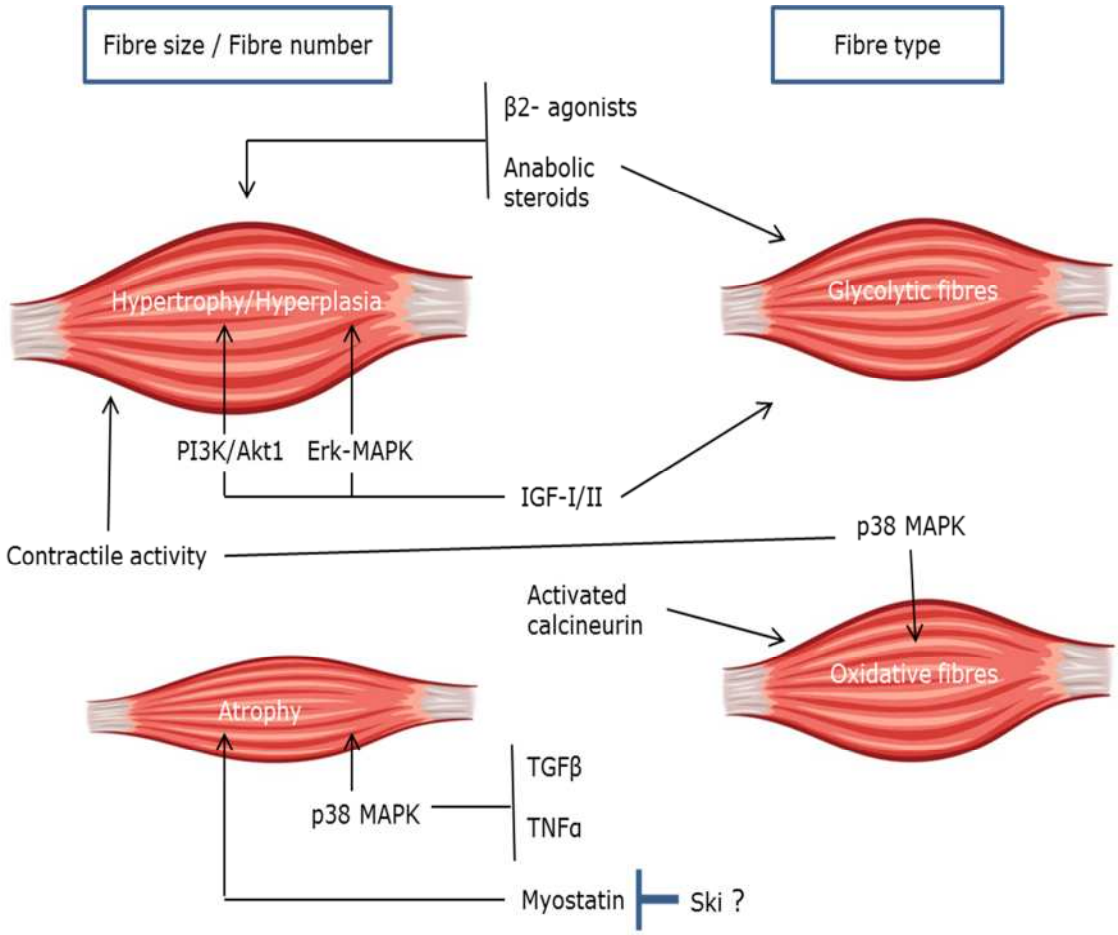


Figure 1-3: Actions of signalling factors and pathways on muscle fibre.

The figure shows the fibre type and fibre number. The role of p38 MAPK activation on phenotype characteristics. The proposed inhibition of myostatin by Ski .

1.6 Growth Promoting Agents and Their Effects on Animals

There are various types of growth promoters which are legally used in various countries in the world to increase the efficiency of animal production. These growth promoters can be divided into four categories; those that increase both growth and consumption of feed, those that improve growth without changing feed consumption, those that do not change growth but reduce feed consumption, and those that increase growth and decrease feed consumption (Dibner and Richards, 2005). The growth promoters

considered in this thesis are those which have an impact on nutrient utilisation within animals. Other growth promoters such as antibiotics have their effects within the gut altering the availability of nutrients available for absorption by altering the intestinal microflora (Dibner and Richards, 2005).

The main categories of growth promoters are those that directly manipulate the endocrine axis to stimulate growth these include those which are steroid, GH-IGF-I or beta-adrenergic based. All of these cannot be legally used in the European Union to enhance growth in animal production systems, but they are used in many countries in the world, such as United States of America and Australia.

The essential mode of action of these agents is to affect one or all of the following; enhance feed efficiency, repartition nutrients into lean growth whilst decreasing fat deposition or increase the rate of weight gain (Gadberry, 2009, Etherton and Bauman, 1998, Ludwig et al., 2012, Callaway et al., 2003, Diarra and Malouin, 2014). Agents that manipulate growth hormone – IGF-I or beta-adrenergic axis have been characterised as manipulating by animal growth by enhancing all of the three main characteristics of growth promoters' action. Although these two groups of agents have similar effects there are distinct differences in their specific mode of action. One of the objectives of the research described in this thesis was to characterise the mechanisms by which these two types of growth promoters influence muscle metabolism by examining changes in gene expression. Therefore the following chapters describe these two agents in more detail.

1.6.1 The Beta-adrenergic Agonists

The beta adrenergic agonists (BA) are organic molecules that bind to β-adrenergic receptors (β-AR) then signal through a secondary messenger cascade to generate a response in cells, which, in the case of skeletal muscle, is hypertrophy (Mersmann, 1998). Following their discovery as a growth promoting agents (Bohorov et al., 1987) they have been utilized to increase the efficiency of animal growth in various countries across the world. This has led to various forms of BA which have manufactured and developed to act as repartitioning agents (Table 1-2).

Table 1-2: Names and source of BA repartitioning agents

BA	**Manufacturer[a]**
BRL-47672	SmithKline Beecham
Cimaterol	American Cyanamid
Clenbuterol[b]	Boehringer Ingelheim
L-644,969	Merck Sharp & Dohme
Ractopamine (Paylean)	Eli Lilly
Ro 16-8714	Roche Pharmaceuticals
Salbutamol[b]	Glaxo

[a]Company name at time of development, [b]Developed for humans, but used in livestock research. Adapted from (Sillence, 2004).

1.6.1.1 β-adrenergic Receptor Subtypes

To understand the physiological functions regulated by β-AR, it is necessary to have knowledge of the β-AR subtypes in different cell types. Three subtypes of β-ARs have been identified; β1-AR, β2-AR and β3-AR (Dixon et al., 1986, Espinasse et al., 1995, Frielle et al., 1987), each with a 65–70% homology in their amino acid sequence composition (Kobilka et al., 1987). Almost every mammalian cell surface has at least one of the three subtypes of β-adrenergic receptors (β-AR) (Mersmann, 1998).

Although β-ARs are present on the surface on most mammalian cells, the distribution and proportion of the three subtypes (β1-AR, β2-AR, and β3-AR) differs between tissues and species (Mersmann, 1998). A study by Bowen et al. (1992) reported that the properties of the β-ARs binding site in sheep and rat display species and depot specific differences. In bovine, radioligand binding studies indicate β2-AR predominantly present on both skeletal muscle and adipocytes, whilst β1-AR was not detected in either tissue (Sillence and Matthews, 1994). A given β-AR subtype has approximately 75% homology across species. For example, the cattle β3-AR has approximately 75% amino acid sequence homology with rat and mouse β3-AR and 85% amino acid sequence homology with human β3-AR (Pietri-Rouxel et al., 1995). The β-AR subtype concentration may alter with the stage of differentiation of a cell. In human, on the undifferentiated cells there are approximately 90% β1-AR, a few β2-AR, and no β3-AR detected, whereas cells after differentiation into adipocytes there are approximately 90% β3-AR, several β1-AR, and very few β2-AR. If the dexamethasone present in the differentiation medium the cells have > 80% β2-AR (Feve et al., 1991, Strosberg, 1992). A study by Van Liefde et al. (1994) reported that only β1-AR and β2-AR are present in cattle adipose tissue. The properties of the three β-AR subtypes are summarized in Table 1-3.

Table 1-3: The Properties of β-adrenergic receptors. Adapted from (Mersmann, 1998).

Item	**β1-AR**	**β2-AR**	**β3-AR**
Prototypical tissue	Rat heart	Hamster trachea	Rat adipocyte
Selective agonist	—	Fenoterol	CGP 12,177
Selective antagonist	CGP 20, 712A	ICI 118,551	SR 59,230A
Glycosylated molecular wt.	65,000	65,000	65,000
No. of amino acids (human)	477	413	408
mRNA, approx. kb	2.8	2.0	2.2
Introns	No	No	Yes
Phosphorylation sites	Yes	Yes	Few or none

1.6.2 β-adrenergic Signalling Pathway

All adrenoceptors belong to superfamily of the guanine nucleotide binding G protein-coupled receptor (GPCR), the biggest class of cell surface receptors in mammals (Fredriksson et al., 2003). It has been accepted that the first step of any expected mechanism of (BA) should be start through the Gs proteins to activate adenylyl cyclase to produce cyclic adenosine monophosphate (cAMP), one of the main intracellular signalling molecules (Mersmann, 1998). The cAMP then can bind to the regulatory subunit of protein kinase A (PKA). This kinase can have a direct effect of phosphorylating proteins. For example it can phosphorylate phosphorylase kinase which then activates phosphorylase that then catalyses glycogenolysis. Other enzymes become inactivated when phosphorylated by PKA. Activation of PKA can also affect gene expression, the transcription factor cAMP response element binding protein (CREB) is phosphorylated via PKA, and this affects the interaction of CREB with specific binding sites in the genome which can influence gene transcription. The transcriptional activity of the CREB increases by increase phosphorylation, providing a potential mechanism action for β-AR agonist-mediated through the

transcription of a selection of genes in the mammalian cells (Strosberg, 1992, Liggett and Raymond, 1993).

The predominant receptor on skeletal muscle is β2-AR and it is through these receptors muscle hypertrophy would be expected to be mediated by BA. Activation of β2-AR is associated with high intracellular cAMP concentrations (Robison et al., 1967). This is the outcome of stimulation of adenylyl cyclase that leads to conversion of ATP into cAMP. The coupling of the β2-receptor to adenylyl cyclase is influenced by trimeric Gs protein, consisting of an α-subunit that stimulates AC and β as well as γ-subunits that transduce other signals (Johnson, 1992). The guanosine diphosphate (GDP) is released from the Gα- subunit as a result of binding of a ligand to the GPCR, this is then followed by GTP binding which activates the Gα- subunit as well as presenting other interaction sites in the Gβγ dimer (Bockaert and Pin, 1999, Gilman, 2015) (Figure 1-4). There are many types of Gα subunits, but it has been demonstrated that β-ARs couple mainly with Gαs and Gαi isoforms to initiate downstream effector pathways such as adenylyl cyclase, phospholipases, and transmembrane protein kinases (Dascal, 2001, Wenzel-Seifert and Seifert, 2000). Once generated by adenylyl cyclase the concentration of cAMP is regulated via the activity of phosphodiesterase isoforms, which convert it to 5'-AMP (Johnson, 1992).

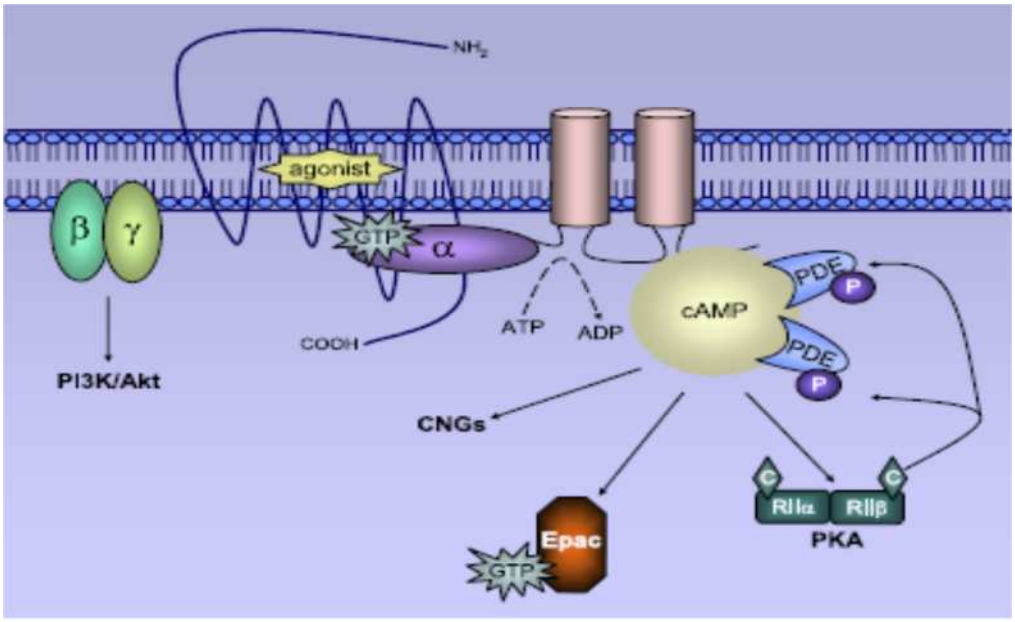


Figure 1-4: The activation of the β-AR through signalling pathway

Both the α- and βY-subunits can activate downstream signalling targets including the PI3K/Akt, Epac, and cyclic nucleotide-gated (CNG) signaling pathways, as results in GTP displacement of GDP from the α-subunit of the G protein, causing a conformational change in the heterotrimeric protein. The cAMP signal is terminated through its hydrolization to 5'-AMP by phosphodiesterases (PDEs). Epac, referred cAMP regulated guanine nucleotide exchange factors. This pathway activation requires the receptor to be activated by PKA and is mediated by βY-subunits of the G protein acting as an assembly scaffold for a number of intracellular proteins, including non-receptor tyrosine kinase cSrc, Raf, and RAS, resulting in the stimulation of MAPK. Reprinted from Lynch and Ryall (2008).

The resulting rise of intracellular cAMP activates protein kinase A (PKA) (Daaka et al., 1997). In spite of the fact that most of the activities of the β2-AR are mediated through Gs proteins and the cAMP-dependent PKA system, β2-ARs can also couple to Gi proteins, stimulating the extracellular signal-regulation kinase and p38 mitogen-activated protein kinase (MAPK) pathways. This pathway activation requires the receptor to be activated by PKA and is mediated by βY-subunits of the G protein acting as an assembly scaffold for a number of intracellular proteins, including non-receptor tyrosine kinase cSrc, Raf, and RAS, resulting in the stimulation of MAPK (Daaka et al., 1997) (Figure 1-5). This process might act to uncouple the β2-AR from the Gs protein and therefore acts to terminate the β2-AR signal and response. Therefore, interactions between both the GTP-bound Gα and the GβY dimer

are capable of stimulating downstream effects (Lynch and Ryall, 2008). There are many downstream signalling pathways that are activated by cAMP , including the PKA signalling pathways, the novel cAMP targeted exchange protein activated directly by cAMP (Epaca, also known as a guanine nucleotide exchange factor) and the cyclic nucleotide-gated (CNG) cation channels (Bos, 2003, Dremier et al., 2003, Tasken and Aandahl, 2004), but PKA is the most common signalling pathway studied β-AR effector in skeletal muscle (Navegantes et al., 2001, Navegantes et al., 2002). Thus PKA plays an important role in cell metabolism and growth (Dremier et al., 2003). Moreover, evidence has been provided that β2-receptor–activated MAPK phosphorylates the glucocorticoid receptor (GR) at a number of proline-directed serine residues in the N- terminal zone of the receptor, resulting in a receptor more sensitive to steroid-dependent activation (Johnson, 2006). In addition, activation of β2-Receptor leads to increased translocation of the GR from the cytoplasm of a cell to the nucleus, which is considered to be a primary step in the anti-inflammatory activity of corticosteroids. This mechanism is associated with the activation of a CCAAT enhancer binding protein (C/EBPα). These mechanisms can potentially be exploited as therapy to asthma because there is a positive interaction between GR and β-AR in the condition (Roth et al., 2002). The BA is not only used to increase animal production but, they can also be used for asthma therapy and prevention of muscle wasting.

Therefore although the well-recognised signalling pathway for β2-AR activation is via PKA activity there are other potential mechanisms by which muscle hypertrophy could be induced by beta-adrenergic agonists.

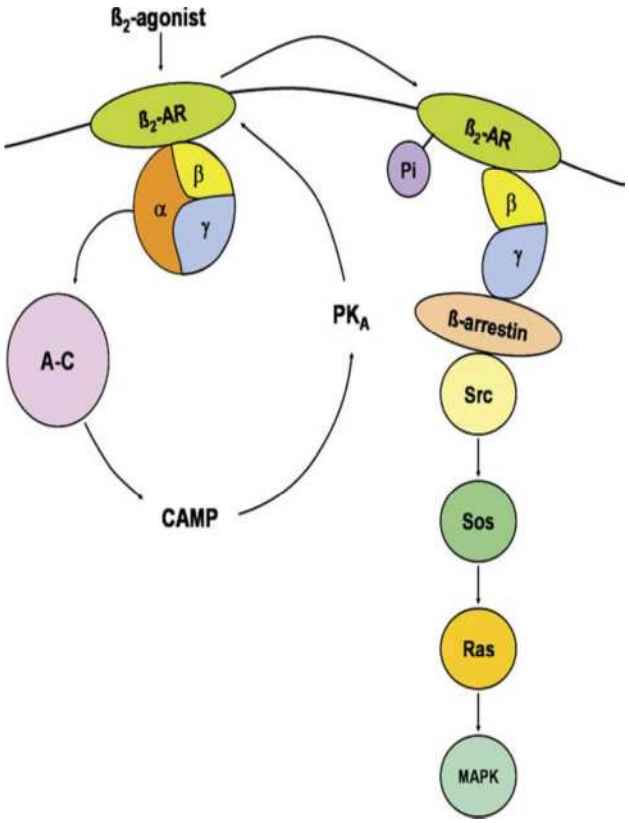


Figure 1-5: Intracellular signalling pathways for the β2-AR.

Activation of the β2-AR by BA causes the α-subunit of the associated Gs protein to dissociate and couple with AC, resulting in production of cAMP and stimulation of PKA. PKA induces phosphorylation (Pi) of the receptor protein and uncoupling of Gs. The phosphorylated receptor, with its residual βY-subunits, then couples to Gi. After the binding of β-arrestin, which acts as an assembly scaffold for Src, SOS, and RAS, the MAPK pathway is activated. β-arrestin is an intracellular protein that participate in agonist-mediated desensitization of G protein coupled receptors and induce specific dampening of cellular responses to stimuli such as hormones. The non-receptor tyrosine kinase Src, SOS, and RAS. Reprinted from Daaka et al.(1997).

1.6.3 Response of β-adrenergic Receptors

The variation of each β-AR subtype across different cell types and species may, in part, be responsible for the different responses to BA treatment. The effects and response of β-AR agonists differ between animal species; bigger effects in sheep and cattle, and significantly weaker effect in chickens, whereas intermediate effects are observed in pigs (Table 1-4). These differences in response to BA are due to many reasons. For example, some species such as broiler chickens have been selected for maximal growth rate while other species such as sheep have not therefore,

have more capacity to increase growth rate. Clear differences in responsiveness exist tissues in the same animal species. For example, in cattle BA (cimaterol) treatment resulted in a significant mass increase of *longissimus dorsi* and *vastus lateralis* whilst no effect on *semitendinosus* (Dawson et al., 1991).

Table 1-4: Effects of oral β-AR agonists in several species[a]

Animal	**Weight gain**	**Feed consumption**	**Gain/feed**	**Muscle**	**Fat**
Cattle	+10	-5	+15	+10	-30
Chickens	+2		+2	+2	-7
Pigs	+4	-5	-5	+4	-8
Sheep	+15	+2	+15	+25	-25

[a]The values indicated as the percentage change. Adapted from (Mersmann, 1998).

1.6.4 BA Induced Muscle Hypertrophy

Beta-agonists may induce muscle hypertrophy through the activation of a series reactions leading to increased protein accretion as a result of its direct binding to skeletal muscle membrane receptors. Alternatively, induced muscle hypertrophy may be due to indirect mechanisms. The BA may activate β-AR of non-muscle cell, resulting in production of hormone(s) or other factor(s) (Figure 1-6). Then these factors may act on the muscle or create an environment conducive to promote protein accretion (Figure 1-6).

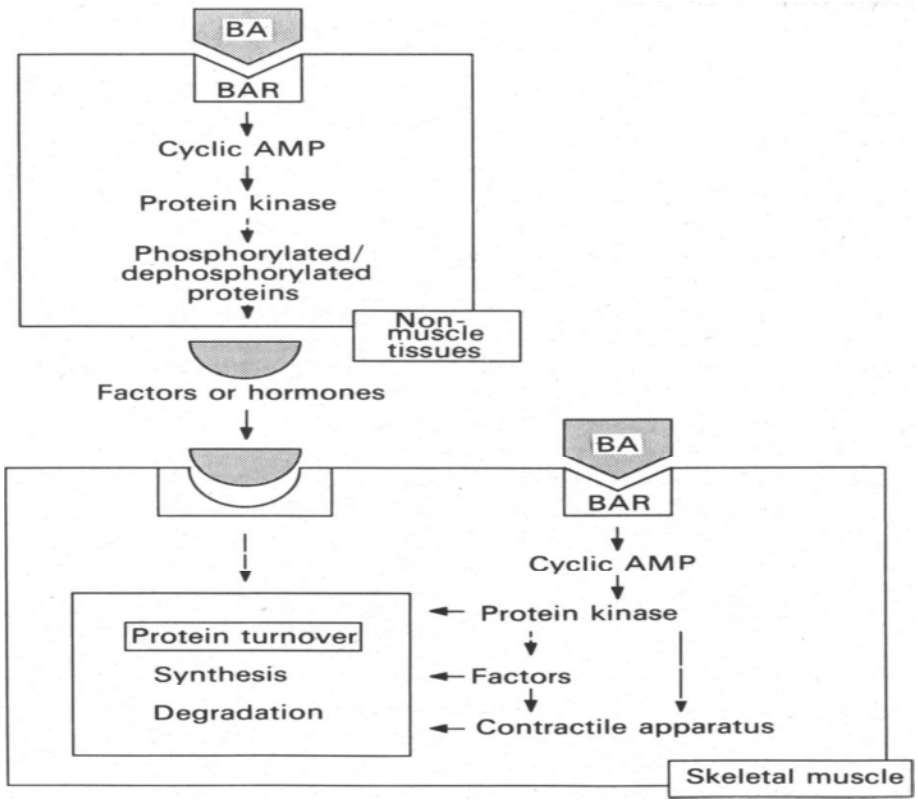


Figure 1-6: Mechanisms of BA induced muscle hypertrophy.

The figure shows BA bind to skeletal muscle membrane receptors directly and activate a series of events leading to protein accretion. Muscle hypertrophy may be due to indirect mechanisms. Reprinted from Yang and McElligott (1989).

BAs are considered the most potent and specific agents that can improve skeletal muscle growth. Increased muscle weight in lambs fed the BA, cimaterol was primarily associated with hypertrophy of fast glycolytic fibre type, particularly type II fibres (Kim et al., 1987). These heavier muscle weights were not the result of carcass weights being higher in cimaterol-fed lambs but was due to a greater cross sectional area of the muscle (Beermann et al., 1986). The β-agonist, cimaterol, fed to lambs for 12 weeks increased the weights of several muscles by 25-30% compared to controls (Beermann et al., 1986, Beermann et al., 1987). The greater muscle weights were found to be the result of increased rate growth or hypertrophy of type II fibres in the SS and ST muscles. In growing lambs treated with clenbuterol, although carcass weights were slightly increased there was a dramatic increase in

skeletal muscle weights (Young et al., 1995). A study by Rothwell and Stock (1985) reported that clenbuterol increased the muscle mass in normal and dystrophic mice (mDX) indicating that clenbuterol can be used to prevent muscle wasting.

Many studies reported that oral administrated of BA in farm animals led to increases in muscle mass as a result of hypertrophy that was associated with an increase in muscle protein synthesis, a decrease in muscle protein degradation, or a combination and balance of both synthesis and degradation (Yang and McElligott, 1989, Kim and Sainz, 1992, Mersmann, 1995, Beermann et al., 1987). Parr et al. (1992) as well as Wheeler and Koohmaraie (1992) reported BA induced muscle hypertrophy was associated with increased activity of calpastatin which could result in the reduction of proteolytic capacity of calpain proteinases. The muscle hypertrophy in response to BA administration appears to be a true muscle growth effect which involves muscle hypertrophy, in contrast with some other types of muscle growth. A study by Beermann et al. (1987) concluded that ST (*Semitendinosus*) muscle from lambs fed cimaterol had a higher RNA concentration compared to controls. They also suggested an increased capacity for protein synthesis due to an increase in the ratio of RNA/DNA. However, both muscle DNA content and muscle weight were 25% greater in lambs fed cimaterol with 12 weeks treatment , but muscle DNA content was 22% less in in lambs fed cimaterol for 7 weeks compared to controls (Beermann et al., 1987), suggesting increased muscle hypertrophy.

1.6.5 BA Reduce Fat Deposition

Beta-adrenergic agonists (BA) such as clenbuterol have strong lipolytic impacts in both humans and animals (Belahsen and

Deshaies, 1992, MacRae et al., 1988, Rothwell and Stock, 1985, Rothwell et al., 1987). Both GH and BA are known as partitioning agents because they partition nutrients and energy toward lean or protein accretion and markedly decrease fat deposition (Bergen and Merkel, 1991). Glycogenolysis and fat mobilisation increased in calves following BA administration (Blum and Flueckiger, 1988). BA can effect lipid metabolism and stimulate lipolysis in cattle (BLUM et al., 1982). BA reduce deposition of body fat by stimulation of lipid mobilization and by inhibition of lipid synthesis (Yang and McElligott, 1989). Due to the stimulated increased skeletal muscle size, these increased fibres may play an important site for adrenergic induced thermogenesis in human and animals (Thurlby and Ellis, 1986). There was no effect of dietary ractopamine on fat deposition in growing gilts (Dunshea et al., 1993). Fat deposition was not affected in growing lambs treated with zilpaterol hydrochloride (ZH) (Avendano-Reyes et al., 2011). These differences may be due to experimental conditions and agent type.

1.7 Growth Hormone (GH)

Growth hormone (GH) is a protein hormone product of the anterior pituitary gland, and it plays a key role in regulating growth and metabolism in many different animals. GH stimulates increases in muscle, and bone growth, increased milk production, as well as decreased fat deposition (Etherton and Bauman, 1998). In the 1920s, GH was first identified as a substance in the pituitary gland that affects the growth rate of rats (Evans and Simpson, 1931). There are different forms of GH depending on the amino acid position at the amino terminus (Wood et al., 1989). Recombinant DNA biotechnology has led to the production of recombinant bovine ST (bST) and porcine ST (pST) that are used commercially to enhance growth and lactation (Etherton and Bauman, 1998).

Compared to BA, which appears to act directly on muscle via β-AR2, the effects of GH appears to be more global as it exerts effects at multiple sites. One of the tissues that GH is known to effect is the liver as it is the main site of GH binding (Pell et al., 1990). Therefore by altering the rate of nutrient absorption GH could not only impact on liver metabolism but change the nutrients that would be available to peripheral tissues such as muscle, thereby influencing its capacity for growth. In addition GH may change the endocrine status within the circulation which would impact on tissues (Pell et al., 1990).

The biological effects of GH can be divided into somatogenic effects (e.g. cell proliferation) and metabolic effects (Rechler and Nissley, 1990). With metabolism of nutrients are being directly regulated by GH or indirectly via the stimulation of IGF-I synthesis (Table 1-5).

Table 1-5: Biological effects of growth hormone in farm animals during growth and lactation. Adapted from Etherton and Bauman (1998).

Tissue	Physiological process affected
Skeletal muscle (growth)	Protein accretion
	↑ Protein synthesis
	↑ Amino acid and glucose uptake
	↑ Partial efficiency of lysine utilization
Bone (growth)	↑ Mineral accretion paralleling tissue growth
Mammary tissue (lactation)	↑ Synthesis of milk with normal composition
	↑ Uptake of nutrients used for milk synthesis
	↑ Activity per secretory cell
	↑ Maintenance of secretory cells
	↑ Blood flow consistent with change in milk synthesis
Adipose tissue	↓ Glucose uptake and glucose oxidation
	↓ Lipid synthesis if in positive energy balance
	↑ Basal lipolysis if in negative energy balance
	↓ Insulin stimulation of glucose metabolism and lipid synthesis
	↑ Catecholamine-stimulated lipolysis
	↓ Ability of insulin to inhibit lipolysis
	↓ Glucose transporter-4 translocation (?)
	↓ Transcription of fatty acid synthase gene
	↓ Adipocyte hypertrophy
	↑ IGF-I mRNA abundance
Liver	↑Glucose output
	↓ Ability of insulin to inhibit gluconeogenesis
Intestine	↑Absorption of Ca, P required for milk or bone (growth)
	↑ Ability of 1, 25 vit. D3 to stimulate calcium-binding protein
	↑ Calcium-binding protein
Systemic effects	↑IGF-I and IGFBP-3
	↓ IGFBP-2
	↑ Acid-labile subunit
	↓ Amino acid oxidation and blood urea nitrogen
	↓ Glucose clearance
	↓ Glucose oxidation
	↓ Response to insulin tolerance test
	↑ Free fatty acid oxidation if in negative energy balance
	↑ Cardiac output consistent with increases in milk output
	↑ Enhanced immune response

1.7.1 GH Regulation

GH plays an important role in body growth and metabolism. Secretion of GH is regulated either by a hypothalamic peptide growth hormone releasing factor (GHF), also known as GH-releasing hormone (GHRH), or growth hormone secretion can be inhibited by somatostatin that inhibits from the pituitary gland (Tuggle and Trenkle, 1996, Leshin et al., 1994). GHRH is the main physiological motivation for secretion and synthesis of GH (Frohman and Jansson, 1986, Mayo et al., 1995, Rivier et al., 1982), while somatostatin (SRIF) is the main hypothalamic peptide that inhibits GH release from pituitary (Olias et al., 2004).

Once GH is secreted by the pituitary gland into the blood, it effects peripheral organs to support production of insulin like growth factor- I (IGF-I; also called somatomedin C)(Cuttler, 1996). GH does have direct effects on growth, but most actions of the GH are mediated by IGF-I. Regulatory feedback of GH production is via GH, IGF-I, GHRH, and SRIF. Other factors are also having a regulatory effect. Neurotransmitters are considered the main components influencing GH axis by acting at the brain and hypothalamus (Etherton and Bauman, 1998). It has been suggested that paracrine/autocrine factors may act within the pituitary to further regulate GH secretion. These numerous elements define the quantity of GH secreted from pituitary gland and the mode of GH secretion (Cuttler, 1996). The liver is the major source of insulin-like growth factor I (IGF-I) production and therefore has an important role in growth and development (Yakar et al., 1999). The regulation of GH excretion is illustrated in (Figure 1-7).

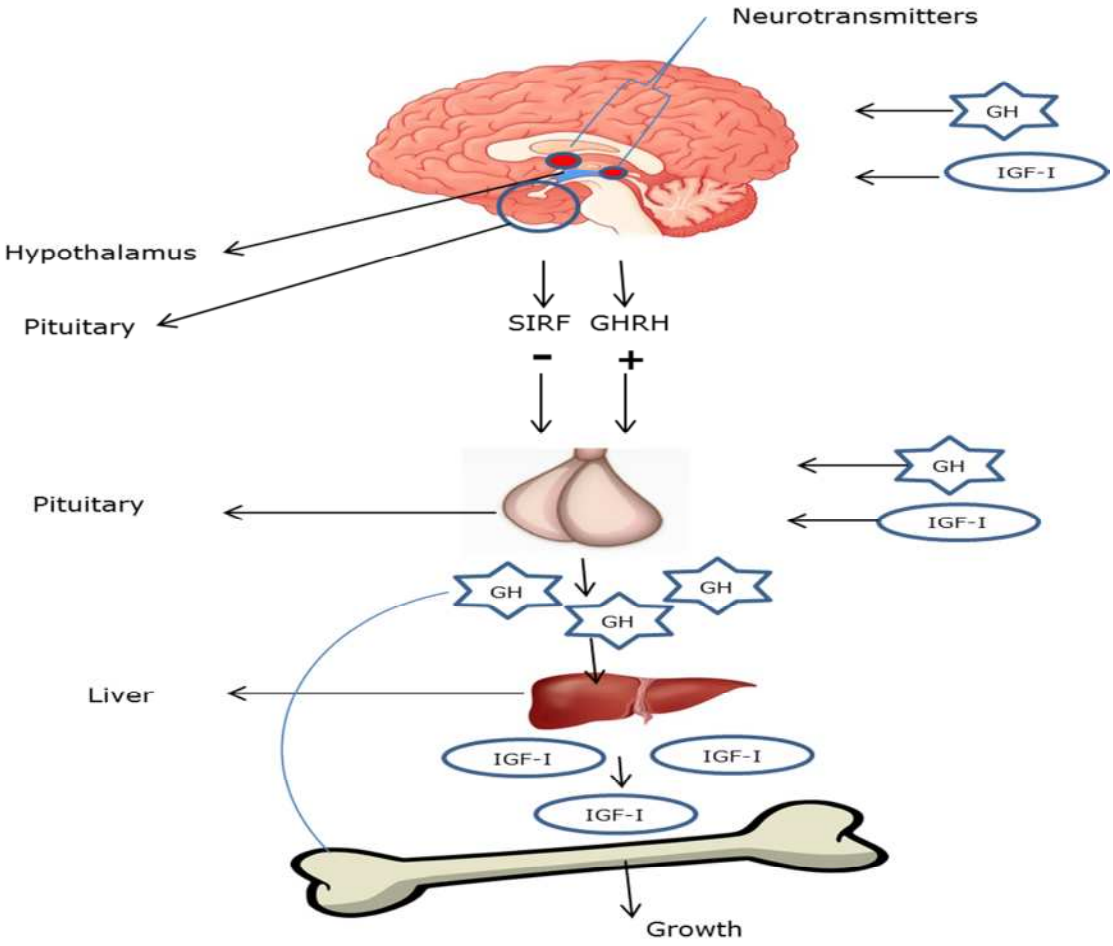


Figure 1-7: Regulation of GH excretion

The figure shows the pituitary GH secretion is under the stimulatory GH-releasing hormone (GHRH), or the inhibitory somatostatin (SRIF). The production of insulin-like growth factor I (IGF-I) in peripheral tissues, IGF-I act to stimulate growth with or without GH.

1.7.2 GH and IGF-I

GH regulates the most important physiological mechanisms, including body growth and development, as well as metabolism of carbohydrate and lipid, directly via activation of the GH-receptors (GHRs) or indirectly via IGF-I, generated mostly by the liver in response to GH stimulation. However, the majority of somatotropic and metabolic actions of GH are mediated by IGF-I, which is released from the liver (Isaksson, 2004, Muller et al., 2003). It appears that GH has direct effects and stimulates increased synthesis of IGF-I in most tissues, especially in the liver, but also

skeletal muscle (Gosteli-Peter et al., 1994). The liver is the major source of serum IGF-I production, administration of GH cause increased expression of IGF-I mRNA and protein (Mathews et al., 1986). IGF-I is the predominant form of IGF expressioned (Nakae et al., 2001). IGF-I is able to act in an endocrine manner but can also be generated in the specific tissues in response to growth promoting stimulus thereby potentially acting in both an autocrine and paracrine fashion. This suggests that IGF-I induced muscle growth may be the result of a response to multiple stimuli via autocrine/paracrine or endocrine action (Loughna et al., 1992, Eppler et al., 2007).

A study by Gosteli-Peter et al. (1994) reported that skeletal muscle in hypophysectomized rats IGF-I mRNA concentrations increased 20-fold after GH treatment whereas IGF-I administration only increased skeletal muscle IGF-I mRNA 2.5-fold. This suggested that regulation of skeletal muscle growth may be associated with the autocrine/paracrine generated IGF-I concentrations rather than the circulating IGF-I. GH treatment does increase IGF-I mRNA concentrations in muscle (Loughna et al., 1992, Lemmey et al., 1997). Muscle hypertrophy is associated with increased muscle IGF-I levels. IGF-I receptor mediates the actions role of IGF-II on growth before birth and IGF-I after birth (Nakae et al., 2001). Although, IGF- I receptor (IGF-IR) and the insulin receptor (IR) have homologous structures they have different biological actions. Most of the effects exerted by IGF-I are mediated by the IGF-IR, which has tyrosine kinase activity and signals through the phosphatidylinositol 3 kinase (PI3K)/AKT pathway. IGF-I can also bind to the IR but with much lower than to the IGF-IR (Pandini et al., 2002). GH treated animals, resulting in an increased in liver and muscle IGF-I mRNA levels (Lemmey et al., 1997) was associated with increased IGF binding proteins (IGFBP) (Eppler et

al., 2007). There are six types of IGF-binding proteins (IGFBPs), that are primarily found within circulation. These IGFBPs are proteins capable of binding IGF-I and IGF-II but not insulin. Most serum IGF-I preferentially binds with IGFBP3 and is found in the acid labile subunit (ALS). Whereas IGFBP6 has a marked preferential binding affinity for IGF-II (Bach et al., 1993). Complexes of IGF-IGFBP can access tissue and leave the circulation if they are not binding with the ALS (Velloso, 2008). IGFBPs can be phosphorylated which affects their affinity for IGFs (Kajantie et al., 2002). It has been suggested by pharmacological and genetic studies in animals that a combination of both GH and IGF-I may be used for optimal therapy for some statural growth in children (Isaksson, 2004).

1.7.3 GH and IGF-I Signalling Pathway with Specific Focus on Muscle

Given that this thesis is predominantly focused on the effects of GH-IGF-I in muscle the description of the signalling pathway will be described, however this does have significant similarities with other tissues. Binding of IGF-I to its receptor induces a modification in the IGF-I receptor tyrosine kinase, resulting in activation of tyrosine kinase, as well as phosphorylation of insulin receptor substrate (IRS), which then leads to activate phosphatidylinositol-3-kinase (PI3K) (Glass, 2003b) (Figure 1-8). The effects of IGF-I on skeletal muscle hypertrophy is associated the PI3K pathway as direct stimulation of PI3K is a strong mediator of mammalian muscle hypertrophy (Murgia et al., 2000, Bodine et al., 2001, DeVol et al., 1990). Activation of PI3K leads to the production of phosphoinositide-3, 4, 5-triphosphate (PIP3) and the subsequent activation of phosphoinositide dependent kinase 1 (PDK1) which

then activates the kinase Akt (Protein kinase B) via phosphorylation at serine 308 (Glass, 2003b). Activation of Akt stimulates protein synthesis through the kinase mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3b (GSK3b), as well as inhibits degradation of protein via repressing the forkhead transcription factor FoxO family (Schiaffino and Mammucari, 2011). Activated mTOR can then activate P70S6K which influences protein translation (Figure 1-8). In addition activated PDK1 can directly activate the protein P70S6K, suggesting that Akt may be unnecessary for signalling to P70S6K (Pullen et al., 1998). However, it is generally well accepted that the Akt and mTOR pathway influences the processes that affect skeletal muscle growth (Pallafacchina et al., 2002). The mTOR activation leads to increased protein synthesis through two ways. First, mTOR activates p70S6K, a positive regulator of protein translation. Second, mTOR inhibits the activity of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (also known as PHAS-1), a negative regulator of the protein eukaryotic initiation factor (eIF-4E) (von Manteuffel et al., 1996, Hara et al., 1997). Glycogen synthase kinase-3b (GSK3b) is another target for Akt that associated with glycogen and protein synthesis in muscle hypertrophy. Activation of Akt would result in the inactivation of GSK3β (Cross et al., 1995). Expression of an inactive form of GSK3β (phosphorylating and an inactivating agent of glycogen synthase) has been reported to stimulate hypertrophy in skeletal myotubes (Rommel et al., 2001). In addition to the effects on protein synthesis the activation of Akt represses the activity of the transcription factor FoxO. This transcription factor is required for the gene expression of the muscle specific ubiquitin ligases, muscle ring finger-1 (MuRF1) and muscle atrophy F-box (MAFbx) (Schiaffino and Mammucari, 2011, Guertin et al., 2006, Li et al., 2007). Both of which have been demonstrated to be essential for

protein degradation or muscle atrophy (Glass, 2003b), therefore their suppressed gene expression would reduce protein degradation and thereby accentuate the effects of the IGF-I signalling pathway on protein synthesis.

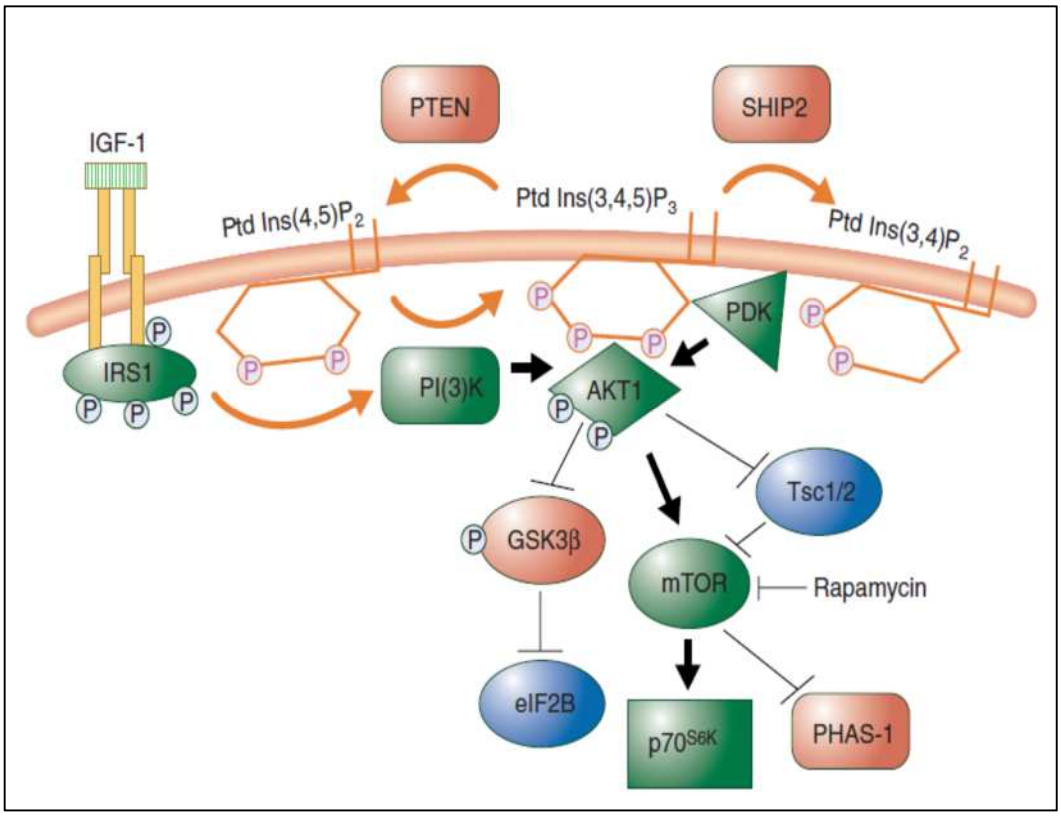


Figure 1-8: Signalling pathways of IGF-I in muscle

This diagram indicates the essential role of the PI3K/Akt cascade. Negative effects of proteins on hypertrophy are indicated in red. Positive effects of proteins on hypertrophy are indicated in green. Blue indicated to that proteins have not been assayed for their role in hypertrophy. Insulin receptor substrate 1 (IRS1), PTEN and SHIP are two different types of phosphatases, Ptd Ins(3,4,5)P3 refer to phosphatidylinositol- 3,4,5-trisphosphate, PDK means 3'-phoshphoinositide-dependent protein Kinase, Glycogen synthase kinase 3β (GSK3β), Tsc1/2 refer to the tuberous sclerosis complex 1 (Tsc1) and Tsc2 proteins that can inhibit mTOR-mediated signalling. PHAS-1 (also known as 4E-BP1), rapamycin is inhibiter of mTOR. Reprinted from Glass (2003b).

1.7.4 Response of GH in Animals

Growth hormone is involved in regulating growth and metabolism in the body (Gouty-Colomer et al., 2010). GH significantly improved the live weight gain, increased protein content and decreased fat deposition in lambs treated for 10 weeks with GH

(Pell et al., 1990). These authors pointed out that the weight gain of the lambs treated with GH was 36% greater than that of controls, also they found increased protein deposition and decreased fat deposition in carcass composition of the lambs. Likewise in steers at high dosage (160mg/wk) of recombinant bovine somatotropin (rbST) increased protein synthesis. The protein in the rib increased by 9.4% and fat decreased by 11.8% at high dosage of recombinant bovine somatotropin (rbST) in steers (Dalke et al., 1992).

There is some evidence that administration of pST to growing pigs increases weight gain by about 15% and muscle growth by over 70% and decreases fat deposition approximately 80%, with decreased feed intake of approximately 12% (Etherton, 2004). Average daily gain in heifers treated with 15 mg/d GH was approximately 18% higher than controls but without affecting in feed intake (Vestergaard et al., 2003). However, feed efficiency dramatically improved in GH treated lambs compared with controls (Pell et al., 1990). Administration of bST may increase growth rate over 25% in heifers (Vestergaad et al., 1995).

GH has a nutrient partitioning effect, redirecting much of the glucose from adipose tissue to muscle. The energy available from glucose metabolism can then be used to stimulate an increase in muscle protein synthesis (Etherton, 2000). However the effect of GH in different muscles depends on differences in fibre type composition and growth impetus (Boge et al., 1995).

1.7.5 Biological Effects of IGF-I

As described IGF-I is a major growth factor predominantly produced via the liver which mediates a large number of the effects of GH on body growth. IGF-I regulates the essential processes

necessary for cell proliferation and differentiation (Brisson and Barton, 2012) and is considered to be one of the most effective growth factors in animal production systems as it effects skeletal muscle mass (Ryall et al., 2010). A positive correlation between plasma IGF-I and growth rate is often observed, suggesting that IGF-I stimulates lean tissue growth (Owens et al., 1993, Owens et al., 1999). Both IGF-I and Ca2+/calmodulin-dependent transcriptional routes are the main pathways regulating muscle growth (Al-Shanti and Stewart, 2009).

Circulating IGF-I from liver, muscle, or fat, presumably stimulates lean tissue growth through endocrine mechanisms or acts via production of IGF-I from somatic tissue, suggesting that IGF-I acts as paracrine growth factor (Brameld et al., 1996, Coleman et al., 1994). Because of the relative high cost of exogenous IGF-I, only a few studies have administered IGF-I to livestock (Sillence, 2004). The insulin-like growth factors (IGFs) family have multiple biological roles in skeletal muscle mass development in the embryo, foetus and adult (Stewart and Rotwein, 1996). Some biological effects of IGF-I are shown below (Table 1-6).

Table 1-6: Some biological effects of IGF-I. Adapted from Etherton (2004).

Increases glucose uptake (in vitro, in absence of IGFBPs)
Promotes wound healing
Stimulates myogenesis
Inhibits apoptosis
Chemotactic
Activation of cell cycle genes
Enhances steroidogenic responsiveness to LH/hCG in Leydig cells
Stimulate progesterone production in granulosa cells
Increases lipid synthesis (in vitro, in absence of IGFBPs)
Stimulates DNA synthesis
Stimulates protein synthesis
Stimulates RNA synthesis
Stimulates cell proliferation

1.7.6 IGF-I induced Muscle Hypertrophy

Skeletal muscle hypertrophy can be regulated by different molecular mechanisms, but the most important and common processes involved are, the activity of satellite cells as well as the rate of the transcription of gene and protein translation both in satellite cells and myofibres. IGF-I has effects on all of these mechanisms, stimulating increases in satellite cell proliferation, expression of actin mRNA in muscle and synthesis of protein (Florini et al., 1996, Chakravarthy et al., 2000). Therefore, increased IGF-I in the body plays a crucial function in mediating muscle hypertrophy (Adams and McCue, 1998).

The growth effects of IGF-I can be influenced by nutrition as IGF-I treatment with a high protein diet (HPD) resulted in a significant increase in muscle protein synthesis compared to those fed a normal protein level (Zdanowicz et al., 1995). A considerable number studies have reported that plasma IGF-I levels and IGFBPs are changed by feeding level, concluding that nutrition is one of the

major regulators of plasma IGF-I (Thissen et al., 1994). However, the response of IGF-I to nutrition varies according to the stage of development and age (Oster et al., 1996, Fliesen et al., 1989). There is a pronounced effect of IGF-I on the utilisation of nutrients, as IGF-I stimulates increased uptake of glucose and amino acids by muscle presumably to support the rate of protein synthesis (Turkalj et al., 1992). There are reported effects of nutrients influencing the GH-IGF-I axis. In pig hepatocytes, there was a reported interaction between glucose and amino acids with GH, which regulated the expression of IGF-I and GH-receptors mRNA (Brameld et al., 1999). It has been suggested that variations in endocrine based stimulation and local changes in the IGF-I system may be necessary to give changes in muscle growth seen when animals have a different nutritional status (Oksbjerg et al., 2004). Slow oxidative fibers have a high binding of IGF-IR in compared to fast glycolytic fibers, indicating that IGF-IR play an important role in growth and development (Louveau et al., 1996). This reflects the rate of protein turnover as it is higher in slow oxidative fibres compared to fast glycolytic fibres. Protein synthesis decreases with increasing age. In the skeletal muscle the number of IGF-I receptors or expression of mRNA along with IGFBP-3 decreased in slow and fast twitch muscles with advancing age (Louveau et al., 1996, Peng et al., 1996). These observations further indicate that changes in muscle protein turnover may be related to GH/IGF-I axis. It is not only nutrient status that alters IGF-I concentrations. A study by Adams and McCue (1998) has demonstrated a positive relationship between the expression of muscle IGF-I and the increase in muscle DNA content in the overloaded muscle. Unlike many growth factors, IGF-I can stimulate both proliferation and differentiation of myoblast cells (Florini et al., 1996).

Both *in vivo* and *in vitro* studies, it has been accepted that local synthesis of IGFs and systemic IGF-I is involved in postnatal muscle hypertrophy and regeneration (Musaro et al., 2001).

1.7.7 Effects of GH and IGF-I on Fat Metabolism

Although IGF-I is known as a mediator of the growth promoting effects of GH there are varying metabolic effects of these two hormones. For example, GH leads to increased insulin and glucose plasma concentrations and reduced sensitivity of insulin (GH caused insulin resistance), whilst IGF-I leads to decreased insulin and GH concentrations and increased insulin sensitivity (Hussain et al., 1994). The lipid metabolism changes induced by GH have been widely studied in animals especially in pigs. GH decreased fat deposition in pigs irrespective of age, sex, or genotype (Louveau et al., 2015). GH treatment decreases lipid synthesis in both pigs and humans adipose tissues (Frick et al., 2002). GH significantly effects adipose tissue and fat metabolism, for example treatment of lambs with GH for 10 weeks decreased fat deposition (Pell et al., 1990). GH treatment decreased the activity of lipoprotein lipase (LPL), a key enzyme in the regulation of the fatty acids flux in adipose tissue, suggesting that GH may participate in regulating the activity of both LPL and hepatic lipase (HL) (Oscarsson et al., 1999).

Insulin like growth factors (IGF-I and IGF-II) are very important in the regulation of fat and muscle in farm animals (Owens et al., 1993). There is a positive correlation between plasma IGF-II and back fat (Owens et al., 1999). The effect of IGF-I on lipogenesis in adipose tissue appears to be through decreased serum insulin levels, rather than direct effects, which reduce the capacity of adipocytes to metabolise glucose. In muscle, IGF-I increased the

glycogen content and insulin-stimulated glucose incorporation into glycogen, indicating that IGF-I has different effects on glucose uptake in adipose tissue and skeletal muscle (Frick et al., 2000). Decreased fat deposition in IGF-I treated animals is thought to be due to decreases in lipogenesis rather than an increase in lipolysis, and includes a reduction of insulin sensitivity in adipocytes (Louveau and Gondret, 2004). Although it is seems clear that the major role of GH is to minimize total body fat, the mechanism by which GH stimulates these effects at the cellular level is still unknown. Thus the role of GH and IGF-I on adipose tissue development and metabolism are still complex (Louveau and Gondret, 2004).

1.8 General Summary

In the next 40 years there will be increases in demand for food and meat consumption in the world which will impact on global food security. Growth promoters may be able to meet this demand by various mechanisms but would ultimately, lead to increased muscle mass and feed efficiency. Some of the growth promoters are more effective than others. For example, BA increases muscle mass more than GH and steroids, whilst GH decreases fat deposition more than steroids. The effect on growth of these agents appears to involve processes across the tissues which have a central metabolic role in nutrient utilization, such as muscle and liver. Although the signalling mechanisms utilised by GH and BA have been identified across a wide variety of cells the distinct mechanisms (metabolic changes) by which these agents have their effects in muscle has not yet been characterised. This is particularly the case for the effects of BA on muscle hypertrophy. Although there is a clear understanding of how these agents will affect glycogenolysis and catabolic processes, it is not yet apparent how they also increase protein accretion. By identifying the processes, such as the co-ordinated regulation of genes, which influence the metabolic changes associated with growth stimulated by GH and BA, it may be possible to manipulate these mechanisms by selection (genetic) or by new agonists to improve feed utilization efficiency.

1.8.1 Hypothesis

- Growth promoting agents, such as beta-adrenergic agonists, (BA) and growth hormone (GH) enhance muscle growth through the modification of metabolism which then impacts on protein accretion. Changes in energy metabolism in the

liver and muscle in ruminants are key to the effectiveness of these growth promoters.

1.8.2 Objectives

The objectives of this study are:

- To examine and compare how the anabolic agents BA or GH effect lamb growth.
- To identify whether the muscle hypertrophy stimulated by the growth promoters BA or GH is associated with changes in the gene expression of distinct metabolic pathways in muscle and liver.
- To determine whether the changes in the gene expression of metabolic pathways stimulated by BA and GH is supported by changes in circulating metabolite concentrations.
- Utilizing cell culture based experiments in the myogenic C2C12 cell line determine if the genes associated with the GH and BA response in sheep are similarly affected during myogenesis, when treated with agents that stimulate BA and GH signalling pathways.

2 Materials and Methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich Ltd (poole, UK) or Fischer Scientific Ltd (Loughborough, UK). Molecular biology reagents were obtained from either Promega (Southampton, UK) or Roche Ltd (Burgess Hill, UK). Specific qPCR reagents as well as some specific molecular biology reagents were obtained either from Roche or from Invitrogen (Paisley, UK). Western blot criterion (TGX) precast SDS-polyacylamide gels (SDS-PAGE) and some specific reagents were obtained from (BioRad, US) and Prime blocking agents and nitrocellulose membrane were obtained from (GE Healthcare Life Sciences, UK) .Water ribonuclease (RNase)/ deoxyribonulease (DNase) free used for molecular biology were purchased from Sigma-Aldrich Ltd.

2.2 Animal Trials

Animal procedures were conducted in accordance with the provisions the UK Home Office Animals (Scientific Procedures) act of 1986. The pig trial was carried out by Dr Kevin Ryan, Dr David Brown and Dr Molebeledi Mareko. The sheep trial was carried out by Dr Krystal Hemmings and Dr Zoe Daniel.

2.2.1 Animals and Sampling (Sheep)

Twin male lambs were kept with their mothers until 53 ± 4 days of age, then when they were weaned and each pair was divided into two age groups, day 60 (D60) and day 120 (D120). The D60 animals were kept for an experiment that was carried out at day 60. The D120 lambs were used for the experiment described in this

thesis. About 28 days before weaning creep feed (Appendix 1A) was started, and then from weaning onwards lambs were given *ad-libitum* access. At seven days before treatment, at day 120, lambs were individually penned with *ad-libitum* access to creep feed. Thirty one of these male lambs at 120 days old age were randomly divided into three groups. Initial body weights were not significantly different between treatments ($P>0.05$). All treatment groups were fed creep feed. The control group (C, n=11) were fed *ad-libitum*; the BA group (n=10) were fed *ad-libitum* the diet containing 10 ppm cimaterol/kg feed and the GH group (n=10) were fed ad-libitum and administered a single subcutaneous injection of bovine growth hormone (3.75mg/kg/BW) on the nape of the neck as described by McLaughlin et al. (1994). After six days animals were slaughtered, and blood was collected in EDTA tubes, frozen as whole blood or as plasma by centrifuge at 3000xg for 10 minutes and both was stored at -40^0C. Liver, *longissimus dorsi* (LD) and *supraspinatus* (SS) muscles were frozen in liquid nitrogen and stored at -80^0C.

2.2.2 Animals and Sampling (Pigs)

Forty five (45) Large White x Landrace gilts (PIC Ltd, UK) Weighing 85(±5) kg were given time to adapt to the feed and environment for 5 days, before being distributed to one of three treatment groups. The control group (C, n=15) were fed ad-libitum a standard commercial diet with high energy and high protein content (Appendix 1B), the β-adrenergic agonist group (BA, n=15) were also fed *ad-libitum* the same diet, but containing Ractopamine^{TM} (at 10mg/kg) and the growth hormone group (GH, n=15) were fed the same commercial diet *ad-libitum* and administered Reporcin^{TM} (10mg) by intramuscular injection on days 0, 2, 4 and 6. After slaughter on day 7, liver weights were recorded

and samples of *Longissimus Dorsi* (LD) muscle and liver were collected and snap frozen in liquid nitrogen.

2.2.3 Statistics

Data were analysed by one way ANOVA (Genstat, edition 15), followed by a Post Hoc Dunnett's test. All data displayed as mean ± standard error of the mean (SEM). Significance was accepted at $P<0.05$.

2.3 Glycogen Assay

Glycogen content was determined as described previously (Dreiling et al., 1987). One gram of crushed sample was homogenised in 4ml of 8% (v/v) perchloric acid (PCA) in a fume hood. One millilitre of supernatant was removed, and then centrifuged at maximum speed for 5 minutes at room temperature. One hundred microliter was transferred of each of the supernatants. To neutralize the solution 1.33% (v/v) PCA: saturated sodium bicarbonate: 0.4M sodium acetate pH4.8; ratio (1.25, 1.25) were mixed. Five milligrams of bovine liver glycogen were dissolved in one millilitre of neutralized PCA solution to give glycogen stock solution. Glycogen standards were prepared containing 0 to 200µl. Six microliters of amyl glucosidase (80Units/ml) (Sigma Aldrich) was added to each of the standard and 10µl to 350µl of neutralized supernatant and incubated at 37^0C for 30 minutes to convert glycogen into glucose. Samples were then heated at 100^oC for 5 minutes to stop enzyme activity and stored on ice to cool. Glucose concentration was determined by incubating 10µl of each standard and sample with 150µl of Enzymatic Glucose Assay (Thermo Electron Corporation, UK). This incubation was carried out in 96

micro-titre plate. The plate was incubated at room temperature for 20 minutes. Absorbance was measured by (Micro plate reader, Model 680XR, Bio-Rad, UK) at 550nm wavelength.

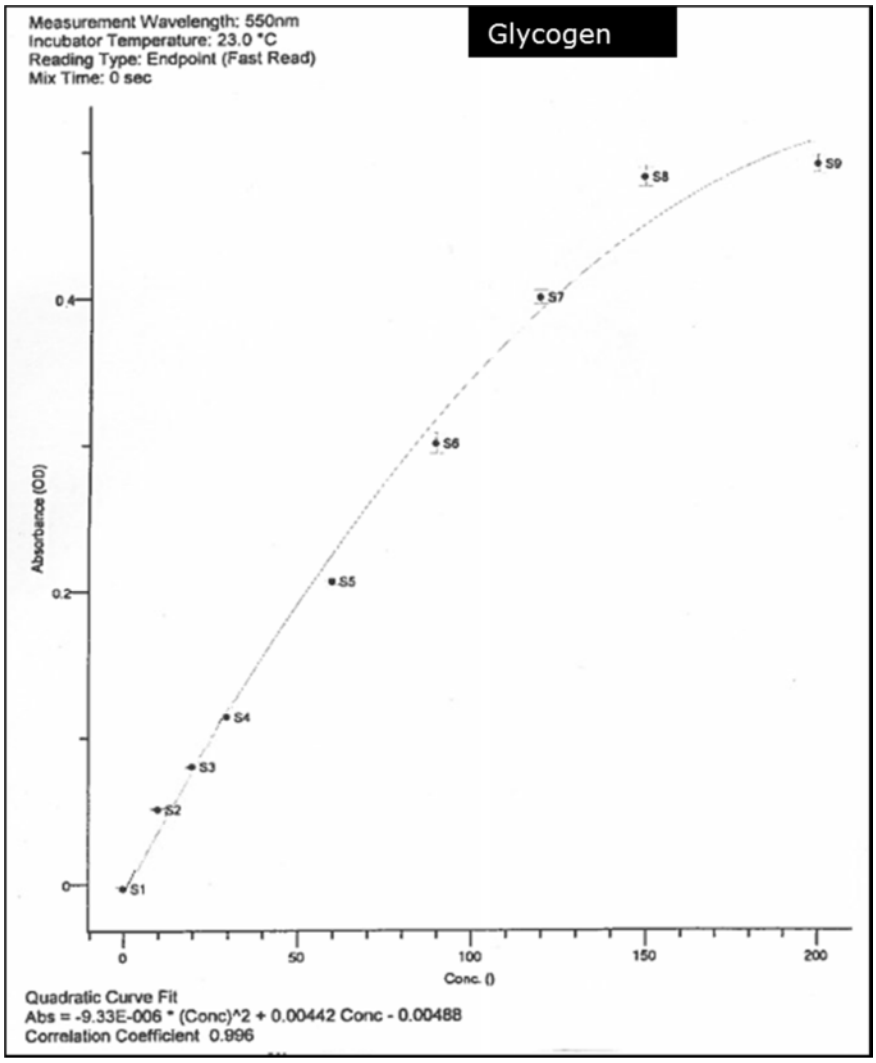


Figure 2-1: Standard curve of glycogen measurement.

The X axis is the glycogen concentration (µg/ml) of standard curve. The Y axis is the absorbance (Optical density). Triplicate for each sample within the range of the standard curve. Error bar means ±SED.

2.4 Determination of Protein Content by Lowry Assay

Protein content of liver samples was determined in triplicate using the Lowry assay (Lowry et al., 1951). One gram of crushed sample homogenized in 3ml of buffer (0.05 % (w/v) Sodium dodecyl sulphate, 0.1M sodium chloride, and 15mM trisodium citrate).

From the stock standard solution of 5mg/ml BSA (bovine serum albumin) in 0.1M NaOH a range of protein standard starting 0 to 60µg was prepared of protein standards (50µl) as well as 0.1M NaOH as blank, were pipetted into a 96 well micro titre plate. Then 50µl of fresh prepared solution 1 (5ml 2%(w/v) Na_2CO_3 in 0.1M NaOH, 0.5ml 1%(w/v) $CuSO_4$, 0.5ml 2%(w/v) KNa tartrate was added and incubated at room temperature for 5 min. After that 50µl of fresh solution 2 was added (5ml 0.1M NaOH, 0.5ml Folin's clocalteu's reagent) and incubated for 20 minutes at room temperature. Absorbance was measured by (Micro plate reader, Model 680XR, Bio-Rad) at 655nm.

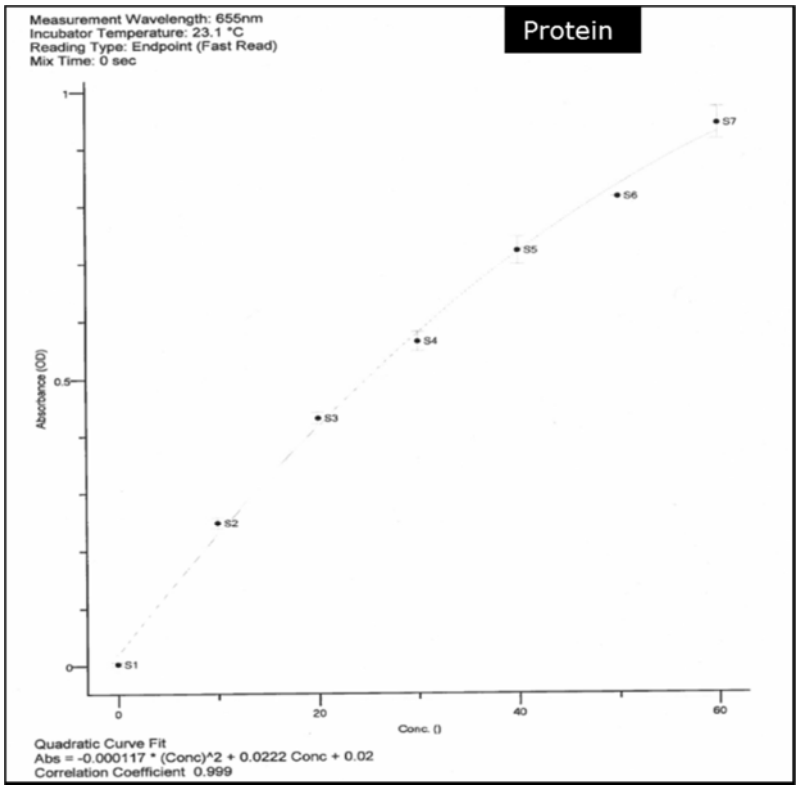


Figure 2-2: Standard curve of protein measurement by Lowry assay

The X axis is the protein concentration (µg/ml) of standard curve. The Y axis is the absorbance (Optical density). Triplicate for each sample within the range of the standard curve. Error bar means ±SED.

2.5 Determination of DNA Content by Hoechst Fluorimetric Method

Cellular DNA content was determined using a modification of the fluorometric DNA assay, described by Rago et al. (1990). Stock solutions of, 10x TNE (100mM Tris-Base, 1M NaCl, 10mM EDTA), 1mg/ml Hoechst dye solution (Sigma) were prepared and 20 µg/ml of fish DNA dissolved in buffer (0.05 % (w/v) Sodium dodecyl sulphate, 0.15M NaCl, 15mM tri-sodium citrate). One gram of crushed tissue was homogenized in 3ml of buffer, then 1ml of homogenized sample placed into (1.5ml) tube and centrifuged at 13000xg at 4-2^{0}C for 10 minutes. One hundred microliter of liquid was diluted to 100 (1:100) in buffer.

Serial dilutions of the stock standard in a range from 20 to 0.3µg/ml were prepared and 100µl of standard, blank and samples (10µl of sample + 90µl of buffer) were added to each well. To each well was added 100µl of dye solution (20µl of 1mg/ml stock dye solution to 15ml of 2x TNE) and the fluorescence assessed using plate reader (Micro plate readers, BMG LABTECH, FLUOstar OPTIMA).

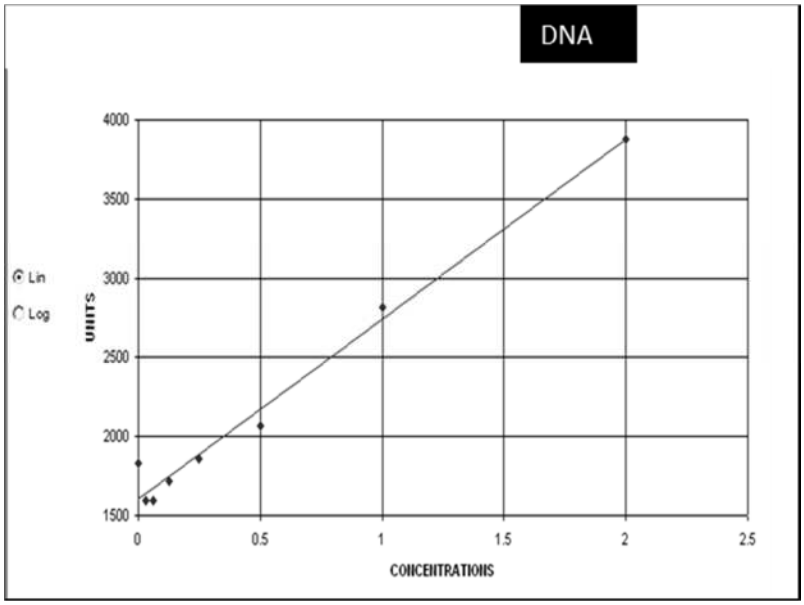


Figure 2-3: Standard curve of DNA quantification

The X axis is the DNA concentration (µg/ml) of standard curve. The Y axis is the fluorescence intensity (Arbitrary Units). Triplicate for each sample within the range of the standard curve. Error bar means ±SED.

2.6 Lipid Extraction of Liver

Liver samples were crushed in liquid nitrogen using pestle and mortar. A bout 1g of crushed sample was weighed into 50ml graduated conical tube. Then 6.3ml of methanol was added to the sample and homogenised for 1 min using a polytron (Kinematica AG). The sample was homogenised again for another 1 min after adding 12.7ml of chloroform, then 4.7ml of 118mM potassium chloride was added to the sample. The extraction solutions were

then mixed thoroughly and centrifuged for 10 min at 1730 x g at room temperature in order to separate the organic and aqueous phases. By using glass pasture pipette, the chloroform containing the lipid in the lower phase was transferred to pre-weighed tube. Then samples were dried under a steam of nitrogen gas and the tubes re-weight to calculate the amount of lipid extracted.

2.7 Total RNA Extraction for QPCR

2.7.1 RNA Extraction

LD, SS muscles and liver were crushed in liquid nitrogen using a pestle and mortar. One millilitre of Trizol (Invitrogen) were added to 100mg of crushed tissues, then homogenised on ice with a polytron for 2x 25 second and transferred to a fresh 1.5ml tube. The homogeniser blade was rinsed in a beaker of distilled water, cleaned and dried between each sample to prevent any cross contamination of RNA. Homogenates were centrifuged at 13000xg (Microfuge 22R centrifuge, Beckman Coulter Inc.) for 10 min at 2-8^{0}C, and the resultant supernatant transferred to a fresh 1.5ml tube where the pellet was discarded. To the supernatant 200µl of chloroform was added and vigorously shaken. The resulting mixture was centrifuged at 13000xg for 10 min at 2-8^{0}C and the upper colourless phase was transferred to a fresh 1.5ml tube, mixed with 250µl of isopropanol and 250µl of high salt precipitation buffer (0.8M sodium citrate/1.2M NaCl) to remove glycogen from RNA. The precipitate was incubated at room temperature for 10 min, then centrifuged as before and the RNA pellet was washed with 1ml of cold 75% (v/v) ethanol, vortexed and re-centrifuged at 13000xg for 10 min at 2-8^{0}C. Ethanol was removed and the pellet air dried for 10 min re-suspending in 40µl of RNase free water. To remove any contaminating gDNA was removed by incubating the

RNA solution with DNase. To the 40µl of total RNA was added 5µl of RQ1 RNase free DNase (Promega, UK) and 5µl of 10x DNase buffer was added to re-suspending pellet in 40µl, mixed and spun for short time (about 10 sec.), then incubated for 30 min at 37^0C.

A 150µl of RNase free water and 200µl of phenol/chloroform/isoamylalcohol (25:24:1) was added to the resulting mixture, vortexed and centrifuged at 13000xg for 10 min at 2-8^0C. The upper aqueous layer containing the RNA was removed carefully to avoid contamination with proteins from the interphase or organic phase components and transferred to a fresh 1.5ml tube, then precipitated by adding 15µl of 3M sodium acetate pH5.5 (NaAc) and 375µl of 100 % (v/v) ethanol at -80^0C for 1-2 hours or overnight. The precipitate was spun at 13000xg for 10 min at 2-8^0C, then RNA was washed with 1ml of 75% (v/v) ethanol, vortexes and re-spun as before. The ethanol was removed, the pellet was air dried and re-suspended in 40µl of RNase free water, then stored at -80^0C for further analysis.

2.7.2 RNA and DNA Evaluation

The concentration of RNA and DNA that was diluted in nuclease free water were measured by NanodroPND-1000 (Thermo Scientific, Wilmington, USA). Ratio of absorbance's at wavelengths 260 /280 and 260/230nm were used to assess the purity of nucleic acids. Ratio reading of 1.8-2.0 at 260/280 was accepted for RNA and 1.6-1.8 for DNA. For all samples the concentration of RNA was normalised to 100ng/µl for subsequent use in cDNA synthesis.

2.7.3 Running Agarose Gel Electrophoresis

To examine and check the total RNA integrity by electrophoresis using a non-denaturing agarose gel. Agarose (0.5g) was added to 50ml (1%w/v) of 1X TAE buffer (40Mm Tris, 1Mm EDTA, 0.12 %(v/v) acetic acid). The percentage of agarose that required to be run depended on the size of fragments. The edges of gel cassette were taped with autoclave tape and a comb was inserted in the apparatus. Agarose was heated in microwave to boiling point until it became clear, left to cool down, and then poured into a gel cassette. The gel was then placed in gel tank electrophoresis apparatus filled with 1X TAE buffer. Samples (10µl) that had been diluted to 100ng/µl, were mixed with 2µl of loading dye (for 6X loading buffer, 50% (v/v) glycerol, 0.2%(w/v) bromophenol blue, 5mM sodium phosphate pH 6.8) then run on a 1%(w/v) agarose gel at 100V for 45-60 minutes. The gel was soaked in a 1µg/ml ethidium bromide for 20-30 minutes to stain nucleic acids, and then soaked briefly in water. The gel image was captured using Gel Doc system and MultiAnalyst (Bio-Rad, UK).

2.7.4 cDNA Synthesis

Total RNA was diluted to 100ng/µl on a 96 well plate; the reverse transcription reaction was carried out to synthesise first strand DNA using a cDNA synthesis kit (Transcriptor First Strand cDNA Synthesis Kit, Roche, Burgess Hill, UK). In the PCR tube, 500ng of total RNA was mixed with 2µl of Random Hexamer primer (60µM) and 6µl of DNase RNase free water (Sigma water) to a final volume of 13µl. Lids which were attached together in strips that allowed the wells in a column to be sealed were placed the plate. The plate was then shook to mix and spun in centrifuge at 12,000xg for 30 sec at 4^0C. Then, the mixture was incubated at 65^0C for 10

minutes in a thermal block cycler of PCR machine to denature RNA secondary structure, then immediately cooled on ice. To 5µl of the annealed primer mix was added 4µl of 5x concentrated. Reverse Transcriptase Reaction buffer, 0.5µl of protector RNase inhibitor (20U), 2µl of deoxynucleotide (1mM) mix, and 0.5µl (10u) of Transcriptor Reverse Transcriptase to a final volume of 20µl. The plate was incubated for 10 minutes at 25^0C followed by 30 minutes at 55^0C, then at 85^0C for 5 minutes and then put on ice. At this point single stranded cDNA was completed and diluted fivefold with 80µl of H_2O added to 20µl first strand cDNA reaction mix to create a stock solution and stored at -20^0C for subsequent real-time PCR analysis.

2.7.5 Real – time PCR

Random primed first cDNA, was diluted 1:4 and a pool of samples created. An aliquot of this pool was taken then serially diluted to produce standard curve. Dilutions were two fold serial dilutions with the most dilute sample being at a 1 in 64 dilution of the original sample. The first strand cDNA for individual samples that were to be assessed for gene expression were further diluted by taking the stock and diluting it to 1:8. Real-time PCR reactions were carried out using a Lightcycler 480 (Roche, UK). Reactions contained the following PCR master mix.

7.5µl 1X SYBR Green Master Mix (Roche, Burgess Hill, UK)

0.45µl Forward Primer (0.3mM)

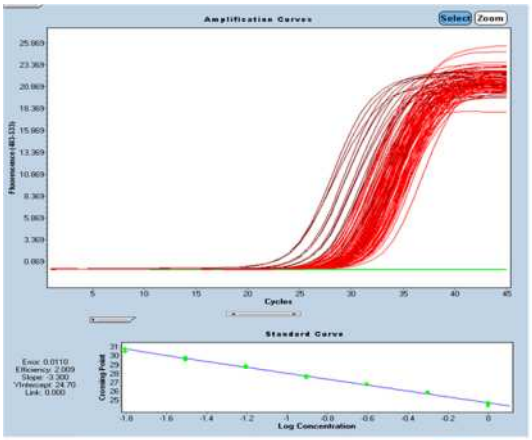
0.45µl Reverse Primer (0.3mM)

1.6µl Water (molecular grade)

A pool of master mix was generated the volume of which was dependent on the number of samples being assayed. Reactions were set up in triplicate in 384 well plates in a final volume of 15µl (10µl PCR master mix + 5µl first strand cDNA sample or diluted pool in every well). Samples and standard curve for different selected genes were ran on Lightcycler 480 PCR machine using the following conditions 50^0C for 2 minutes, 95^0C for 10 minutes, and then 40 cycle of 95^0C for 15 seconds, 60^0C for 1 minute and 72^0C for 1 second.

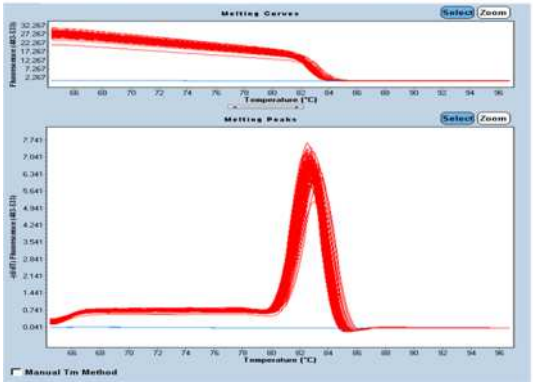
Melting curve analysis was used to make sure that samples were not contaminated and producing only one product and a linear standard curve with acceptable efficiency of 2 and slope of – 3.3 see Figure 2-4.

A



- Standard curve using pool of cDNA with serial dilutions (1:8) cDNA samples (brown lines)
- Expression of sample cDNA within the range of standard curve
- PCR linear slope check
- Optimal gradient =-3.3
- Optimal efficiency =2.0

B



- Melt curve analysis of samples produced only one amplicon

Figure 2-4: Outputs from the Roche LightCycler380.

The figure (A) shows a standard curve of serial dilutions of the template cDNA, linearity of standard curve confirmed by slope and efficiency in Q-PCR reaction to quantify mRNA. The standard curve generated confirms the linearity and permits to quantify mRNA abundance. The figure (B) shows melt curve analysis of the samples. Only one amplicon was produced in the PCR reaction.

2.8 Oligreen for Determining cDNA Quantity

An alternative to the use of a reference gene to normalise gene expression is through use of oligreen (Invitrogen, Paisley, UK). Identification of a reference gene can sometimes be problematic as experimental interventions can often cause significant shifts in global gene expression which makes it difficult to identify a gene whose expression does not change due to treatment. The oligreen protocol utilises a fluorescent chemical that specifically binds with single stranded DNA molecules (Rhinn et al., 2008), therefore it is possible to normalise the gene expression to total first strand cDNA concentrations. In all the gene quantification procedures described in this thesis the concentration of extracted total RNA was assessed after DNase treatment, then normalised to the same concentration which was then followed by the samples being assessed by gel electrophoresis for quality as well as quantity before first strand cDNA was generated. A working solution of oligreen sDNA solution was made by diluting 1:200 in 1x TE buffer (e.g. 5µl oligreen into 1ml 1x TE buffer). A standard curve was made by a serial dilution standard curve from a pool of cDNA. To 5µl of each aliquot of the cDNA standard curve was added 5µl of working oligreen reagent, likewise for 5µl of each sample's first strand cDNA on a 384 well plate. The 384 plate was spun the then incubated in dark for 5 min and run on Roche Lightcycer 480. The data from LC 480 was used to generate a standard curve and thereby determine the relative quantity of cDNA for each sample.

2.9 Primer Design Bioinformatics

For target genes to be measured by Real-time PCR, forward and reverse primers were designed from gene specific sequences for the gene of interest. Target genes were searched in NCBI (National Centre for Biotechnology Information) database. Due to the lack of verified sequences for ovine sequences for target genes were often expression Sequence Tag (EST) cDNAs or partial length cDNAs. To identify these conserved sequences between species a multiple sequence alignment was carried out of mRNA/cDNAs by Clustal W2. The cross species conserved region of target cDNA was then subjected to the standard nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the ovine specific areas of the NCBI sequence database. The ovine sequences identified were then subjected to multiple sequence alignment to verify their identify, in some instances composite cDNA sequences were generated utilising common sequence identified within the multiple ovine cDNAs identified for each target.

After the target cDNA was identified the potential exon boundaries were identified via EnsEMBL database either using ovine sequences or a composite cross species approach. The target ovine cDNAs were used to design forward and reverse, qPCR primers using Primer Express (Applied Biosystems) using the default parameters for Taqman Quantification. Outputs from the software were amplicon sequences, primers/probes, as well as parameters, such as secondary structure. Sheep primers used in this study are shown in Appendix 2. Primers were manufactured by Sigma (UK); Primers were re-suspended to 100mM, and then diluted to 10mM by adding nuclease-free water. Next, primers were checked with Real-time PCR using SYBR-Green master mix to ensure only one amplicon was being produced in the PCR reaction.

2.10 Western Blotting Analysis

2.10.1 Extraction of Protein

Frozen liver and muscle samples were crushed to powder in liquid nitrogen using a pestle and mortar. Crushed sample (200mg) was weighed in 2ml tube and homogenized in 1ml of extraction buffer (15mM NaCl, 50mM 2-(4-hydroxy ethyl)-piperazineethanesulfonic acid (HEPES), 2.5mM ethylenediamine tetra acetic acid (EDTA), 10% (v/v) glycerol and 1% (v/v) Triton X100 (Sigma), and immediately before use 1 tablet of protease inhibitor (Protease inhibitor Cocktail Tablets, EDTA-free, EASYpack, Roche) and phosphatase inhibitor (Halt Phosphatase Inhibitor Cocktail 100x, Thermo) were added per 10ml of buffer. Samples were homogenised on ice at high speed for 2x 30 seconds using a polytron (Kinematica, Switzerland). Fifty microliters of whole homogenate were added to 950µl of 0.1M NaoH, mixed and stored at -20^{0}C for protein determination by Lowry assay. Also, 200µl of whole homogenate was added to equal volume of 2xSDS mix (2ml glycerol, 1.25ml 1M Tris/Hcl, pH6.8, 4ml 10% (w/v) sodium dodecyl sulphate, 0.145g/ml Dithiothreitol (DTT) and 0.01% (w/v) bromophenol blue in 10ml, mixed and frozen at -20^{0}C for western blot analysis. The rest of the sample was centrifuged at 4^{0}C for 10 min at 13000xg (Microfuge 22R centrifuge, Beckman Coulter Inc.). Then 50µl of supernatant added to 950µl 0.1M NaoH, mixed and stored at -20^{0}C for protein determination by Lowry assay. To 500µl of supernatant equal volumes of 2xSDS mix were added, before being mixed thoroughly and stored at -20^{0}C until use for western blot analysis.

2.10.2 Sample Preparation

After determination of protein concentration of the samples by Lowry assay. Samples were diluted with 1xSDS mix to make all samples in the equal concentration. Therefore, samples were thawed, mixed and boiled on a heating block at 100^0C (Techne, Dri-Block, DB-2A) for 5-10 min then centrifuged for 3 min before dilution.

2.10.3 Protein Separation by SDS-PAGE

Protein samples diluted in SDS-mix were separated by4-15% Criterion (TGX) precast SDS-polyacylamide gels (SDS-PAGE) (BioRad, US). Prepared samples in SDS mix were boiled on a heating block at 100^0C for 5 min and centrifuged at maximum speed 15000xg at room temperature. The seal at bottom of gel was removed and the apparatus assembled with tank filled with 1x of running buffer (0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS. Samples and prestained molecular weight marker (Precision plus Protein Standards, Bio-Rad) were loaded onto the gel. Gels were then run for 45-60 min at 200V in running buffer or until the bromophenol blue migration dye had migrated to the bottom of the gel. The prestained marker, had molecular weights ranging from 10-250KDa, and was used to estimate migration of the protein of interest to be detected.

Separated proteins on the gels were transferred onto nitrocellulose membrane by western blotting. A nitrocellulose membrane (Amersham Protran, Supported 0.45µM, GE Healthcare Life Sciences) was soaked in distilled water for 10 min. The SDS-PAGE gel and the nitrocellulose membrane were soaked in western blotting buffer (400mM glycine, 25mM Tris-base, and 5% (v/v) isopropanol) for 10 min before setting up the western blot

apparatus. The gel cassette was prepared by lying on the black section (bottom) a sponge soaked in western blot buffer, followed by 2 sheets of 3MM Whatman chromatography paper. On to this was placed the gel which was covered by nitrocellulose and rolled out to make sure there were no bubbles. Onto this was placed another 2 sheets of 3MM Whatman chromatography paper and then the other sponge on the top of the cassette were putted (Figure 2-5). The cassette was closed and placed into the western blot apparatus with the black side of the cassette next to the black side of the apparatus (the cathode pole) to migrate protein to the anode (BioRad, UK). The ice reservoir was placed into apparatus or alternatively a cooling coil was inserted and connected the water supply. The apparatus was then filled with blot buffer and a stirrer placed in the bottom of the reservoir, to allow the reservoir buffer to be circulated. The apparatus was then run at a constant current of 350mA for 1-2 hours. This allowed the proteins to transfer onto the nitrocellulose membrane.

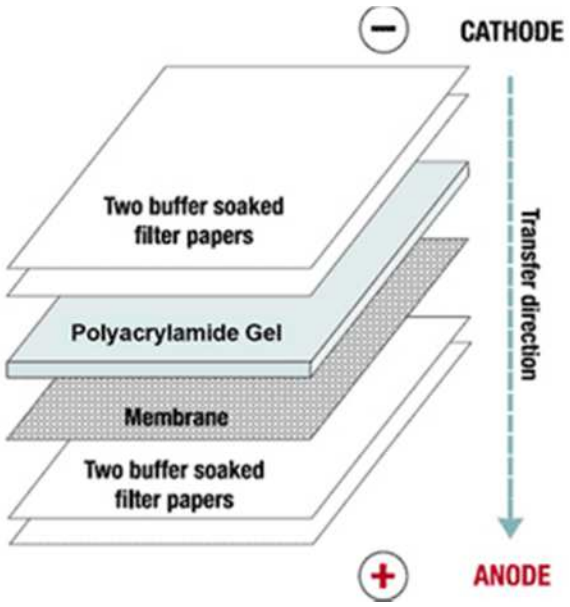


Figure 2-5: Western blotting transfer process.

The cassette prepared,2 sheets of 3 MM What- man chromatography paper, then the gel was laid on the 3 MM paper, covered by nitrocellulose and rolled out to make sure there

was no bubbles, after that another 2 sheets of 3 MM paper, transfer direction of protein from the cathode to anode. (Source: kollewin.com).

2.10.4 Western Blotting Detection

After transferring, the apparatus was taken apart and the excess nitrocellulose was trimmed from around the gel whilst it was still on top of the gel and then carefully removed from the cassette. The nitrocellulose was stained with ponceau Stain (0.5% (w/v) ponceau S, 5% (w/v) Trichloroacetic acid) for 1 min then destained with water to make sure that the proteins were transferred to the blot. The membrane was distained with 1x TBS-T buffer (50mM NaCl, 100mM Tris, 1% (v/v) Tween-20) until the stain was completely removed. To prevent non-specific binding to the membrane it was blocked using 2% (w/v) ECL Prime blocking reagent (GE Healthcare Life Sciences, UK) in 1x TBS-T buffer for 1 hour at room temperature. The chosen primary antibody (Appendix 3) was diluted to the appropriate concentration in 2% (w/v) of Prime blocking agent in 1x TBS-T buffer was added to nitrocellulose membrane and for overnight at 4^0C on a platform shaker. The primary antibody was removed and the membrane washed with 1x TBS-T for 2x 30 sec, 1 min, and 3x 5 min with changes of 1x TBS-T to remove unbound antibody. The membrane was incubated with the appropriate secondary antibody diluted to the concentration required in 2% (w/v) Prime blocking agent in TBS-T for 1 hour at room temperature. The second antibody was then removed and the membrane was then washed again as described above. The secondary antibodies were either anti-rabbit or anti-mouse conjugated with IgG horse radish peroxidase (HRP) (GE Healthcare Life Sciences). The membrane had excess washing solution removed using 3MM paper and immediate exposed to the detection solutions.

2.10.5 Western Blotting Development

An equal volume of reagent A (Luminol solution) and B (Peroxide solution) (GE Healthcare Life Sciences, Italy) were mixed together, (5mls per 100cm^2 of membrane), pipetted to cover the membrane and then incubated at room temperature for 5 min. The detection solutions were removed from the membrane and placed into cling film with air bubbles being rolled out before being placed in a cassette. In the dark room the high performance chemiluminescence film (Hyper film, ECL, GE Healthcare Life Sciences, UK) was exposed to the membrane in cassette in the dark room, then developed in 1x Kodak x-ray developer solution (Calumet Photographic Company, UK), then washed in water and fixed in 1x IIford Hypam rapid fixing solution (Calumet Photographic Company, UK). Once dried the film was annotated to indicate the position of the molecular markers. Bands intensities of western blots were quantified using Gel Doc software imaging system (BioRad, UK). Data were expressed and analysed relative to the protein concentration of each sample.

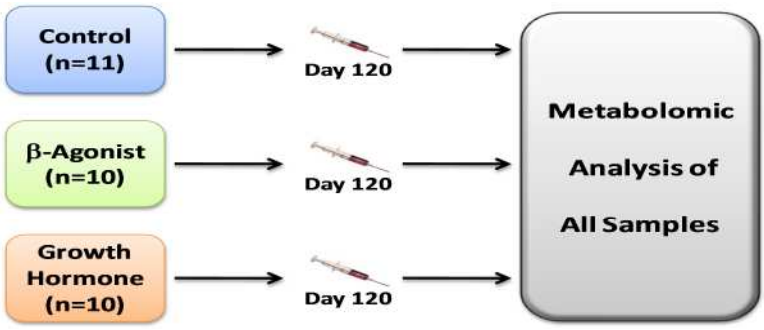
2.10.6 Stripping Membrane

To investigate more than one protein on the same western blotting membrane, primary and secondary antibodies were removed using a stripping buffer. In this project two stripping buffers were used. The first one was called mild stripping buffer (0.4M glycine), 0.1% (w/v) SDS, and 1% Tween20, pH 2.2. Sufficient volume of buffer was used to cover the membrane and it was incubated at room temperature for 5-10 min. Then buffer was discarded from the membrane and fresh stripping buffer applied and incubated for 5-10 min. The stripping buffer was then removed and incubated in phosphate buffered saline) (PBS for 2x 10 min incubations.

Following this the membrane was washed with 1x TBS-T for 2x 5 min, at which point the membrane was ready for the blocking agent stage of the antibody probing procedure. The second membrane stripping method used a commercially available blot stripping buffer (Thermo Scientific). The blot was washed with 1x TBS-T or PBS for 10 min at room temperature, and 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 as mentioned previously (chapter2).

2.11 Analysis of the Metabolomics Profile of the Plasma of Lambs Treated in the Trial was Carried Out by Metabolon, Inc. (NC 27713, www.metabolon.com)

Plasma samples from all the 31 male lambs from the trial (placebo (n=11), BA (n=10) or GH (n=10)) were collected as described in chapter 2. The frozen samples were sent to Metabolon and analysed using their biochemical profiling platform.



At the time of analysis, samples were thawed and utilizing metabolon's standard protocol small molecules were extracted from the blood.

An aliquot was taken from each of the plasma sample, and then pooled for the creation of "Client Matrix" (CMTRX) samples. These CMTRX samples act as reference sample to allow comparison of sample runs. They were injected throughout the platform run and served as client-specific technical replicates Figure 2-6. All experimental and CMTRX samples were split for analysis on the GC/MS and LC/MS/MS platforms.

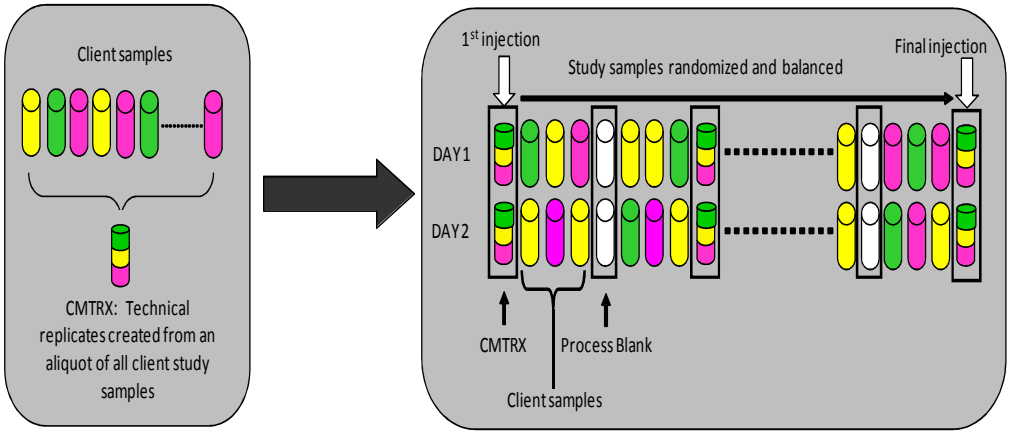


Figure 2-6: Process of client-specific technical replicates.

A small aliquot of each client sample (indicated coloured cylinders) was pooled to create a CMTRX technical replicate sample (multi-coloured cylinder). This is was injected periodically throughout the platform run to act as a standard to allow comparison between column runs.

All processed samples were spaced equally among the injections for every day and the entire client samples were distributed at random during each day's run on whatever analytical process was being carried out. Data were collected over multiple platform run days and thus, 'block normalized' by calculating the median values for each run-day block for each individual compound. Missing values (if any) were assumed to be below the level of detection for

that biochemical with the instrumentation used and were imputed with the observed minimum for that particular biochemical.

Prior to injection into the mass spectrometers, a number of internal standards were added to each experimental sample and process standard (CMTRX). Variability in whatever analytical platform was being used was measured by calculating the median relative standard deviation (RSD) for the standards. These standards consisted of the added internal standard which represents the total variability within the process for the actual experimental samples, whilst selected endogenous biochemicals were also used a standard which indicated the variability of the endogenous metabolites within these samples. For endogenous biochemical sample standards the RSD was 9% whilst for internal standards this variation was 4%.

Quality Control Sample (Matrix)	Median RSD
Internal Standards	4%
Endogenous Biochemicals	9%

The total number of biochemicals that identified in this study, known (named/ matched a named structure in our reference library), and unknown (unnamed/ not currently match a named biochemical in our reference library) was shown in Table 2-1.

Table 2-1: The total number of biochemical substances identified in this study

Compound Classification	
Total	***405***
Named / Identified	277
Unnamed	128

2.12 Cell Culture

2.12.1 Mouse Cell Culture

Mouse C2C12 myoblasts were grown in growth medium DMEM (Dulbecco's modified eagle's medium) (DMEM; Sigma Aldrich, Poole, UK) supplemented with 10% foetal bovine serum (FBS; Invitrogen, Paisley, UK), 1% (w/v) penicillin and streptomycin (P/S; Invitrogen, Paisley, UK), at 37^0C and 5%CO_2. The cells were grown then passaged. Once seeded on to the appropriate plates cells were grown to 60-70% confluence in growth medium then the media was replaced by differentiation medium (DMEM, 2% (v/v) horse serum (Invitrogen, Paisley, UK) and 1% P/S). This was defined as day 0. Differentiation media was changed every 2 days in order to maintain pH and refresh nutrients required for the formation of myotubes.

2.12.2 Splitting Cells (Passaging)

When C2C12 become confluent they will begin to differentiate. Therefore, splitting cells were necessary to keep them in the proliferative state (less than 70% confluence). Once cells had reached ~60% confluence all culture medium was removed from the plate or flask and washed with 10ml pre-warmed PBS (37^0C). Two millilitres of warm Trypsin-EDTA (Invitrogen, Paisley, UK) were added to flask, rocked gently to ensure Trypsin-EDTA covered all cells then incubated at 37^0C for 3-4 minutes. Cells cultured in a flask was knocked to dislodge the cells so they could be observed under a microscope to ensure the majority of cells had detached, and then 8ml of growth medium containing 10% foetal bovine serum was added to culture and mixed by pipetting up and down several times. The cell suspension was then transferred to a 20ml universal tube and centrifuged at 1000rpm for 5 minutes.

Supernatant was removed and the resulting cell pellet was re-suspended in 10ml of growth medium. A $1/10_{th}$ of the cells were transferred to a new T75 flask, and 13-14ml of growth medium was added. At this density cells took 2 days to a reach sub-confluence and required splitting again.

2.12.3 Counting Cells

C2C12 myoblasts in suspension were counted using a haemocytometer. The haemocytometer and its coverslip were prepared by spraying them with 70% ethanol and wiping dry. The haemocytometer and coverslip were secured with a small quantity of moisture. A small volume of cell suspension was then placed in the haemocytometer chamber. The number of cells per ml of culture medium was calculated which then allowed the total number of cells to be determined in the total volume of cell suspension media. To determine the volume of cell culture required for seeding densities, the formula below can be used.

(Desired cell number/Total cell number) X Volume of cell suspension=Volume required

This volume was added to the volume of medium required for the flask or plate to be used, and then mixed well before seeding.

2.12.4 Freezing Cells

Mouse C2C12 myoblasts were frozen in liquid nitrogen to keep stocks of cell lines and used later. Pelleted cells obtained from the passaging step as described above were re-suspended in FBS containing 10% DMSO in cryovials, and then transferred to a polystyrene container at -80^0C for 24 hours before being transferred to liquid nitrogen.

2.12.5 Cells Harvest

The media was removed from the cells to be harvested for RNA. Then 200µl of RNase-free phosphate buffered saline (PBS) was added to the cells, where the cells were scraped and collected directly into a sterile tube, snap frozen on dry ice and stored at -80^{0}C until RNA extraction was carried out.

2.12.6 RNA Isolation from Cells

The total RNA was extracted from cells using an RNA isolation kit (High Pure Isolation Kit for RNA isolation, Roche, UK); lysis-Binding buffer (400µl) was added to the cells that were re-suspended in 200µl RNase free PBS, vortex for 15 sec. The high filter tube from the kit was combined with the collection tube and entire sample was pipetted into the upper reservoir filter tube. The entire tube assembly then was centrifuged for 15 sec. at 8000xg. The filter tube was removed from the collection tube and liquid which had passed through the column was discarding. The filter tube and collection tube were then re-combined. Onto the glass filter fleece in the upper reservoir of the filter tube 100µl of DNase incubation buffer containing DNase I was added (as directed in the protocol). This was then incubated for 15 min between 15 to 25^{0}C. Wash buffer I (500µl) was added to the upper reservoir of the filter tube assembly then centrifuged for 15 esc. at 8000xg. The flow-through was discarded. Wash buffer II (200µl) was added to the upper reservoir of the filter tube assembly then centrifuged for 2 min at 13000xg to remove any residual wash buffer. The collection tube was discarded and the filter tube inserted into a sterile 1.5 ml micro centrifuge tube. Elution buffer (50-100µl) was added to the upper reservoir of the filter tube and centrifuged for 1 min at 8000xg to collect the eluted RNA. The RNA in the microfuge tube

was either sent directly to the nanodrop to measure the RNA concentration for each sample or stored at -80^{0}C.

3 Effects of Growth Promoters on Lamb and Pig Carcass Characteristics

3.1 Introduction

Increase growth by partitioning nutrients into lean tissue and increasing nutrient utilisation efficiency are important goals of animal production systems. Improved ratio of lean to fat deposition in animals is associated with improved feed efficiency. The net benefits of this may involve lower production costs, improve product quality, decrease excretion of nitrogenous waste into the environment, as well as reduced grazing pressure. The growth promoters, BA and GH, induce muscle hypertrophy which is associated with changes in nutrient mobilisation and utilization (Bell et al., 1998). The effects of BA on growth animal appear to be mediated via direct stimulation of β-Adrenergic Receptors (β-ARs) on muscle cell membranes. In the case of GH the muscle growth effects appear to be via GH stimulating liver or muscle to produce IGF-I which is released into the circulation which then stimulates the growth effect.

In this study, two growth-promoting agents, cimaterol (a beta-adrenergic agonist) and bovine growth hormone (recombinant bovine growth hormone), were administered to growing lambs. In addition two commercially available growth promoters, Ractopamine (beta-adrenergic agonist) and Reporcin (recombinant growth hormone), were administered to growing pigs to compare their effects on growth and metabolism. The growth promoters' (GH & BA) effects on increasing muscle mass are thought to be associated with changes in metabolism of the liver and muscles, but this may be different and dependent on the animal species. The research presented in this chapter of thesis sought to determine the relationship between muscle and liver, particularly

the role of the liver, as it is central to the metabolism of the nutrients being utilised for lean growth and thereby has an important role in nutrient partitioning. Therefore, the objective of this study was to compare the effects of GH and BA on the liver and muscles of growing lambs and pigs, with a specific focus on protein and glycogen metabolism.

3.2 Results

3.2.1 Effects of Growth Promoting Agents on the Growth Characteristics of Sheep and Pig

There were no significant differences in body weight of sheep treated for six days with BA or GH relative to controls, probably due to the short time of growth promoter administration. However BA significantly increased the muscle weight in both SS and ST with a trend for an increase in *Vastus Lateralis* (VL) compared to control. There were no effects of growth promoting agents on the *Vastus Intermedius* (VI) muscle between groups (Table 3-1).

GH significantly increased liver weight whilst BA significantly decreased LD muscle glycogen concentration compared to controls (Table 3-1). BA, but not GH, significantly increased the LD muscle protein per unit wet weight whereas there were no significant effects observed in the total protein in whole LD muscle compared to control. In contrast BA significantly increased the total protein in whole SS muscle whilst there were no differences in protein concentration per unit weight of SS muscle compared to control. This was due to an increase in SS muscle weight in animals treated with BA (Table 3-1).

Table 3-1: The effects of 6 day treatment of sheep with growth promoters (GPs) on carcass characteristics

Measurement (Day 120)	**Control (n=11)**	**BA (n=10)**	**GH (n=10)**	**SED**	**P. Value**
Body weight (kg)	43.00	46.39	44.30	1.638	0.122
SS (Supraspinatus) (g)	98.50	**116.40***	98.00	6.32	0.009
LD (Longissimus Dorsi) (g)	592	667	632	35.2	0.108
ST (Semitendinosus) (g)	112.40	**131.20***	120.40	6.55	0.023
VL (Vastus Lateralis) (g)	164.50	180.30	163.80	7.5	0.058
VI (Vastus Intermedius) (g)	51.70	55.70	52.40	3.58	0.487
Liver wt (g)	928	926	**1101***	54.9	0.004
IGF-I (ng/ml)	972	964	**2092****	126.5	<0.001
Glycerol (µg/ml)	8.16	9.38	9.40	2.425	0.826
LD muscle glycogen (mg/g)	21.63	**18.33***	24.21	1.33	<0.001
LD muscle protein (mg/g)	65.51	**58.40***	62.27	2.322	0.017
LD total protein (g)	38.73	38.97	39.23	2.434	0.979
SS muscle protein (mg/g)	36.27	36.06	35.34	1.248	0.744
SS total protein (g)	3.58	**4.20**	3.47	0.270	0.027

*significantly different to control (P<0.05), ** significantly different to control (P<0.01) (Dunnett test)

In the pig study, there were no significant effects relative to control of BA and GH treatments for 7 days on body weights or carcass weights (Table 3-2). Although there was no significant effect of BA and GH on whole SS weights there was a trend for an increase (P<0.062) in the weight of the *Vastus Lateralis* muscle (Table 3-2).

GH treatment for seven days significantly increased liver weights compared to control groups, whilst there were no significant effects of BA. In BA there was a significant decrease in LD muscle glycogen content compared to control.

Table 3-2: Effects of 7 day treatment of pigs with GPs on carcass characteristics

Measurement	Control (n=15)	BA (n=15)	GH (n=15)	SED	P. Value
Initial body wt (kg)	87.07	86.9	86.03	2.11	0.871
Final body wt (kg)	93.63	94.00	91.70	2.22	0.543
Carcass weight (kg)	74.96	75.62	73.59	1.93	0.567
ST wt (g)	434.21	454.58	417.46	21.23	0.231
VLat wt (g)	340.62	376.87	342.51	16.56	0.062
Liver weight (kg)	1.57	1.55	**1.86****	0.067	<0.001
LD glycogen (mg/g)	6.57	4.78	6.69	0.741	0.015

**significantly different to control (P<0.01) (Dunnett test)

IGF-I is one of the most effective growth factors that regulates skeletal muscle mass by increasing protein synthesis and decreasing protein degradation (Ryall et al., 2010). GH, but not BA significantly increased (P<0.001) plasma IGF-I concentration (ng/ml) in lambs treated with bovine GH (Table 3-1).

Glycerol is important substrate for lipolysis and energy metabolism. There was no significant effect of GH or BA on plasma glycerol concentration (µg/ml) observed in any treatments relative to control (Table 3-1).

3.2.2 Effects of Growth Promoting Agents on Sheep and Pig Liver DNA, Glycogen, Protein and Lipid Content

3.2.2.1 DNA Quantification

Growth is an increase in tissue or organism size due to an increase in cell number (hyperplasia), or due to an increase in cell size (hypertrophy). Therefore, in this study, DNA content was measured to determine if the increase in liver size was due to an increase in DNA (increase in cell number) or due to hypertrophy. There was no significant difference observed of growth promoting

agents in DNA content per unit liver weight compared to control, whilst GH treatment significantly increased the total DNA in whole liver compared to control groups (Table 3-3). In pig, GH treatment significantly decreased liver DNA content per unit weight tissue compared to control groups, whilst there were no differences observed in the total DNA in whole liver between BA and GH treatments (Table 3-4).

3.2.2.2 Liver Glycogen Determination

In sheep administered GH for six days there was a significant increase in the total glycogen in whole liver compared to BA and control groups. There were no significant effects of GH or BA treatment on liver glycogen per unit tissue or per unit DNA compared to control (Table 3-3). GH treatment significantly increased the total liver glycogen content in pigs compared to control groups, but there were no differences observed in the total glycogen in whole liver between BA and control (Table 3-4). In the pig study, GH dramatically increased glycogen content per unit DNA compared to control (Table 3-4).

Table 3-3: Effects of 6 day treatment of sheep with GPs on liver DNA, glycogen, protein and lipid

Measurement	Control (n=11)	BA (n=10)	GH (n=11)	SED	P. Value
Liver weight (g)	928	926	**1101****	54.9	0.004
DNA (mg/g liver)	11.22	11.32	12.26	0.579	0.158
Total DNA in whole liver (g)	10.42	10.48	**13.45****	0.803	<.001
Glycogen (mg/g DNA)	2.40	2.43	2.65	2.66	0.755
Total glycogen in whole liver (g)	24.95	25.05	**35.31**	4.342	0.037
Glycogen (mg/g liver)	26.91	26.79	32.11	3.962	0.33
Protein (mg/g DNA)	11.71	11.75	10.91	0.446	0.125
Total protein in whole liver (g)	121.35	121.90	**146.36***	8.266	0.007
Protein (mg/g liver)	130.61	131.84	133.19	4.878	0.868
Lipid (mg/g liver)	208.08	201.87	232.89	15.26	0.132
Total lipid in whole liver (g)	185.45	185.87	**256.98***	22.96	0.01

**significantly different to control (P<0.01), *significantly different to control (P<0.05) (Dunnett test)

3.2.2.3 Liver Protein Determination

In sheep study, GH treatment significantly increased the total protein in whole liver compared to BA and control groups (Table 3-3), but the protein content in liver per unit of tissue, protein or DNA was not affected between treatments (Table 3-3). In pigs, there was a trend for lower for protein content in liver per unit tissue for animals treated with GH, but there were no differences in the total protein in whole liver or per unit DNA between the groups (Table 3-4).

3.2.2.4 Lipid Determination

Lipid concentration was measured to determine the relationship between lipid content and liver weight. Previous reports had described a negative correlation between fat and water content (Kim et al., 1988). Tissue with low fat has higher water content than tissue containing high levels of fat. There were significant decrease in water and fat content within muscle treated with cimaterol whereas protein content significantly increased,

suggesting that cimaterol increased protein concentration in muscle in addition to increasing muscle mass, and these factors contributed to higher protein deposition (Kim et al., 1989).

In sheep there were no significant effects of GH or BA treatment on lipid content in liver per unit tissue compared to control, whilst GH treatment significantly increased the total lipid in whole liver compared to control treatments (Table 3-3). Unfortunately, lipid content of the pig was not determined.

Table 3-4: Effects of 7 day treatment of pigs with GPs on liver DNA, glycogen and protein content

Measurement	**Control (n=15)**	**BA (n=15)**	**GH (n=15)**	**SED**	**P. value**
Liver weight (kg)	1.57	1.55	**1.86****	0.0696	<0.001
DNA (mg/g liver)	33.2	31.57	**26.82****	1.913	0.007
Total DNA in whole liver (g)	53.05	48.33	50.31	4.542	0.561
Glycogen (mg/g DNA)	1.01	1.30	**1.81****	0.1485	<0.001
Total Glycogen in whole liver(g)	52.15	57.95	**85.49****	4.881	<0.001
Glycogen (mg/g liver)	32.84	38.13	**46.31****	2.288	<0.001
Protein (mg/g DNA)	4.00	4.122	4.268	0.1402	0.172
Total Protein in whole liver (g)	208.10	197.70	213.40	19.22	0.616
Protein (mg/g liver)	131.00	129.20	113.90	8.151	0.076

**significantly different to control (P<0.01) (Dunnett test)

3.3 Discussion

Improving the efficiency of animal production is very important for the world animal production sector, especially in countries which have to meet their increasing consumption of meat despite the likelihood there will be limitations on the feed available and increasing problems with the environmental waste generated (Sillence, 2004). An improvement in feed efficiency could be through the use of growth promoters such as BA and GH. Considerable research has been done to improve the effectiveness of BA by decreasing their negative effects on meat tenderness, reducing their adverse impact on treated animals, and increasing their effectiveness of impacting on animal functions during the period of exposure (their efficacy) (Sillence, 2004). In both studies, although there were no significant effects of BA and GH on body weight many studies have reported a positive effect of growth promoters on growth rate (Owens et al., 1999, Byrem et al., 1998, Beermann et al., 1987, Kim et al., 1989). The lack of effect in our studies are probably due to a short time of administration of the growth promoters (6 or 7 days in sheep and pigs respectively) compared to the longer periods (28 days or greater) used in other studies (Pell et al., 1990, Dunshea et al., 2002).

Many studies reported that orally administrated BA in farm animals led to increased muscle mass as a result of hypertrophy that was associated with an increase in muscle protein synthesis, a decrease in muscle protein degradation, or a combination of both (Kim and Sainz, 1992, Mersmann, 1995, Yang and McElligott, 1989, Beermann et al., 1987). In this study, BA, but not GH significantly increased the muscle weight in both SS and ST with a trend for an increase in (VL) compared to control. This increase was associated with hypertrophy of type II fibres (Hemmings et al., 2009, Hemmings et al., 2014). BA-treated animals produced a significant

decrease in muscle glycogen concentration (mg/g muscle). This was associated with increased size and proportion of fast glycolytic muscle fibres which would be expected to have an increased capacity to metabolize glycogen (Maltin et al., 1990), and utilize glucose as an energy source and the capacity to produce lactate (Hamby et al., 1986).

Previous studies have reported a significant decrease in water and fat content in muscle of lambs treated with the BA cimaterol, which was associated with an increase protein content (Kim et al., 1989). Many studies have indicated protein accretion increased from muscle in animals fed BA (Emery et al., 1984, Smith et al., 1989, Beermann et al., 1987). Increased muscle weight in lambs fed BA was primarily associated with hypertrophy of fast glycolytic fibre type, particularly type II fibres (Kim et al., 1987, Hemmings et al., 2014). These heavier muscle weights were not the result of carcass weights being higher in cimaterol-fed lambs. Cross sectional area of muscles was greater in cimaterol-fed lambs compared to controls (Beermann et al., 1986). The greater muscle weights reported in the literature were found to be the result of increased rate of growth or hypertrophy of type II fibres in the SS and ST muscles. Cross-sectional area of type II fibres increased approximately 50% in SS and LD muscles in lambs fed cimaterol, suggesting that the increase of muscle mass was due to hypertrophy of type II fibres only (Kim et al., 1987, Hemmings et al., 2014). In Friesian bulls muscles, cimaterol increased the cross-sectional area of fibres Type I and Type IIB, but there was no change observed in fibres Type IIA (Vestergaard et al., 1994). Skeletal muscle hypertrophy causes an increase in muscle mass and cross-sectional area and this is associated with increased protein content in myofibrillar proteins (Al-Shanti and Stewart, 2009).

A study by Byrem et al. (1998) provided evidence that cimaterol has a direct action on accretion of protein in skeletal muscle. In cattle treated with BA there was a significant increase in LD and VL muscles mass whilst there no effect observed in ST muscle mass (Dawson et al., 1991), which was reported to be due to differences in muscle fibre type of different muscles. The change in fibre a type is from slow oxidative towards fast glycolytic fibres and is associated with increased in fibre diameter. Such changes in pigs (RAC) treated pigs appear to influence meat quality and tenderness (Aalhus et al., 1992).

In our pig study the effect of BA on muscle was not as strong as in sheep due to species differences and the type of BA used. The effects and response of β-AR agonists are different between animal species; there are stronger effects in sheep and cattle, less of an effect in chickens, whereas an intermediate effect is observed in pigs (Mersmann, 1998).

Although β-ARs are present on the surface on most mammalian cells, the distribution and proportion of subtypes (β1-AR, β2-AR, and β3-AR) differs between tissues and species (Mersmann, 1998). In the bovine, radioligand binding studies indicate β2-AR is the predominant receptor in both skeletal muscle and adipocytes, whilst β1-AR is not detected in either tissue (Sillence and Matthews, 1994). Ractopamine binds to both β1- and β2-AR, but binding to the β1-AR subtype has been reported not to stimulate cAMP, whereas production of the second messenger cAMP occurs when ractopamine binds to the β2-AR (Mills et al., 2003). This suggests that BA binding to β-AR receptors does not always activate adenylyl cyclase, thereby such receptor occupancy is acting as an ”antagonist” (Mills, 2000). Properties of the β-AR binding site in sheep and rat display species and depot specific differences (Bowen et al., 1992).

Other action of BA that could contribute to enhance muscle growth is an increase in blood flow to the muscle (Mersmann, 1987).

β-adrenergic receptors play a critical role in skeletal muscle growth and development through mechanisms that control protein synthesis and degradation (Ryall et al., 2010). Protease activities are often measured as an indicator for protein degradation in muscle (Mersmann, 1998). It has been reported that BA induced muscle hypertrophy may be associated with increased activity of calpastatin, the specific endogenous inhibitor of calpain, which results in reduced proteolytic capacity (Wheeler and Koohmaraie, 1992, Parr et al., 1992, Storr et al., 2012). This is supported by the observation that a muscle hypertrophy condition in lambs (callipyge) is associated with increasing activity of calpastatin, suggesting that protein degradation may be reduced (Koohmaraie et al., 1995). There is increased muscle DNA content associated with callipyge, indicating increases in satellite cell proliferation as well as an increased capacity for muscle protein accretion. These findings suggest that the callipyge condition is associated with both a reduction in protein degradation rate and increased capacity for protein synthesis (Koohmaraie et al., 1995).

In contrast, GH has a wider effect whole body effect on metabolism than BA (Reeds et al., 1986, Reeds and Mersmann, 1991, Beermann et al., 1987). As well as influencing skeletal muscle hypertrophy it can cause cardiac hypertrophy too (Deshaies et al., 1981). GH could increase the rate of nutrient absorption which impacts on liver metabolism leading to an increase liver size or change in the composition of nutrients. In addition GH may change the endocrine status which would impact on tissues (Pell et al., 1990). It has been found that an injection of GH causes increases in serum IGF-I concentration which is associated with increased liver IGF-I mRNA expression (Wu et al., 2010). Administration of

GH to growing animals increases weight gain, muscle growth and decreases fat deposition (Etherton, 2004, Campbell et al., 1988, Caperna et al., 1990, Chung et al., 1985, Sève et al., 1993) and an increase liver weight (Pell et al., 1990).

The liver is the major source of IGF-I and provides direct evidence of its important role in tissue growth and development (Yakar et al., 1999). Young et al.(1995) described how plasma IGF-I was unaffected in growing lambs treated with clenbuterol, indicating that circulating IGF-I does not play an important role in BA stimulated muscle hypertrophy. In the present study, the observed effects of GH treatments on liver weights was possibly due to directly regulated metabolism of nutrients by GH or indirectly via stimulation of IGF-I synthesis, which led to increase liver cells size. BA treatment had no effects on liver weights. Several studies have indicated that BA had no effects on liver weights as they are very specific in repartitioning protein accretion to skeletal muscle (Spencer and Oliver, 1996, Reeds and Mersmann, 1991). Increases in liver weights treated with GH in the current study, appear to be due to an increase in glycogen, and possibly associated water content, rather than an increase in cell number or protein content. In the current pig study, protein content in the livers treated with GH and BA were unaffected, but there was a trend of lower GH, this may due to increased liver weight as a result of GH stimulated increase in glycogen. Total protein in sheep whole liver decreased with BA compared to GH, which may be due to protein being shifted towards muscle. In agreement with this suggestion, Reeds et al. (1986) reported that BA increased metabolic rate and shifted proteins from other tissues towards skeletal muscle. Also Pell et al. (1990) suggested that increased metabolism of amino acids targeted towards muscle anabolism may be lead to decreased liver amino acids that are provided from peripheral tissues. Also in the

current pig study, significantly decreased liver DNA (mg/g liver) contents were observed in GH treatment compared to control groups. However, the lower concentration of DNA may be due to the dilution effect caused by the hypertrophy of liver cells rather than a real change of DNA content per gram liver because there were no differences observed in the total DNA in whole liver between treatments. BA decreased muscle DNA content possibly as result of hypertrophy (Reeds et al., 1986, Beermann et al., 1987).

A study by Beermann et al. (1987) reported that cimaterol fed lambs increased muscle RNA and protein synthesis, whereas plasma insulin and insulin-like growth factor-I (IGF-I) concentrations were decreased, whilst there was no significant effect of treatments observed on plasma glucose concentration at 6 or 12 wk. Studies have reported that plasma IGF-I concentrations were not significantly changed by cimaterol (O'Connor et al., 1991, Chikhou et al., 1991). Another study in yearling steers also found that IGF-I concentration was not affected by ractopamine-HCl (RAC) treatment (Winterholler et al., 2008). Plasma concentration of insulin was decreased in sheep treated with BA (Mohammadi et al., 2006). In this study, plasma IGF-I significantly decreased with cimaterol, whilst dramatically increased in lambs treated with GH for 6 days. This is in agreement with other studies where significant increases in plasma insulin and total IGF-I concentrations in lambs treated with GH were observed (Pell et al., 1990).

The effect of GH in different muscles depends on large differences in fibre type composition and growth impetus (Boge et al., 1995). GH has a nutrient partitioning effect; it redirects much of the glucose from adipose tissue to muscle. This process is very important because it can stimulate an increase in protein synthesis in muscle which is supported by the energy diverted from glucose

(Etherton, 2000). Circulating IGF-I from liver, muscle, or fat, presumably stimulates lean tissue growth through endocrine mechanisms or act via production of IGF-I from somatic tissue, suggesting that IGF-I act as paracrine growth factor (Brameld et al., 1996, Coleman et al., 1994).

3.4 Conclusion

The BA effect in muscle was stronger than GH, indicating BA specifically effects muscle. BA was more effective in sheep than pig. While GH had greater effects on whole body metabolism (GH increased liver weight). Administration of GH in pigs increased liver weight and this appears to be due to an increase in glycogen and possibly associated water content rather than an increase in cell number or protein content. In sheep treated with GH the increased liver weights were associated with increases in glycogen, protein and lipid content. The research described in the next chapter sought to determine if the growth promoters (BA and GH) given to sheep have differing effects on the gene expression in muscles (LD and SS) with intrinsically different fibre type. In addition analysis was carried out on liver to determine whether at the same time these growth promoters were having an effect on this non muscle tissue which is responsible for the provision of substrates that are utilised by muscle for energy metabolism.

4 Effects of Growth Promoters on Gene Expression

4.1 Introduction

As indicated in chapter 3 it appears GH and BA have differential effects on metabolism that lead to effects on muscle mass over a relatively short time frame (6-7 days) compared to the industry standard treatment period of livestock (28 days). Therefore a transcriptome microarray approach was used to help identify potential metabolic pathways that mediated these changes. By using this approach it would allow the identification of critical processes that could potentially be manipulated in order to stimulate an increased efficiency in growth rate in livestock species. Muscle (*longissimus* dorsi) (LD) samples (n=3 from each treatment group) from the growth promoter sheep trial described in the previous chapter were taken and subjected to transcriptome analysis utilizing microarrays to investigate the effects of 6 day treatment with BA or GH. Although there were no significant effects of growth promoters on LD muscle weight (P=0.108), there was a numerical increase in LD weights in lambs treated with BA (667g) compared to control and GH (592 and 632g, respectively). This muscle was selected for transcriptome analysis as it is commercially important and reflects a fibre type that is common across most of the muscles in sheep.

Muscle hypertrophy, which is associated with changes in nutrient mobilisation and utilization, is one of the characteristics of growth promoters, such as BA and GH (Bell et al., 1998). The ultimate aim of this study was to investigate the mechanisms that mediate these effects in sheep treated with BA or GH for 6 days, by examining the changes in skeletal muscle transcriptome. Recent

research has demonstrated that hyperplastic growth and a switch to glycolytic metabolism in some cancers is associated with up regulation of the serine synthesis pathway. The serine produced can then be utilised by additional biosynthetic pathways (Amelio et al., 2014). Therefore, the aim of this study was to investigate whether the muscle hypertrophy stimulated by growth promoters was associated with increased gene expression of the metabolic pathway responsible for serine biosynthesis.

4.1.1 Microarray Analysis

As part of the sheep growth promoter trial LD gene expression was examined using a transcriptome microarray (work carried out by Dr Zoe Daniel). At the time the trial was carried out there were a limited number of transcriptome microarrays available and few sheep studies had been carried out using this technology. Therefore a technique was devised to utilise the human Affymetrix GeneChip transcriptome array, which is a fully annotated array of the human transcriptome, and therefore has a greater coverage of the genome than the other production-animal arrays. Previous work in our labs, in collaboration with the National Arabidopsis Stock Centre (NASC), University of Nottingham who run an Affymetrix microarray analysis service, had developed a methodology which uses a cross-species approach to examine the sheep transcriptome utilising the Affymetrix Human U133+2 GeneChip array (Graham et al., 2011). Using this approach the effects of growth promoters on the LD was examined on a subset of animals from the trial (n=3 per treatment group). Sample selection was based on highest quality RNA isolated from each group. Analysis of gene expression to identify genes with significantly altered gene expression was carried out by Dr Neil Graham (NASC).

Of the gene transcripts that were affected, based on a 1.5 fold change which was significant ($p<0.05$), 477 gene transcripts were altered by BA relative to the control group, with 248 increased and 229 decreased. For GH relative to control, 316 gene transcripts were affected, with 168 increased and 148 decreased. There were 54 transcripts altered by both treatments, with 27 increased and 26 decreased by both agents and 1 transcript being affected in the opposite direction by each treatment Figure 4-1. The list of transcripts affected (up- and down-regulated expression) by BA and GH treatment identified by the cross species transcriptome microarray are shown in Appendix 4 A and B.

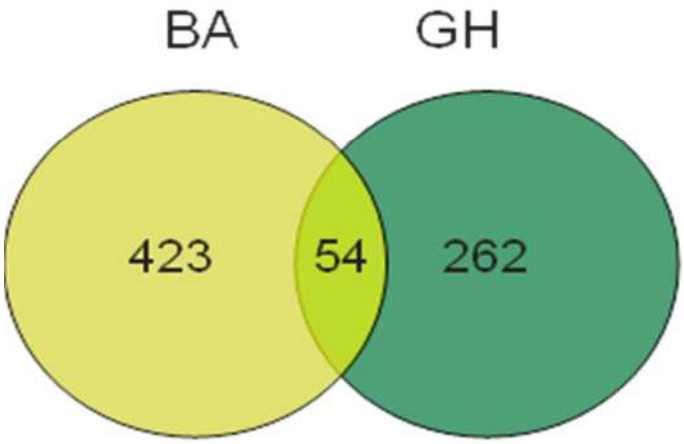


Figure 4-1: The effects of 6 day treatment with GH and BA on the sheep LD transcriptome.

The diagram show the total number of genes (both up and down) affected relative to control treatment group (n=3 per group).

4.1.2 GeneSpring and Ingenuity Pathway Analysis Software

Both GeneSpring and Ingenuity Pathway Analysis were utilised to analyse the gene expression data generated from the arrays. Utilising the list of genes significantly altered in response to treatment, these software packages give an indication of specific

signalling pathways (canonical pathways) that these genes are involved with and the function of the pathways. This bioinformatics approach indicates potential biological mechanisms (Ganter and Corner, 2007). This work was carried out by Dr Neil Graham and Shulan Gong (an MRes student).

4.1.3 Effects of Growth Promoters on the Expression of Genes in Sheep LD Muscle Associated with the TCA Cycle.

The IPA analysis mapped several genes that were "Up or down-regulated in the BA group" to the canonical Citrate Cycle (TCA cycle) pathway (5 genes were down-regulated and 1 gene was up-regulated) (Figure 4-2 and Table 4-1). None of the genes affected by GH treatment relative to control were identified as being associated with the TCA cycle.

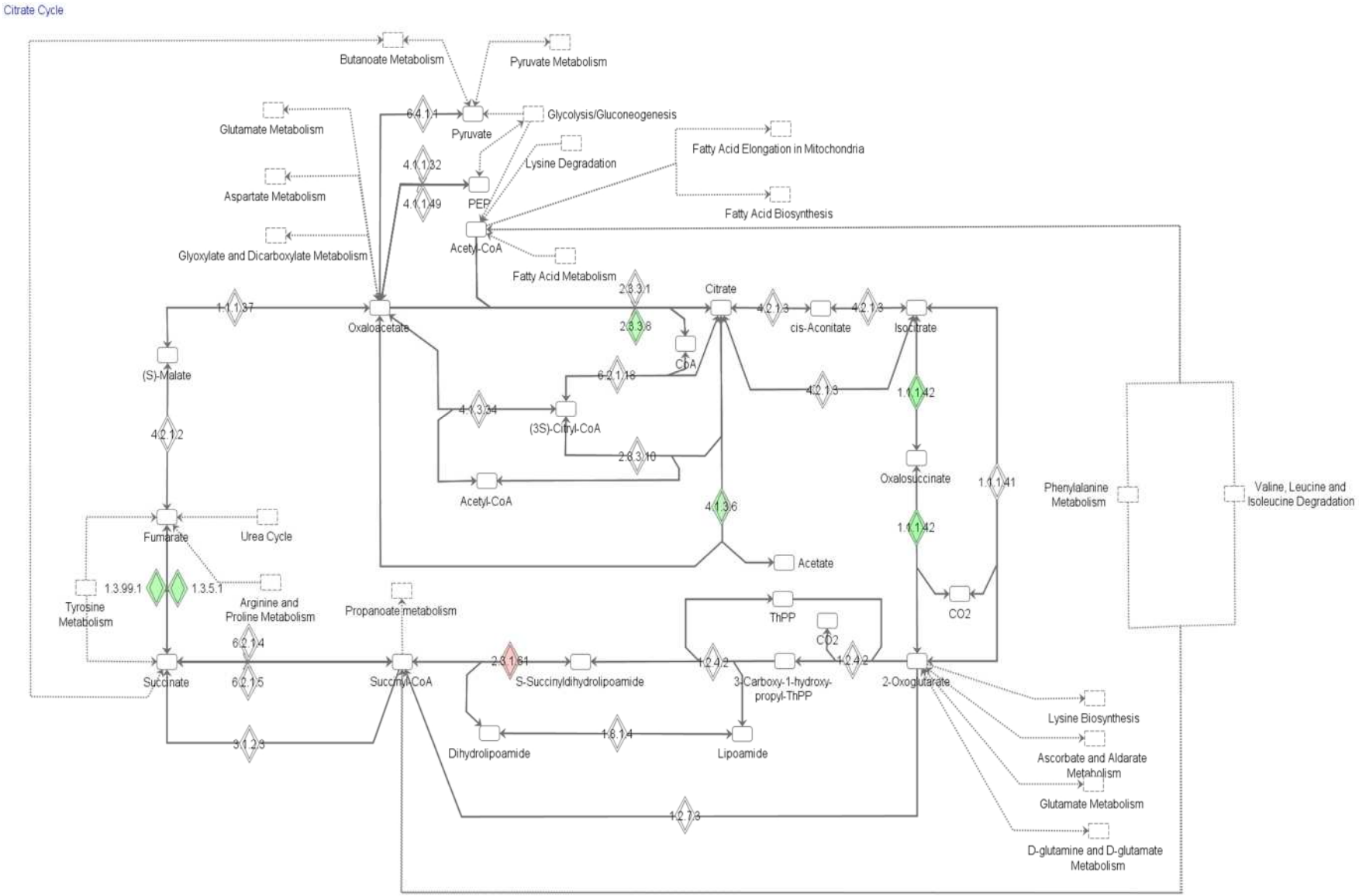


Figure 4-2: The effect of BA treatment on the gene expression in longissimus dorsi of sheep treated for 6 days; effects on the TCA canonical pathway.

The effect of BA relative to control was identified using IPA analysis. Red symbols indicate the gene was up-regulated by BA relative to the control, green indicates the gene was down-regulated by BA relative to the control. White indicates genes not affected by treatment.

Table 4-1: Sheep LD muscle genes associated with the TCA cycle whose expression were either up or down regulated by BA relative to control in response to 6 day treatment. Enzyme Commission numbers (EC) for the TCA cycle enzymes are indicated.

Regulation relative to control	**EC[1] number**	**Name**
down	1.1.1.42	isocitrate (NADP) dehydrogenase
down	1.3.5.1	Complex II, fumarate reductase complex, menaquinol:fumarate oxidoreductase
down	1.3.99.1	Complex II, Succinate INT Dehydrogenase
up	2.3.1.61	dihydrolipoamide succinyltransferase
down	2.3.3.8	acetyl-CoA:oxaloacetate acetyltransferase (isomerizing, ADP-phosphorylating)
down	4.1.3.6	citratase, citrate aldolase, citrate oxaloacetate-lyase

[1] Enzyme Commission (EC) enzyme number, Adapted from (Gong, 2010).

4.1.4 Identification of Sheep LD Gene Networks Influenced by Growth Promoters

Genes that were significantly affected by either BA or GH relative to control ($P<0.05$ and 1.5 fold change) were analysed by GeneSpring and IPA to determine the networks that they are associated with.

For genes that were affected by GH relative to control, there were no clearly no clearly distinct networks generated (Figure 4-3). For genes significantly affected by BA relative to control there were several networks identified, but two of them were considered important as they were associated with protein and amino acid synthesis. These networks were associated with AKT1 and PSAT1 (

Figure 4-4, Figure 4-5, Figure 4-6, and Figure 4-7). The AKT1 pathway is very important because it is associated with muscle hypertrophy and skeletal muscle differentiation (Glass, 2003a, Stitt et al., 2004, Chang, 2007, Rommel et al., 2001, Bodine et al., 2001). The AKT1 pathway had more association/interactions than the PSAT1 network. The protein product generated from the PSAT1 gene is part of the serine synthesis pathway, which utilizes the glycolytic intermediate, 3-phosphoglycerate, to make serine via a series of steps. Serine is a non-essential amino acid that is required in growth. In fact, some studies report that increased expression of PSAT is associated with growth and cell proliferation in cell cultures (Baek et al., 2003, Vi et al., 2008). Given that the canonical pathway analysis identified an increase in the expression of genes associated with glycolysis with BA treatment, the indication that the serine synthesis pathway may also be affected suggests there may be a relationship between the apparent switch in energy metabolism towards glycolysis and biosynthetic processes. Therefore, the next stages of the project were to investigate whether the serine synthesis pathway was being up-regulated, and whether there were associated changes in genes which might influence the availability of intermediates required for the biosynthetic pathway. Thus the study sought to determine whether up-regulation of the serine biosynthetic pathway was associated with BA stimulated muscle hypertrophy.

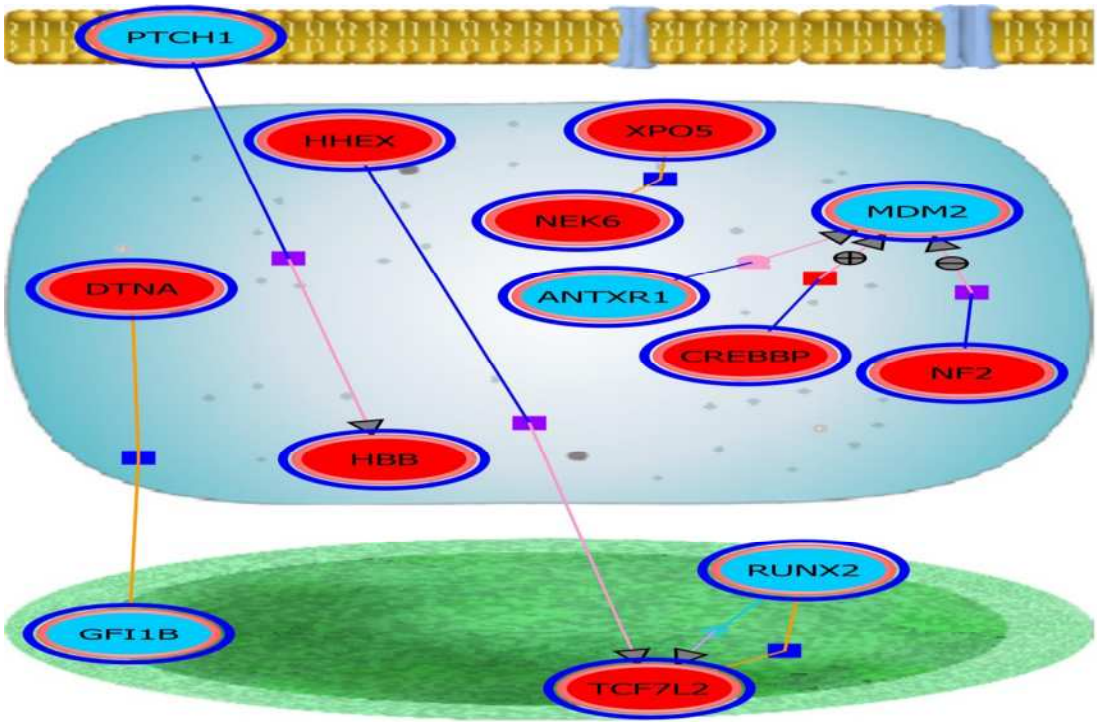


Figure 4-3: The effects of GH on the gene expression in *longissimus dorsi* of sheep treated for 6 days.

The figure shows the pathway according to the Gene Ontology. The plasma membrane is indicated by the yellow bar whilst blue represents the cytoplasm, green represents the nucleus. Red circles show the genes up-regulated by GH relative to the control, blue circles shows the genes down-regulated by GH relative to the control. Patched homolog 1 (PTCH1), growth factor independent 1B transcription repressor (GFI1B), anthrax toxin receptor 1 (ANTXR1), proto-oncogene, E3 ubiquitin protein ligase (MDM2), runt-related transcription factor 2 (RUNX2), dystrobrevin, alpha (DTNA), hemoglobin, beta (HBB), hematopoietically expressed homeobox (HHEX), transcription factor 7-like 2 (TCF7L2), exportin 5 (XPO5), NIMA-related kinase 6 (NEK6), CREB binding protein (CREBBP) and neurofibromin 2 (NF2).

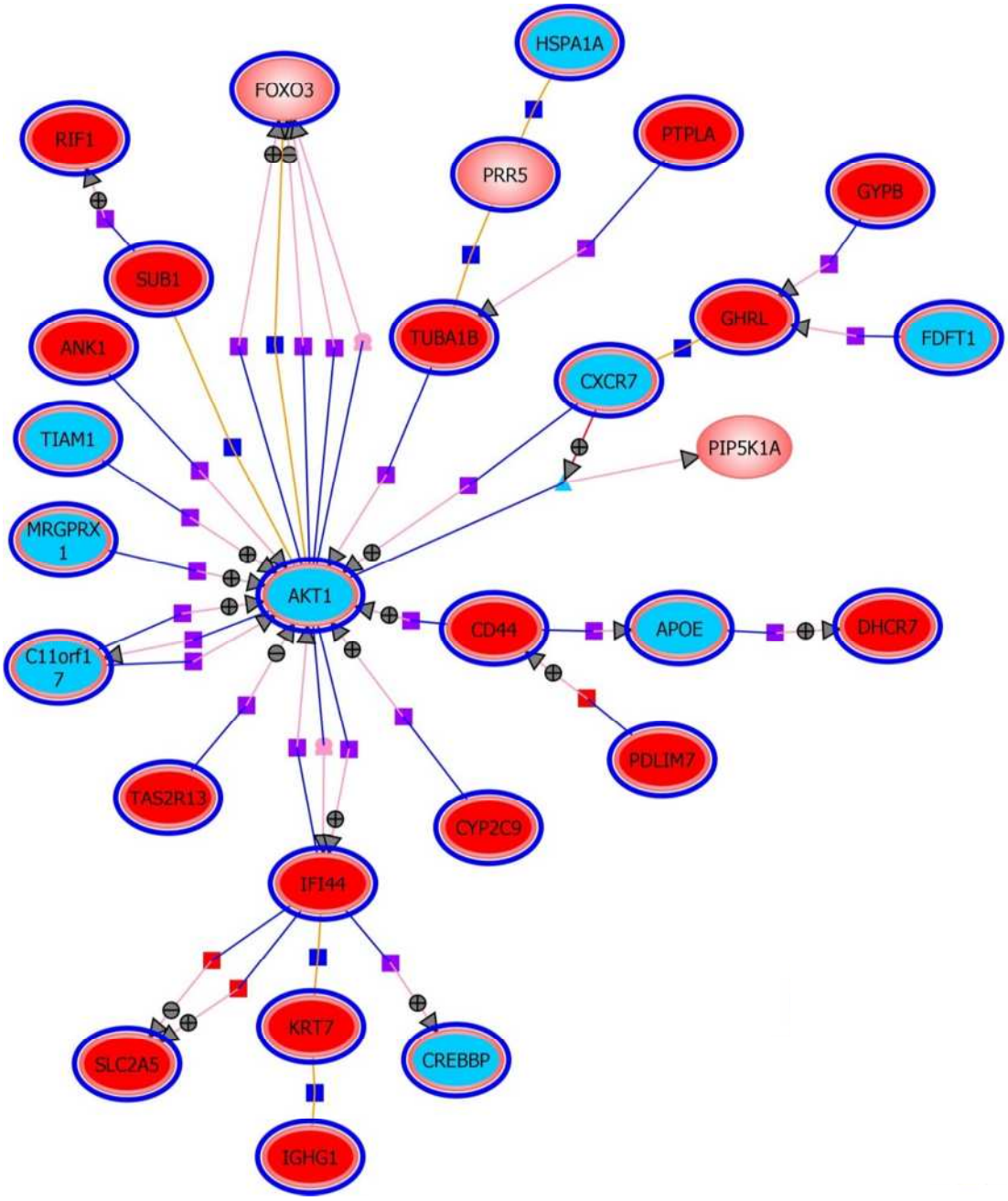


Figure 4-4: The effects of BA on the gene expression in longissimus dorsi of sheep treated for 6 days; effects on the AKT1 gene expression network (GeneSpring analysis).

The network identification of genes significantly changed in BA treatment relative to control was carried out using GeneSpring analysis. Red circles show the genes up-regulated by BA relative to the control, blue shows the genes down-regulated by BA relative to the control. V-akt murine thymoma viral oncogene homolog 1 (AKT1), forkhead box O3 (FOXO3), replication timing regulatory factor 1 (RIF1), SUB1 homolog, transcriptional regulator (SUB1), ankyrin 1, erythrocytic (ANK1), cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9), interferon-induced protein 44 (IFI44), taste receptor, type 2, member 13 (TAS2R13), tubulin, alpha 1b (TUBA1B) and CREB binding protein (CREBBP).

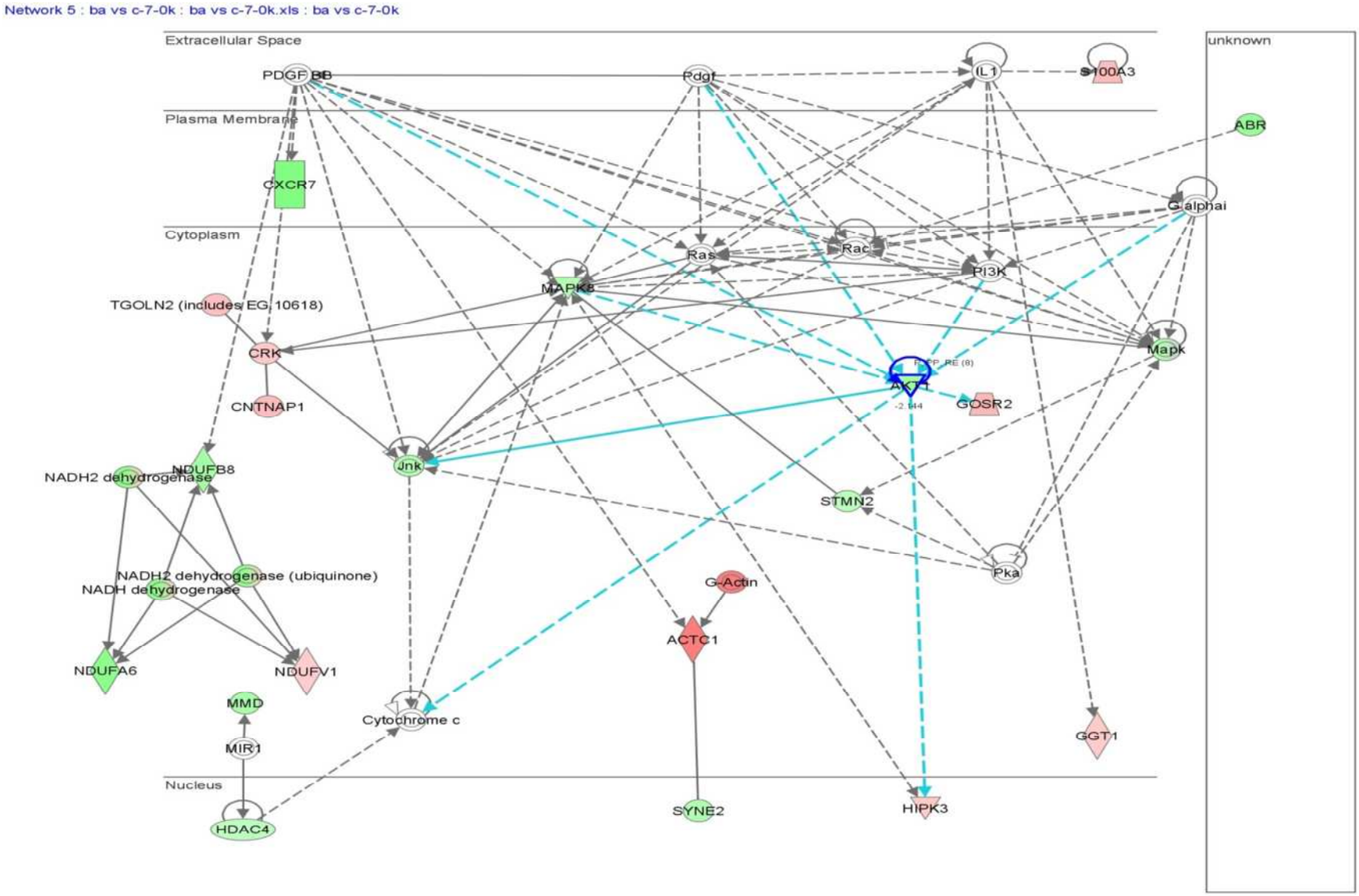


Figure 4-5: The effects of BA on the gene expression in longissimus dorsi of sheep treated for 6 days; effects on the AKT1 gene expression network (IPA analysis).

The network identification of genes significantly changed in BA treatment relative to control was carried out using IPA analysis. Red indicates the genes up-regulated by BA relative to the control; green indicates the genes down-regulated by BA relative to the control. White shows the molecule is not affected by the treatment, but was added from Ingenuity Knowledge Base in order to complete the network. AKT1 and its relationship lines were blue dashed lines to refer to indirect interactions, while the blue solid lines show direct interactions. Adapted from (Gong, 2010)

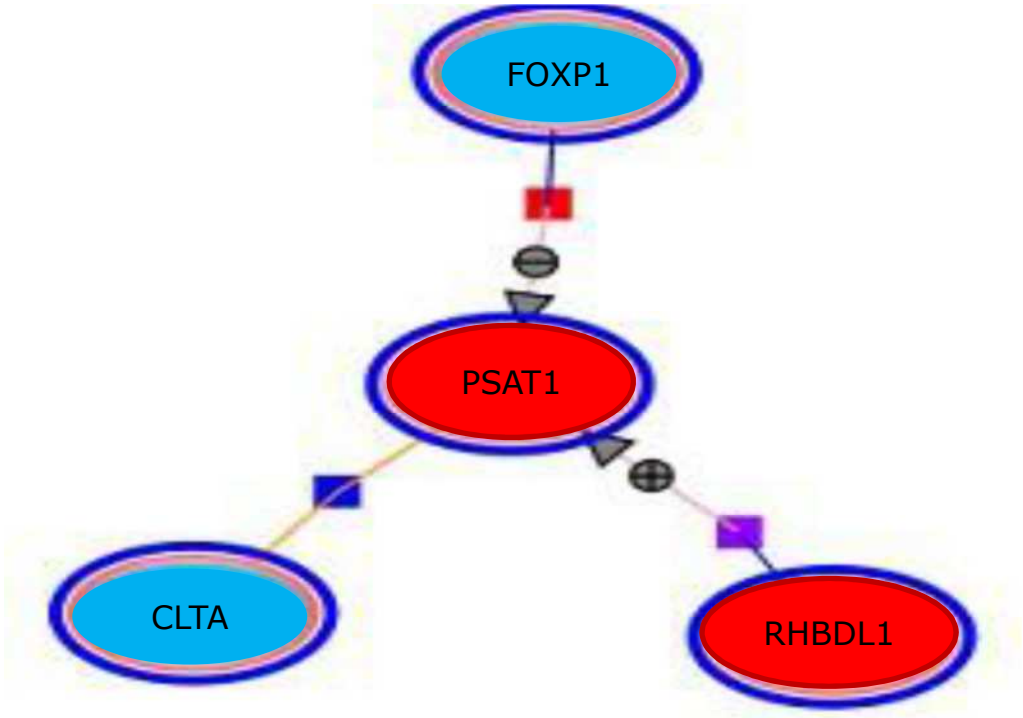


Figure 4-6: The effects of BA on the gene expression in longissimus dorsi of sheep treated for 6 days; effects on the PSAT1 gene expression network (GeneSpring analysis).

The network identification of genes significantly changed in BA treatment relative to control was carried out using GeneSpring analysis. Red circles show the genes up-regulated by BA relative to the control, blue shows the genes down-regulated by BA relative to the control. Phosphoserine aminotransferase 1 (PSAT1), rhomboid, veinlet-like 1 (RHBDL1), Forkhead box (FOX) transcription factor family (FOXP1) and clathrin, light chain A (CLTA).

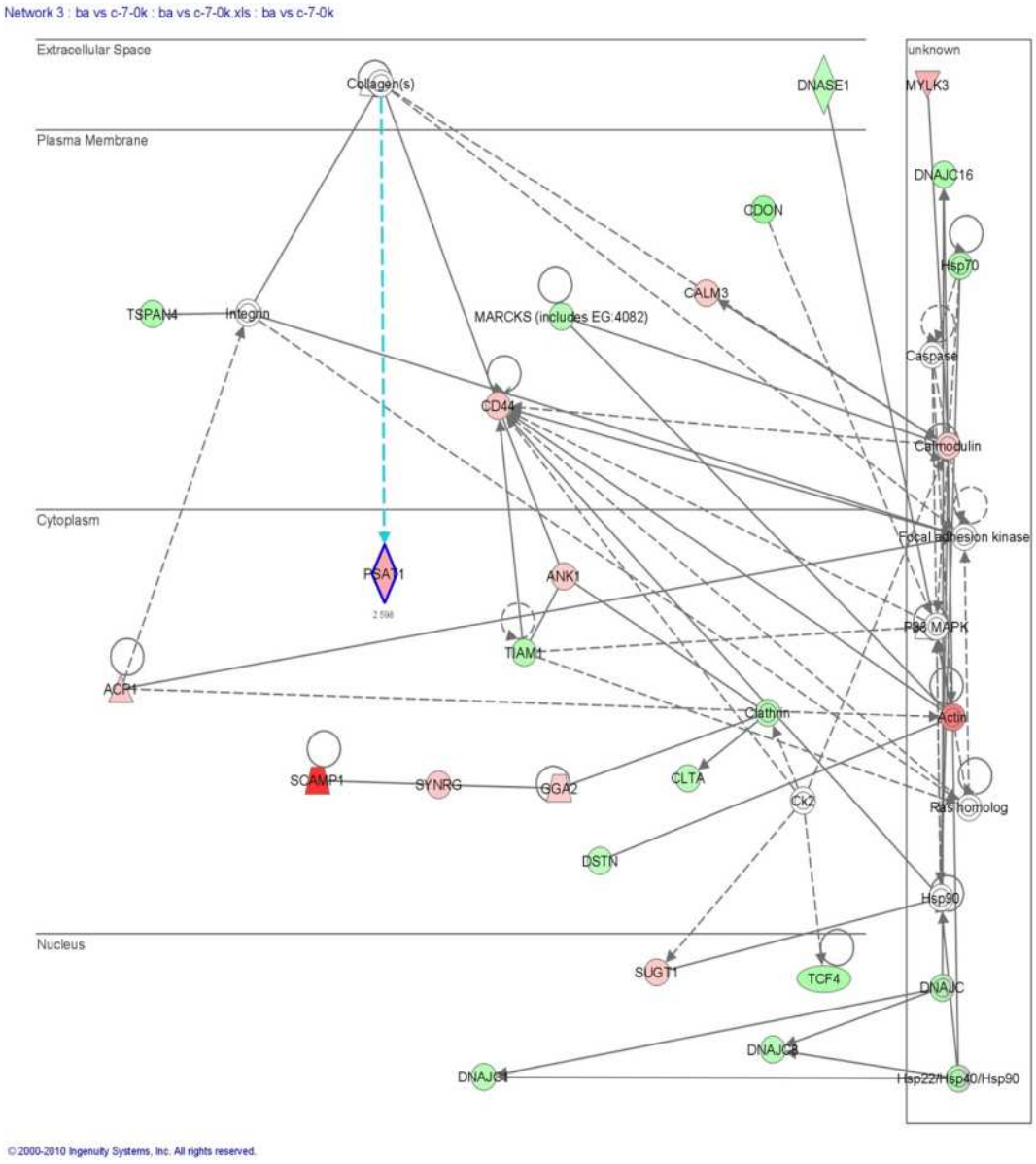


Figure 4-7: The effects of BA on the gene expression in longissimus dorsi of sheep treated for 6 days; effects on the PSAT1 gene expression network (IPA analysis).

The network identification of genes significantly changed in BA treatment relative to control was carried out using IPA analysis. Red indicates the genes up-regulated by BA relative to the control; green indicates the genes down-regulated by BA relative to the control. White shows the molecules not affected by the treatment, but added from Ingenuity Knowledge Base in order to complete the network. PSAT1 and its relationship lines were blue dashed lines, to refer to indirect interactions. Adapted from (Gong, 2010).

4.1.5 List of Genes Identified in this Study

From the list of genes identified as having altered expression due to growth promoter treatment it was apparent that the gene expression of specific metabolic processes were being influenced

by growth promoters. Of particular interest was the BA induced increased gene expression of phospho-serine amino transferase 1 (PSAT1), which is part of the pathway that utilizes intermediates of glycolysis to synthesize serine (Figure 4-8). Therefore further quantitative reverse transcriptase PCR (qRT-PCR) analysis was carried out on a group of genes (Appendix 2), which were identified by GeneSpring or IPA analysis pathways, to confirm whether these genes were affected by treatment on all of the samples from trial, rather than the subset (n=3, for each treatment) which had been selected for transcriptome microarray analysis.

As part of this study further examination of the genes that appeared to be affected by growth promoter treatment were analysed. Gene expression analysis (GeneSpring) indicated more pathways affected by BA than GH treatment. Transcriptome analysis of selected samples indicated the novel observation that an enzyme involved in serine synthesis was up regulated by BA treatment. The high expression of the three enzymes involved in serine synthetic pathway (PHGDH, PSAT1 and PSPH) has previously been described in cancer cells, indicating that serine synthetic pathways may play a critical role in tumorigenesis (Pollari et al., 2011). Muscle hypertrophy, which is associated with changes in nutrient mobilisation and utilization, is one of the characteristics of growth promoters, such as BA and GH (Bell et al., 1998). Recent research has demonstrated that hyperplastic growth and switch to glycolytic metabolism in some cancers is associated with upregulation of the serine synthesis pathway, which then is utilised by additional biosynthetic pathways (Amelio et al., 2014). BA decreased the gene expression of oxidative respiration pathways (TCA cycle and oxidative phosphorylation) as well as appearing to increase an enzyme involved in the serine

biosynthetic pathway along with increased expression of genes encoding glycolysis enzymes.

For the further analyses by qRT-PCR, two muscles were examined the *longissimus dorsi* (LD) *and supraspinatus* (SS). These muscles have different fibre type composition, LD being a fast glycolytic muscle whilst SS has a significantly higher proportion of slow fibres (Hemmings et al., 2009). Previous reports have indicated that growth promoters such as the BA induce a shift toward a glycolytic fibre type. Therefore analysis was carried out to determine if the growth promoters had differing effects on the gene expression in muscles with intrinsically different fibre types. In addition, analysis was carried out on liver to determine whether the growth promoters were having an effect on this non-muscle tissue, which is responsible for the provision of substrates that are utilised by muscle for energy metabolism.

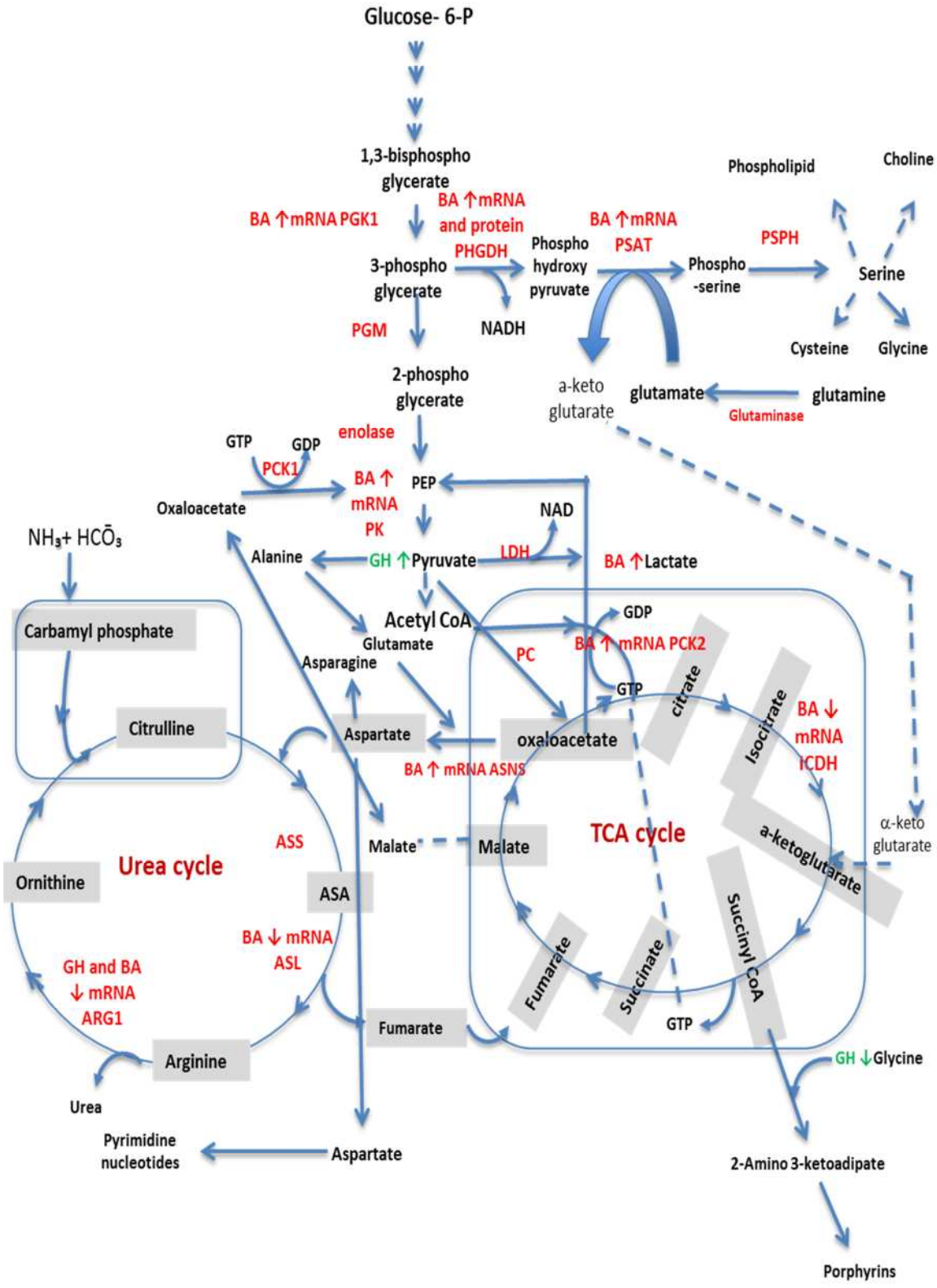


Figure 4-8: The integration of the metabolic pathway for glycolysis, serine synthesis, TCA and urea cycles. In addition those enzymes which are potentially involved in gluconeogenesis are indicated.

The diagram shows the glycolysis associated pathway which uses the glycolytic intermediate, 3-Phosphoglycerate, to synthesise serine and its derivatives. The TCA and urea cycles provide intermediates for many biosynthetic processes, which are indicated.

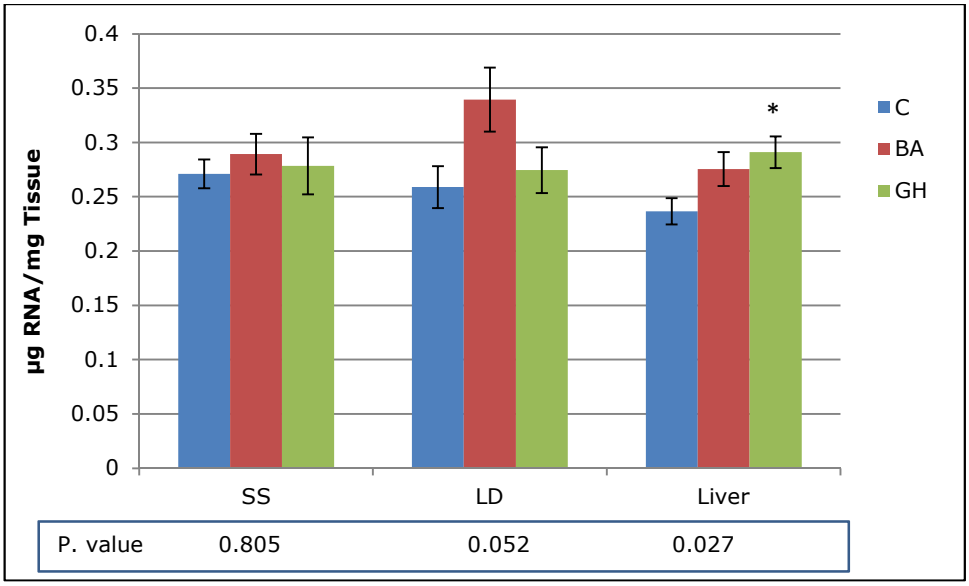
The positions of selected enzymes associated with the pathways are shown. Text in red indicates enzymes or metabolites affected by BA whilst green indicates those affected by GH. Phosphoglycerate kinase 1 (PGK1), phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), phosphoserine phosphatase (PSPH), phosphoglycerate mutase (PGM), phosphoenolpyruvate (PEP), pyruvate kinase (PK), lactate dehydrogenase (LDH), phosphoenolpyruvate carboxykinase (PCK; cytosolic PCK1, and mitochondrial PCK2), pyruvate carboxylase (PC), asparagine synthetase (ASNS), argininosuccinate synthase 1 (ASS1), arginase 1 (ARG1), and argininosuccinate lyase (ASL).

4.2 Effect of GPs on RNA Quantity and Quality

Total RNA was extracted from tissue samples from sheep treated with growth promoters for 6 days using the methods as described in material and methods. There were no significant effects of treatment on RNA concentration per unit tissue in SS and LD muscles ($P<0.805$, $P<0.052$, respectively) (Figure 4-9 A). In liver, treatment of sheep with GH for six days significantly increased ($P<0.027$) the RNA concentration per unit tissue relative to the control group (Figure 4-9 A).

Ratio readings of 1.8-2.0 for the 260nm/280nm were deemed acceptable for RNA quality. There were no significant differences in the 260/280 ratio in SS, LD muscles and liver in lambs treated with BA or GH for six days ($P<0.310$, $P<0.536$, $P<0.885$, respectively) (Figure 4-9 B).

A



B

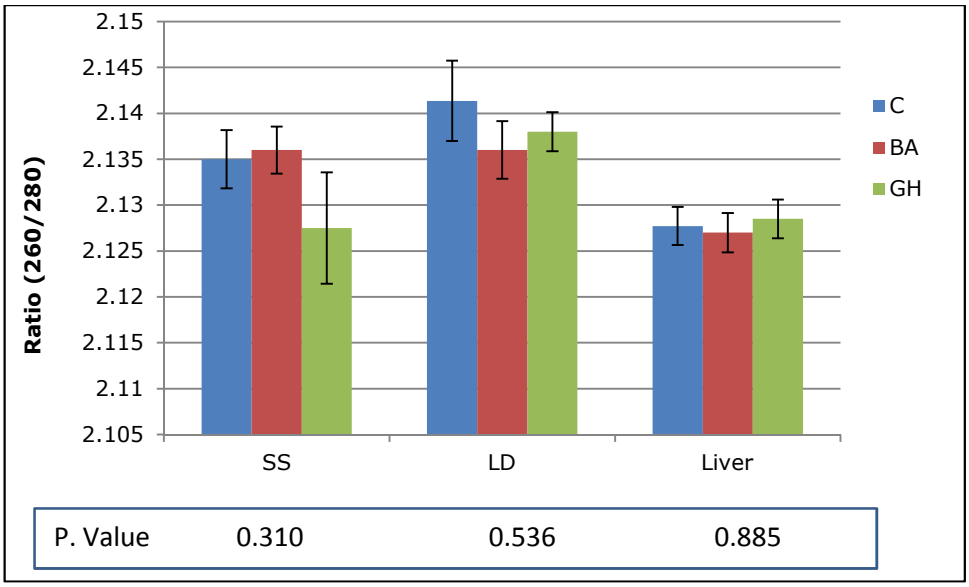


Figure 4-9: Effects of GH and BA on sheep (A) total RNA concentration and (B) 260/280 ratio in SS, LD muscles and Liver.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA p values are indicated and error bars are ± SEM (n=11 for C and n=10 for BA and GH). * indicates a significant difference compared to control (P<0.05) measured by Dunnett's test.

Non-denaturing agarose gel electrophoresis was used to examine and check the total RNA integrity (Materials and Methods) (Figure 4-10).

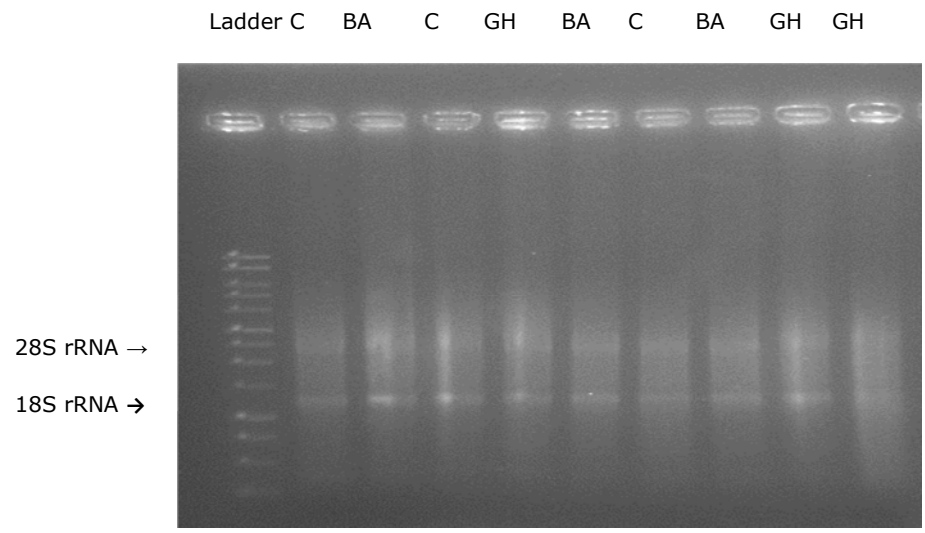


Figure 4-10: Effects of GH and BA on sheep total RNA integrity in sheep LD muscle by non-denaturing agarose gel electrophoresis-representative image.

Treatments: C (control), BA (beta agonist) and GH (growth hormone). The RNA ladder indicates size of bands during electrophoresis. S =Svedberg unit (sedimentation coefficient).

4.3 Normalising Factors for Reverse Transcriptase PCR:Oligreen

To normalise gene expression when carrying out qRT-PCR analysis the commonly adopted procedure is to normalise to expression of a reference gene. This gene's expression should not be influenced by the treatment. A common gene utilised is cyclophillin, but other genes can be utilised such as β-actin (ACTB) and ribosomal protein, large, P0 (RPLP0) which were also tested in this study. The total RNA for all the samples from the sheep growth promoter trial were normalised for RNA concentration then subjected to first strand cDNA synthesis using random hexamers. For all potential normalisation genes there were no significant differences between treatments (Figure 4-11, Figure 4-12 and Figure 4-13), but the variability was quite large at times. An alternative to using a reference gene is oligreen (Rhinn et al., 2008). Oligreen is a dye which emits fluorescence when bound to single-stranded DNA (ssDNA) (Reyderman and Stavchansky, 1996). This technique

makes an assessment of the quantity of cDNA to allow normalization for cDNA used in the PCR. Lundby et al. (2005) analyzed the data from human skeletal muscle RNA to determine cDNA synthesis using oligreen and suggested that this dye gave a potential alternative normalization process compared to the use of reference genes for gene expression studies. When examining oligreen data there were no significant effects (P>0.05) of growth promoting agents on oligreen observed in any tissues used in this study, plus the variability was reduced (Figure 4-14).

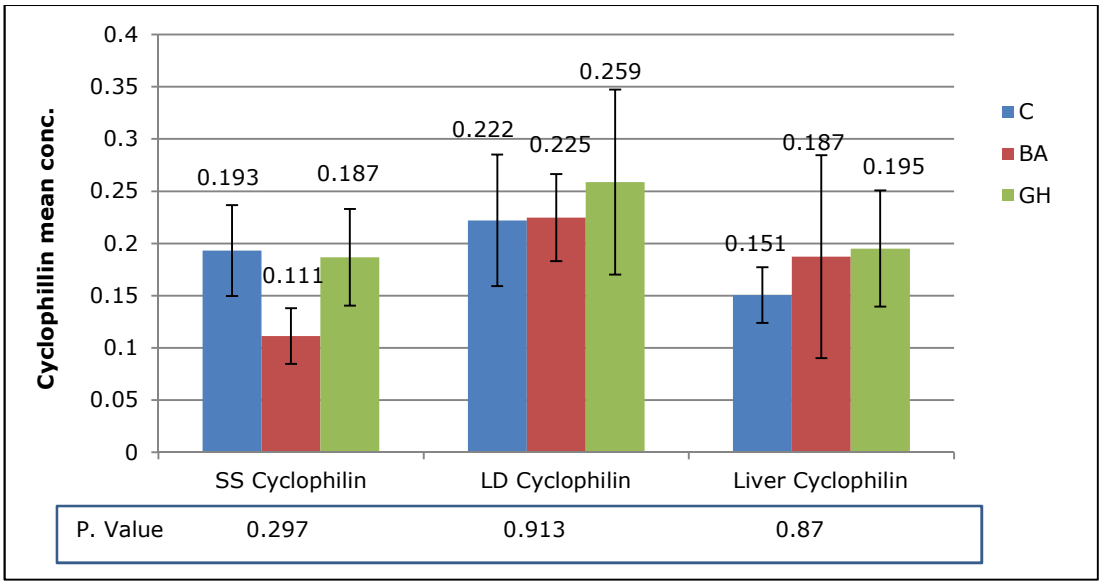


Figure 4-11: Effects of GH and BA on cyclophillin mRNA expression in SS, LD muscles and Liver.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. The error bars are ± SEM (n=11 for C and n=10 for BA and GH). Statistical comparison was made between treatments (one way ANOVA) within the same tissue, not between tissues. There were no significant differences (p>0.05) between treatments.

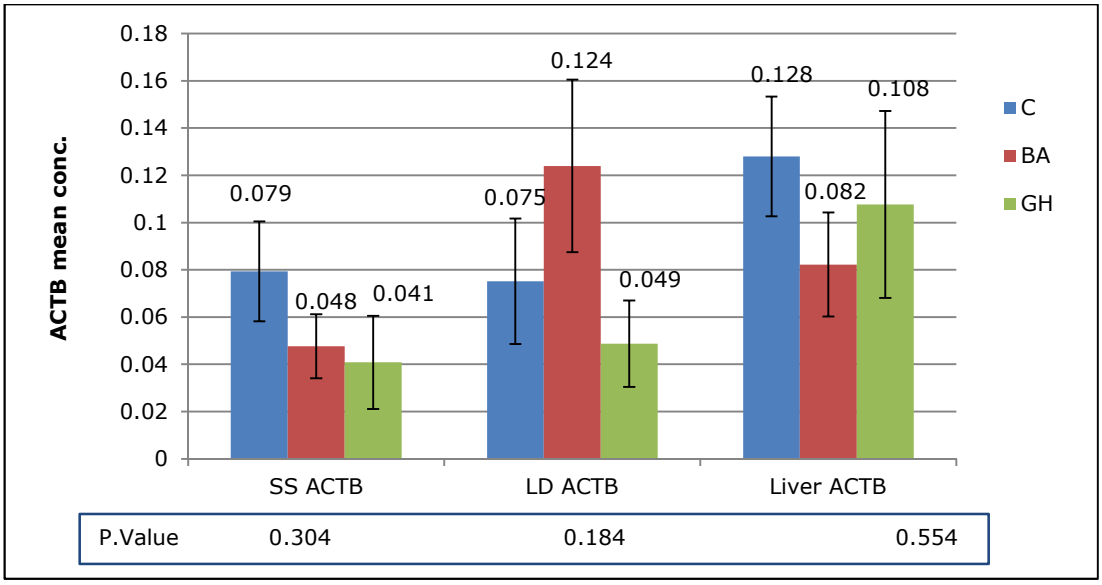


Figure 4-12: Effects of GH and BA on β-actin (ACTB) mRNA expression in SS, LD muscles and Liver.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. The error bars are ± SEM (n=11 for C and n=10 for BA and GH). Statistical comparison was made between treatments (one way ANOVA), within the same tissue, not between tissues. There was no significant difference (p>0.05) between treatments.

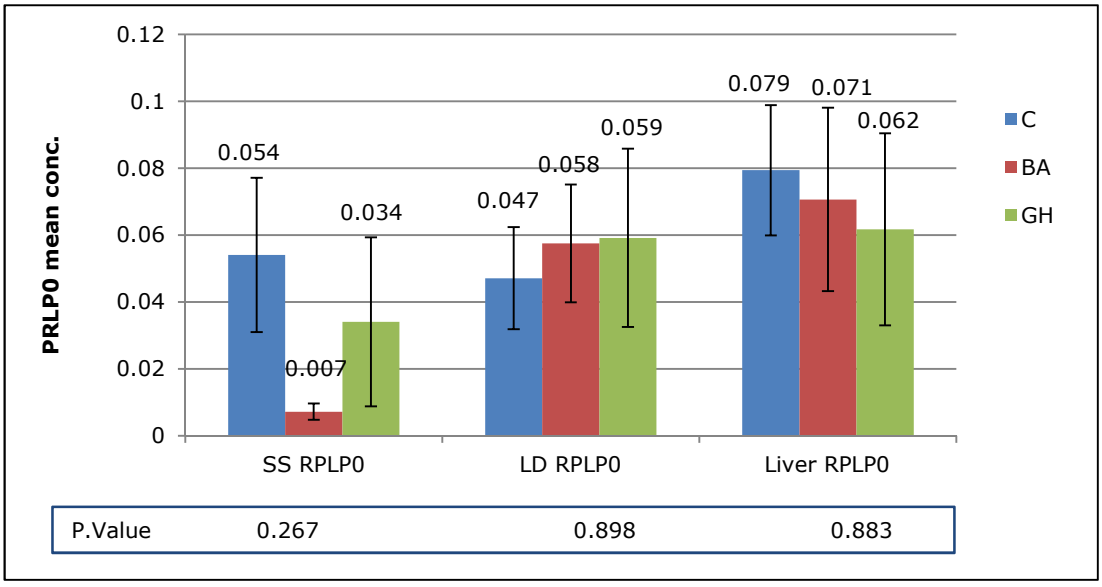


Figure 4-13: Effects of GH and BA on Ribosomal protein, large, P0 (RPLP0) mRNA expression in SS, LD muscles and Liver.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. The error bars are ± SEM (n=11 for C and n=10 for BA and GH). Statistical comparison was made between treatments (one way ANOVA), within the same tissue, not between tissues. There was no significant difference (p>0.05) between treatments.

When examining the variance in the data obtained for reference gene expression there appeared to be a high degree of variance across all treatment groups, although there was no significant difference between treatment groups (Figure 4-11, Figure 4-12 and Figure 4-13), whereas the variance in the oligreen data was much less (Figure 4-14). Therefore, the expression of all metabolic genes in this project were normalised to oligreen, rather than using a reference gene.

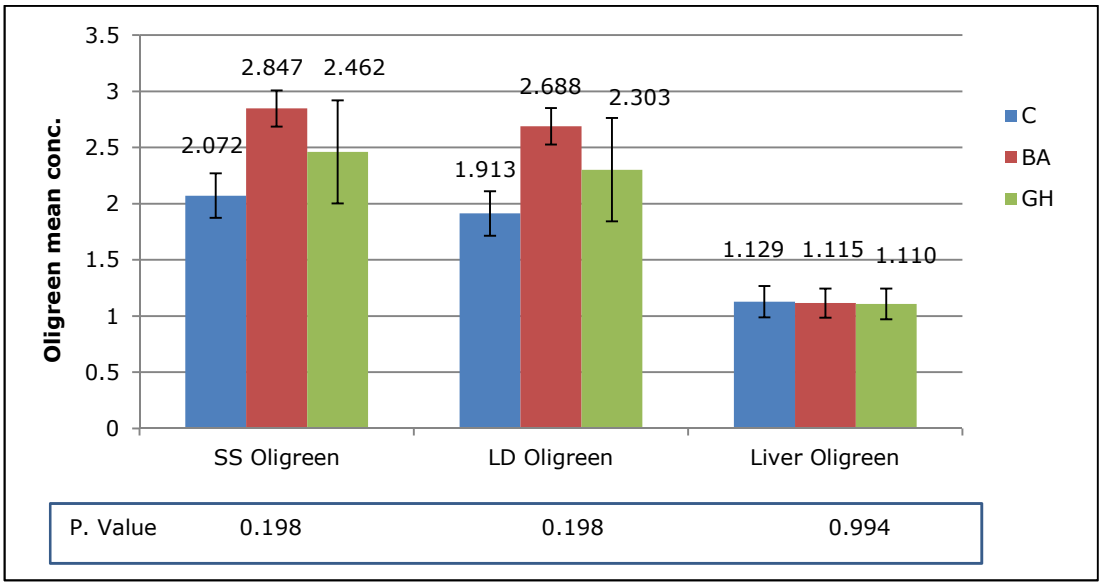


Figure 4-14: Effects of GH and BA on oligreen concentrations (cDNA quantity) in SS, LD muscles and Liver.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. The error bars are ± SEM (n=11 for C and n=10 for BA and GH). Statistical comparison was made between treatments (one way ANOVA), not between tissues. There was no significant difference ($p>0.05$) between treatments.

4.4 Effects of BA and GH on Expression of Genes Associated with Serine Biosynthesis Pathway

4.4.1 Effects of GPs on mRNA Expression

Phosphoglycerate Kinase 1 (PGK1) has an important role in the production of 3-phosphoglycerate (3-PGA) from 1, 3

bisphosphoglycerate, the former being the glycolytic intermediate utilised for serine biosynthesis. It appeared that BA, but not GH, significantly (P<0.05) increased the expression of PGK1 mRNA in the SS muscle compared to control (Figure 4-15). There was also no effect on PGK1 mRNA in the LD muscle found in lambs treated with BA compared to control (Figure 4-15). There was no change in PGK1 expression observed in the liver (Figure 4-15).

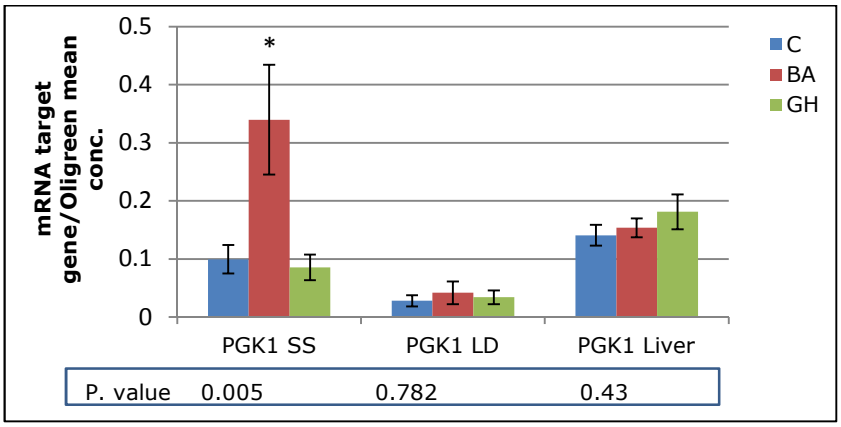


Figure 4-15: Effects of GH and BA on PGK1 (Phosphoglycerate Kinase1) mRNA expression in SS, LD muscles and liver.

RNA expression normalised to oligreen concentrations. Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA p values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant difference between treatments (P<0.05) measured by Dunnett's test.

Phosphoglycerate dehydrogenase (PHGDH) catalyses the first step in the serine synthesis pathway by oxidizing 3-PGA to 3-phosphohydroxypyruvate (PHP), using NAD+/NADH as cofactor. PSAT catalyses conversion of phosphohydroxy pyruvate into 3-phosphoserine, which is then dephosphorylated by phosphoserine phosphatase to form serine (Sugimoto and Pizer, 1968) (Figure 4-16). In the SS and LD muscles the expression of PHGDH mRNA increased (P<0.05) significantly in BA treated lambs relative to control (Figure 4-17 A and B, respectively), but there was no effect in liver (P>0.05) (Figure 4-17 C). There was no effect of GH.

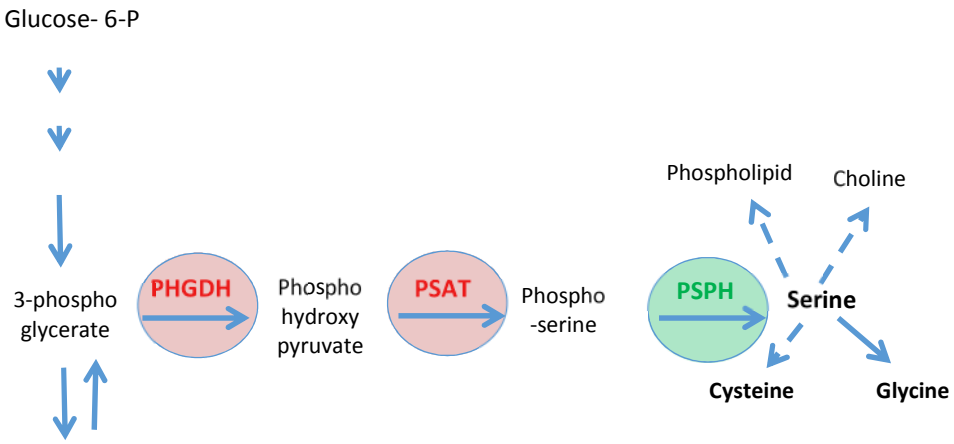


Figure 4-16: Serine synthesis pathway

Phosphoglycerate dehydrogenase (PHGDH) catalyses the first step in serine synthesis by oxidizing 3-PGA to 3-phosphohydroxypyruvate (PHP), Phosphoserine aminotransferase 1 (PSAT1) catalyses the conversion of phosphohydroxy pyruvate to 3-phosphoserine, which is then dephosphorylated by phosphoserine phosphatase (PSPH) to form l-serine. Red circle means significantly increased by BA, green circle means no significant difference.

There was a significant increase in PSAT1 mRNA expression in the SS and LD muscles of BA treated lambs compared to control ($P<0.05$), but there was no effect on the liver, nor any effect of GH on any tissue (Figure 4-17 A, B and C). The expression of phosphoserine phosphatase (PSPH) was not affected by either growth promoting agent in lambs treated for six days (Figure 4-17).

The most important role of serine hydroxyl methyl transferase (SHMT) in serine biosynthesis is to convert serine to glycine. Data in this study concluded that mRNA expression of SHMT1 was unaffected by growth promoting agents on SS and LD muscles as well as liver (Figure 4-17).

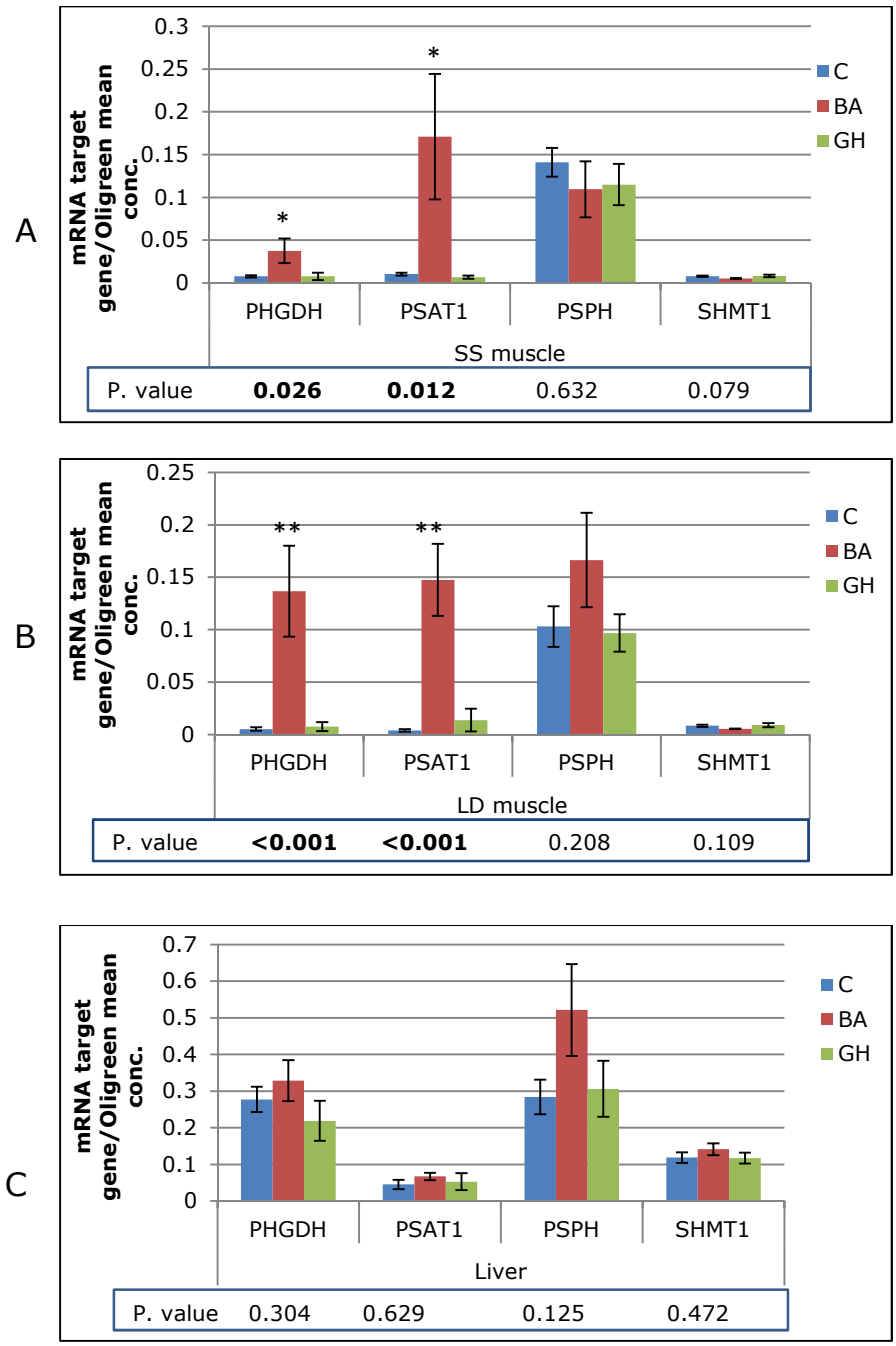


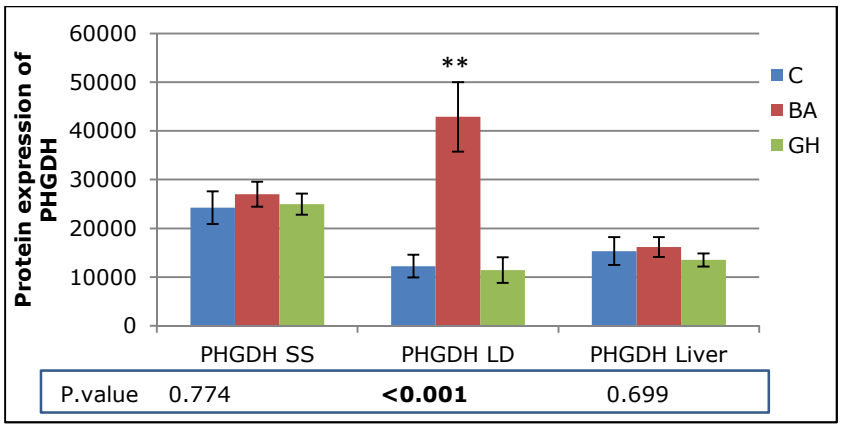
Figure 4-17: Effects of GH and BA on SS (A) and LD (B) muscle and liver (C) gene expression associated with serine biosynthesis pathway.

RNA expression normalised to oligreen concentrations. Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. Selected genes measured were: Phosphoglycerate Dehydrogenase (PHGDH), Phosphoserine Aminotransferase 1 (PSAT1), Phosphoserine Phosphatase (PSPH) and Serine Hydroxymethyltransferas 1 (SHMT1). One way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant difference between treatments relative to control measured by Dunnett's test.* P<0.05, ** P<0.001.

4.4.2 Effects of GPs on Protein Expression

Treatment with BA, but not GH, significantly increased the expression of PHGDH protein compared to control in LD muscle. There were no significant effects of treatment on PHGDH protein expression observed in SS muscle or in liver (Figure 4-18).

A



B

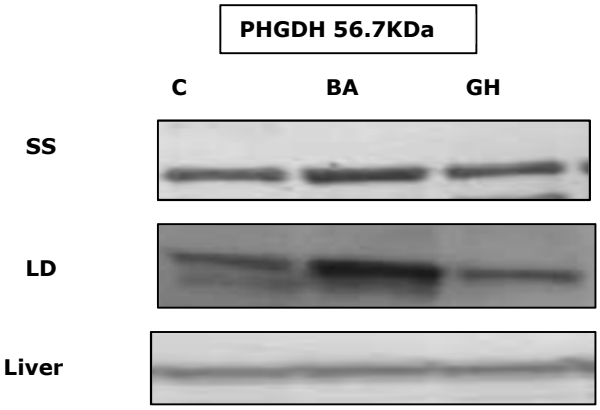


Figure 4-18: Protein expression of PHGDH by western blott analysis in SS, LD muscles and liver.

Western blot PHGDH band intensity (A) and representative western blot bands (B) are shown. Total protein loaded in each well is the same (samples normalised for protein concentration). Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA p values are indicated error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star mean there was a significant difference between treatments relative to control measured by Dunnett's test. ** P<0.001.

4.5 Effects of BA and GH on Transcripts Associated with Energy Metabolism

4.5.1 Effects of GPs on mRNA Expression of Enolase3 and Isocitrate Dehydrogenase2 (ICDH2)

Enolase3 was measured because it is an important glycolytic gene involved in the dehydration of 2-PG to PEP (Donoghue et al., 2005). Enolase3 is a metabolic gene that is used as a marker of glycolytic gene expression. Isocitrate dehydrogenase2 (ICDH2) was used as a marker of oxidative gene expression. Previously our laboratory (Dr Krystal Hemmings) reported changes in these two genes in lamb skeletal muscle treated with BA for 6 days. Enolases expression may be used as a marker of weight loss metabolism (Almeida et al., 2010). Enolase3 and ICDH2, displayed noteworthy correlation with the type IIB and type I MyHC mRNA isoforms *in vivo*, respectively, suggesting these genes may be essential candidates for controlling and monitoring metabolic changes in skeletal muscle associated with contractile characteristics (Brown, 2014). Studies have reported that lower concentrations of enolase are associated with reduced capacity of glycolytic substrate phosphorylation (Donoghue et al., 2005), whilst higher concentrations of the enzyme are implicated in a higher rate of glycolysis to maintain and support ATP production (Jia et al., 2007). There were no significant effects ($P>0.05$) on enolase3 mRNA expression in any tissue used in this study (Figure 4-20 A).

Isocitrate dehydrogenase2 (ICDH2) plays a key role in tricarboxylic acid (TCA) cycle regulation in mammalian tissues, it catalyses the conversion of isocitrate and NADP to alpha-ketoglutarate and NADPH (Figure 4-19). In the current study, there was a significant effect on ICDH2 mRNA expression in the SS and LD muscles ($P<0.05$), with a significant decrease in BA treated lambs

compared to controls (P<0.05) (Figure 4-21 A and B). There was no effect of treatment on the ICDH2 mRNA expression in the liver (Figure 4-21 C). The expression of ICDH2 was unaffected by GH-treatment in any tissue used in this study.

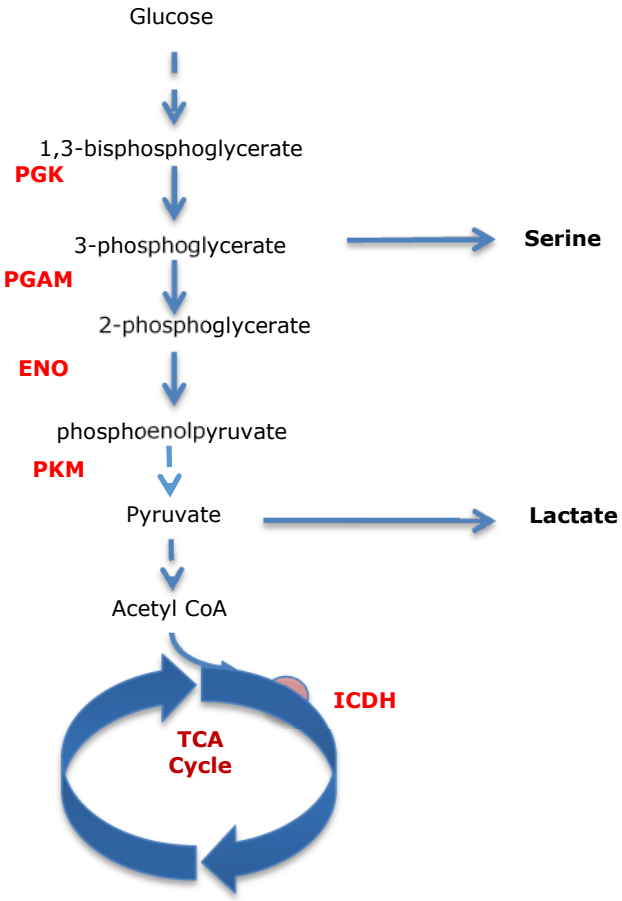
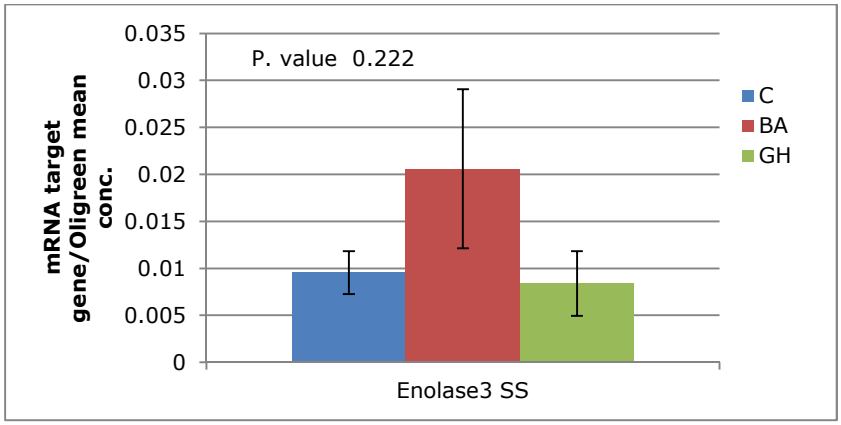


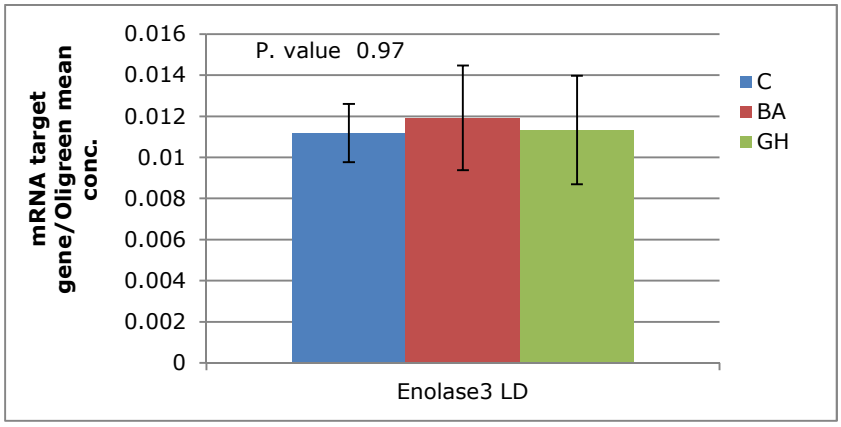
Figure 4-19: Glycolysis pathway and ICDH expression in the TCA cycle.

Phosphoglycerate kinase (PGK), Phosphoglycerate mutase (PGAM), Enolase (ENO), pyruvate kinase muscle isozyme (PKM), and isocitrate dehydrogenase (ICDH).

A



B



C

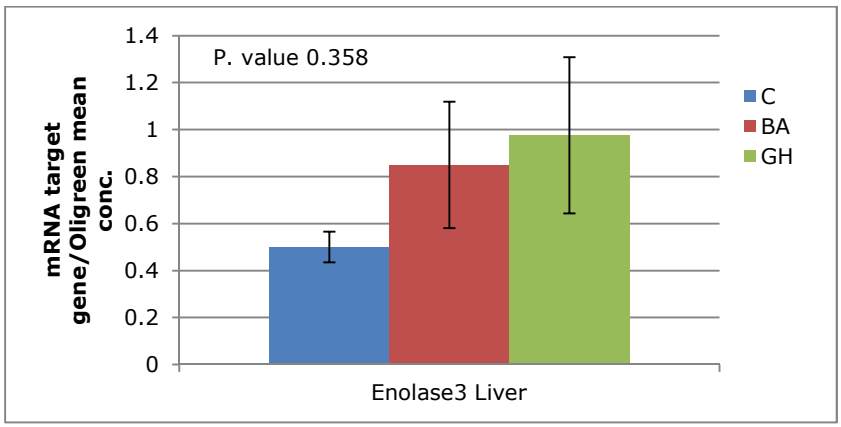


Figure 4-20: Effects of GH and BA on Enolase3 mRNA expression in SS (A) and LD (B) muscles and liver(C).

RNA expression normalised to oligreen concentrations. Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA p values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

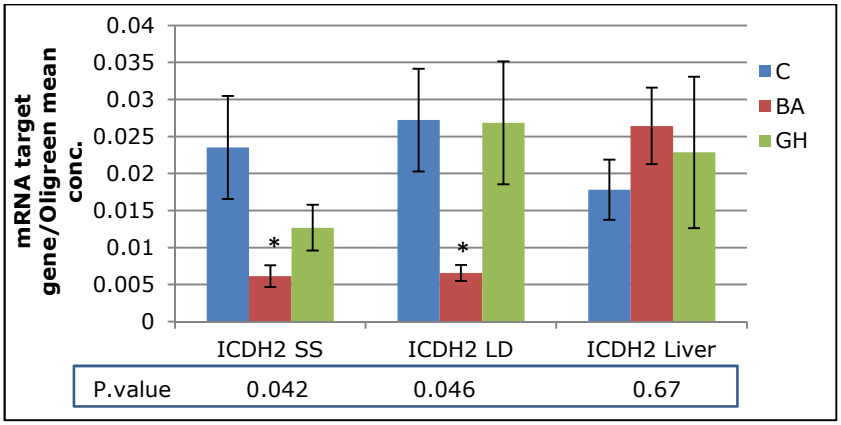


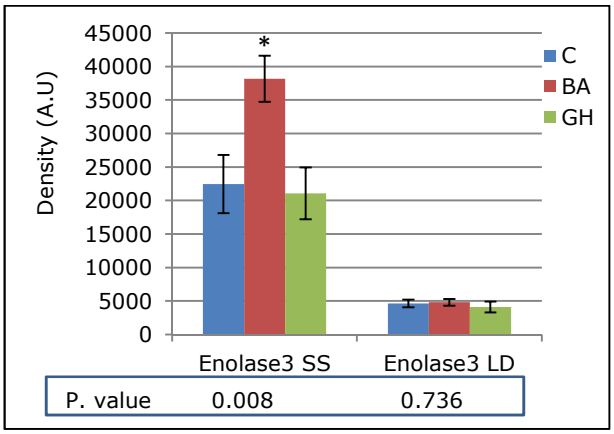
Figure 4-21: Effects of GH and BA on ICDH2 mRNA expression in SS, LD muscles and liver.

RNA expression normalised to oligreen concentrations. Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One way ANOVA p values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test.* $P<0.05$.

4.5.2 Effects of GPs on Protein Expression of Enolase3

There was a significant effect of treatment observed on protein expression of enolase3 in SS muscle ($P<0.008$) relative to control. The lambs treated with BA had significantly higher expression compared to control, but the expression of enolase3 protein was not affected by GH compared to control. There was no significant effect of either growth promoter on the protein expression of enolase3 in LD muscle (Figure 4-22 A and B) relative to control. As expected there was no expression of enolase3 protein detected in the liver, as this protein is predominantly expressed in muscle.

A



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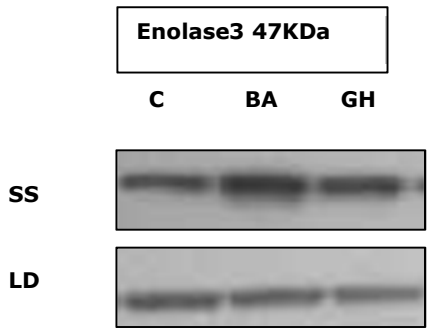


Figure 4-22: Protein expression of enolase3 (A and B) by western blotting analysis in SS and LD muscle.

Western blot enolase3 band intensity (A) and representative western blot bands (B) are shown. Total protein loaded in each well is the same (samples normalised for protein concentration). Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One way ANOVA p values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test.* P<0.05.

4.5.3 Effects of GPs on mRNA expression of PKM

Pyruvate Kinase Muscle (PKM) was examined in this study because of its important role in contributing to the serine synthetic pathway (Ye et al., 2012). The PKM gene consists of 12 exons and 11 introns and has two isozymes named M1 and M2. PKM2 differs from PKM1 only by the mutually exclusive splicing of one exon (exon 9 included in PKM1 and exon 10 in PKM2) (Figure 4-23). There was a significant effect of treatment on the expression of PKM in SS (P<0.003) and LD (P<0.04) muscles (Figure 4-24 A and B), but not liver (Figure 4-24 C). BA, but not GH, significantly increased (P<0.05) the expression of total PKM mRNA in both SS and LD muscles compared to control. BA significantly increased the expression of PKM1 and PKM2 mRNA in both SS (P<0.019 and P<0.003, respectively) and LD (P<0.001 and P<0.024) relative to control, whilst GH did not (Figure 4-24 A and B). There was no effect of treatment on liver PKM expression (Figure 4-24 C).

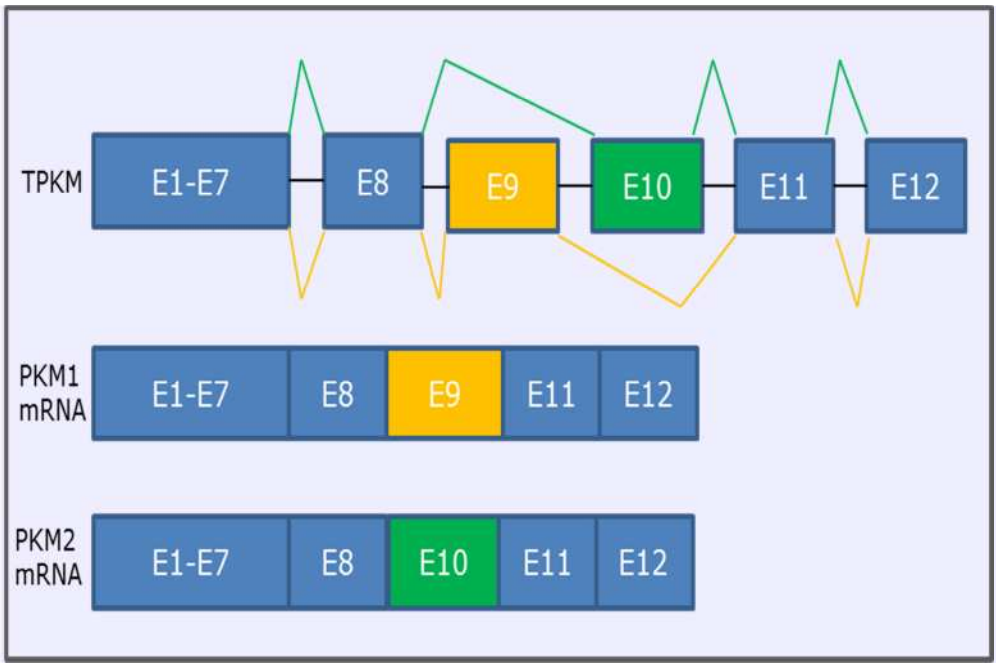
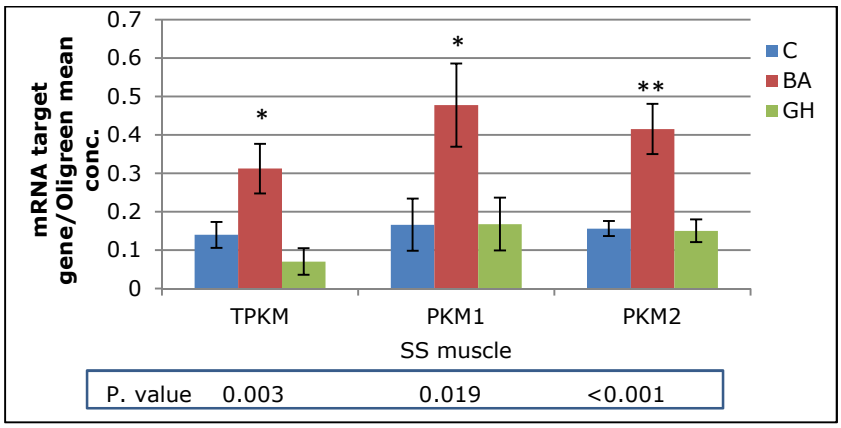


Figure 4-23: Alternative splicing of PKM.

Total pyruvate kinase muscle (TPKM) gene consists of 12 exons. PKM1 and PKM2 transcripts are splice variants which have mutually exclusively splicing of exons 9 or 10, with PKM1 retaining exon 9 and PKM2 retaining exon 10.

A



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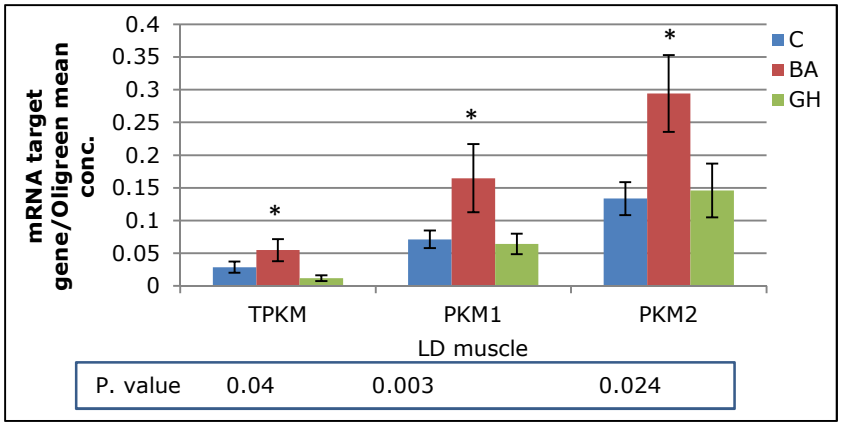

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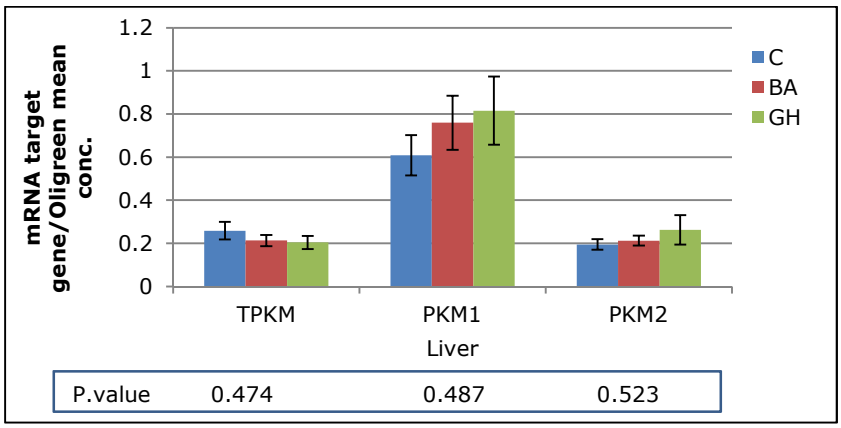


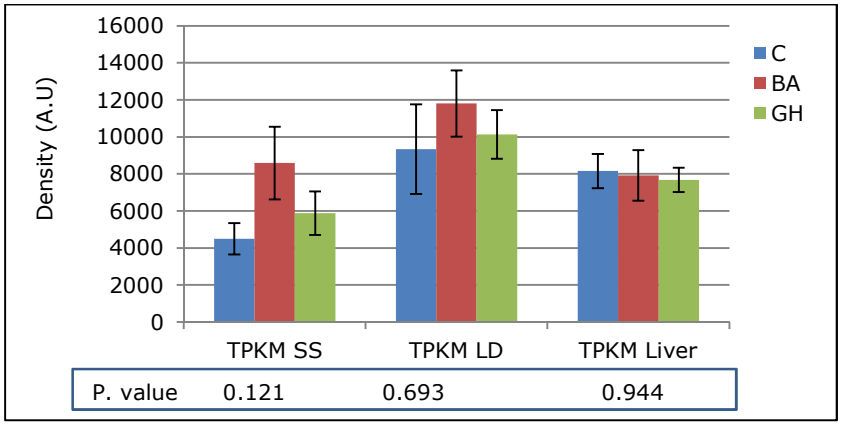
Figure 4-24: Effects of GH and BA on SS (A) and LD (B) muscle and liver (C) gene expression associated with glycolysis pathway.

RNA expression normalised to oligreen concentrations. Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. Selective gene measure were: Total Pyruvate Kinase Muscle (TPKM), Pyruvate Kinase Muscle1 (PKM1) and Pyruvate Kinase Muscle2 (PKM2).One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant difference between treatments relative to control measured by Dunnett's test. * P<0.05, ** P<0.001.

4.5.4 Effects of GPs on Protein Expression of Total PKM

The expression of total PKM protein was unaffected in liver, SS or LD muscles (Figure 4-25 A and B) by six day treatment of lambs with BA or GH.

A



B

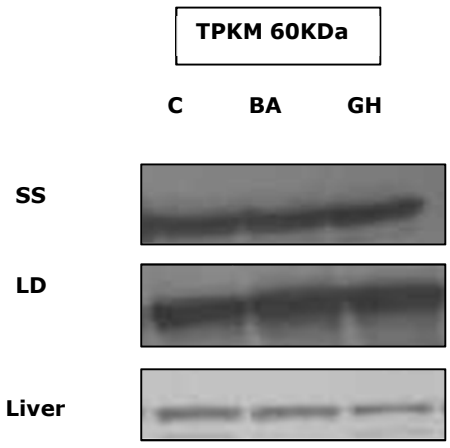


Figure 4-25: Protein expression of total pyruvate kinase muscle (TPKM) by western blot analysis in SS, LD muscles and liver.

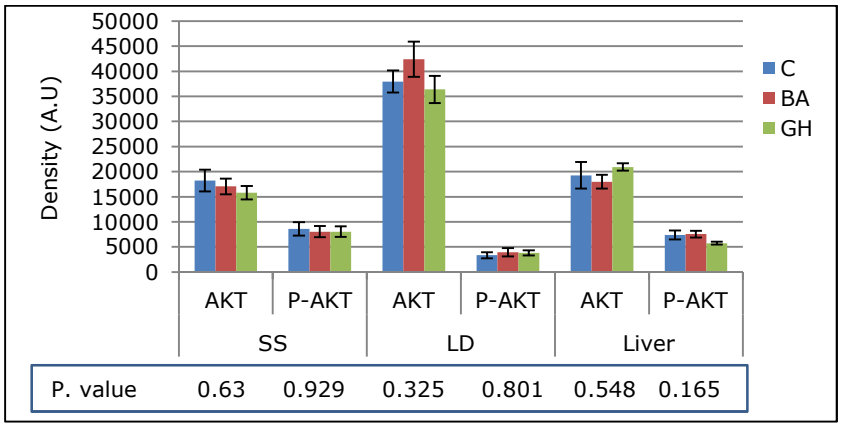
Western blot TPKM band intensity (A) and representative western blot bands (B) are shown. Total protein loaded in each well is the same (samples normalised for protein concentration). Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA p. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

4.5.5 Effects of GPs on Protein Expression of AKT and P-AKT

Microarray and subsequent pathway analysis identified the AKT1 pathway in sheep as being affected by treatment. This pathway has an important role in muscle hypertrophy and skeletal muscle differentiation (Bodine et al., 2001, Rommel et al., 2001, Glass, 2003a, Stitt et al., 2004, Chang, 2007). The v-akt murine thymoma viral oncogene homolog (AKT, also known as protein kinase B), is a serine/threonine-specific protein kinase which regulates multiple cellular processes such as proliferation, differentiation, apoptosis and glucose metabolism (Cantley, 2002), It is the major downstream target of phosphoinositide 3-kinase (PI3K). The PI3K/Akt signalling pathway has an important function in the response to growth factors, especially insulin-like molecules, and its dysregulation is a specific feature of diabetes and many types of cancer (Wilson et al., 2007, Morgensztern and McLeod, 2005).

PI3K activation activates Akt which in turn activates mTOR. Therefore Akt is an activator of many of the signalling pathways associated with growth. In many tumours, such as breast cancers, the PI3k/Akt/mTOR signalling pathway is high phosphorylated and leads to decreased apoptosis and increased growth (Morgensztern and McLeod, 2005). The expression of protein kinase B AKT protein was not affected in lamb SS and LD muscle in response to treatment. There were also no significant effects of either agents on AKT protein in the liver (Figure 4-26 A and B). The quantity of P-AKT was also not affected in SS and LD muscles or liver in lambs treated with BA (cimaterol) or GH for a short time (6 days) (Figure 4-26 A and B).

A



B

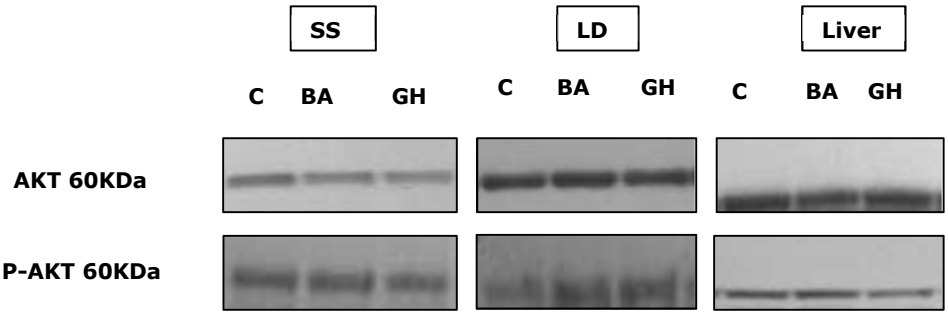


Figure 4-26: Protein expression of AKT and P-AKT (Phosphorylated AKT) by western blot analysis in SS, LD muscles and liver.

Western blot AKT and P-AKT specific band intensity (A) and representative western blot bands (B) are shown. Total protein loaded in each well is the same (samples normalised for protein concentration). Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA p values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

4.6 Effects of BA and GH on Gene Expression Associated with Gluconeogenesis

4.6.1 Effects of GPs on mRNA Expression

Gluconeogenesis (GNG) is a metabolic pathway that leads to synthesis of glucose from non-carbohydrate substrates such as pyruvate, lactate, glycerol and gluconeogenic amino acids. The main site of gluconeogenesis is the liver, where it is involved in generating glucose to maintain blood glucose levels during the fasted state.

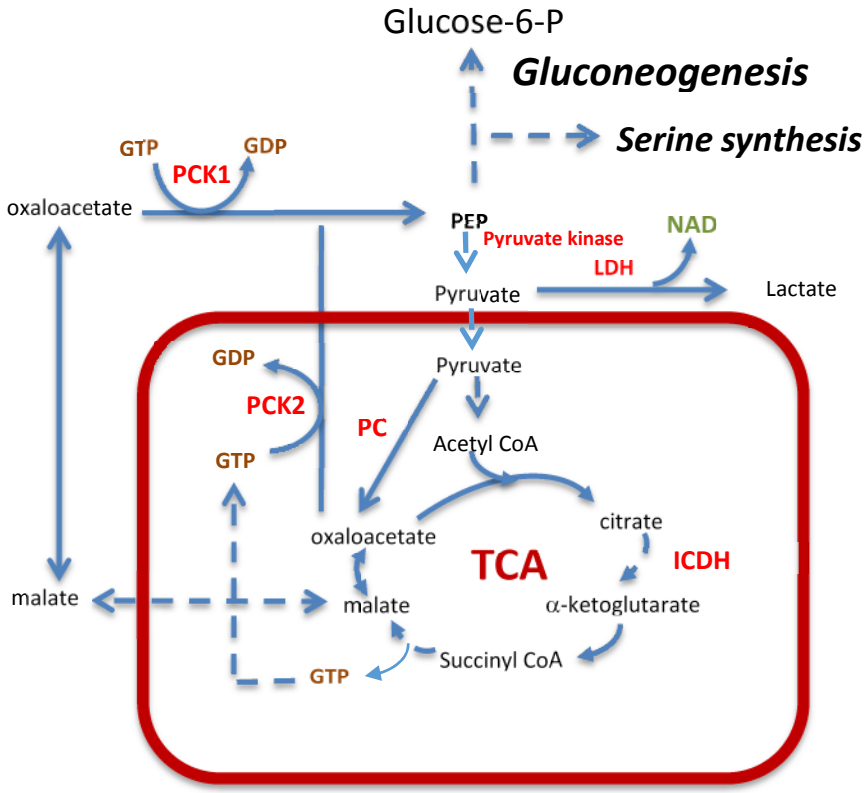


Figure 4-27: Gluconeogenesis and TCA cycle indicating the role of PCK1 and PCK2

The diagram shows the role of PCK1 and PCK2 in gluconeogenesis. Phosphoenolpyruvate (PEP), lactate dehydrogenase (LDH), pyruvate carboxylase (PC), isocitrate dehydrogenase (ICDH), phosphoenolpyruvate carboxykinase cytosolic (PCK1) and mitochondrial (PCK2).

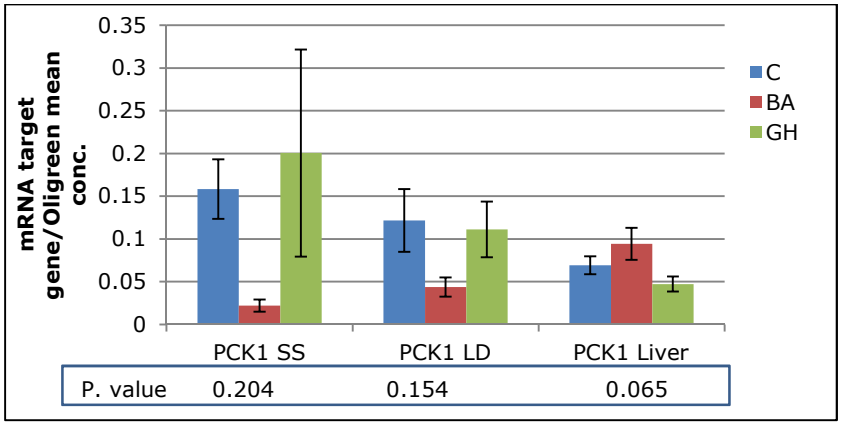
Phosphoenolpyruvate carboxykinase (PCK) has a critical role in the regulation of gluconeogenesis. There are two isoforms of PCK, a cytosolic form (PCK1) and a mitochondrial form (PCK2) (Figure 4-27). In the present study, there were no significant changes of growth promoting agents on PCK1 mRNA expression in LD and SS muscles in lambs treated with BA or GH. However, there was a trend for an effect of treatment on PCK1 mRNA in liver ($P<0.065$), with BA having the highest value and GH the lowest (Figure 4-28 A).

PCK2 is the mitochondrial isoform of PCK. This isoform is expressed in liver and has been described as being involved in liver gluconeogenesis and the TCA cycle, alongside PCK1. In other

tissues it appears to operate independently of PCK1, having direct effects on TCA cycle flux in non-gluconeogenic tissues (Mendez-Lucas et al., 2013).

PCK2 has a role in gluconeogenesis and TCA cycle function cooperating with PCK1 in regulating glucose and lipid metabolism in the liver (Mendez-Lucas et al., 2013). Unlike PCK1, there was a significant effect of treatment on PCK2 mRNA expression ($P<0.011$) in LD muscle, with BA significantly increasing its expression compared to control, whilst there was no significant effect (Figure 4-28 B) in SS muscle and liver. Activation of PCK2 activity could potentially act to provide glycolytic intermediates, which could be used in serine synthesis (Mendez-Lucas et al., 2014). PCK2 was recently identified as a gene whose expression is increased in response to endoplasmic reticulum (ER) stress, this perhaps is mediated by the transcription factor, activating transcription factor 4 (ATF4), which is one of the factors that can mediate the ER stress response.

A



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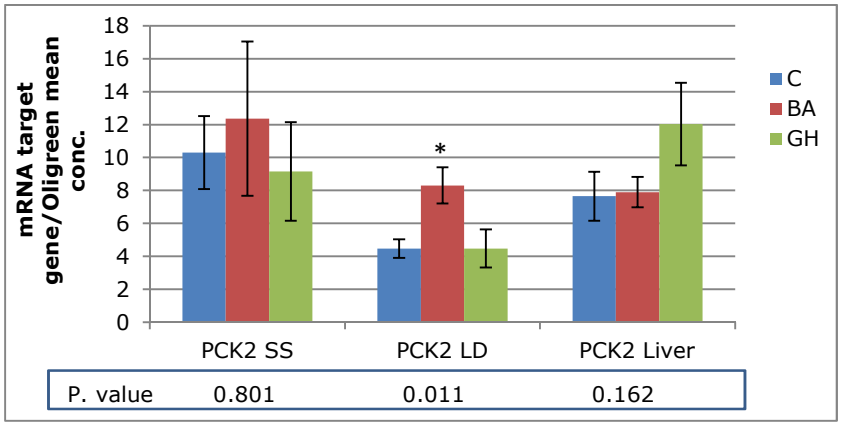


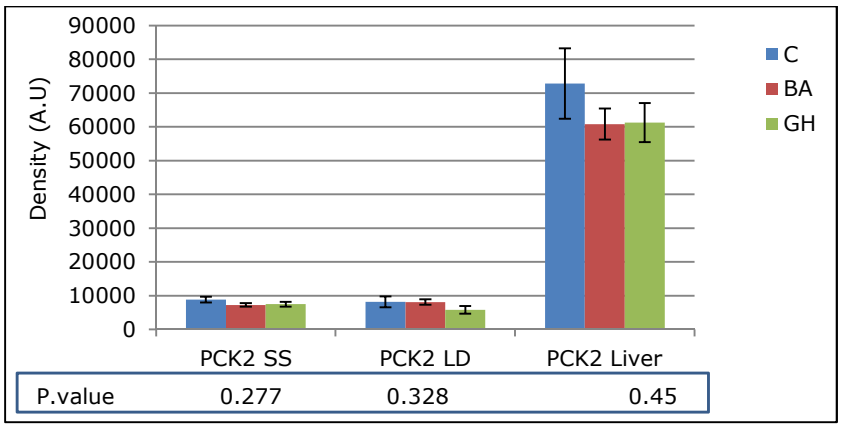
Figure 4-28: Effects of GH and BA on mRNA expression of PCK1 (A) and PCK2 (B) in SS, LD muscle and liver.

RNA expression normalised to oligreen concentrations. Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. Phosphoenolpyruvate carboxykinase 1 (soluble) (PCK1) and Phosphoenolpyruvate carboxykinase 2 (PCK2). One-way ANOVA p. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant difference between treatments relative to control measured by Dunnett's test. * P<0.05.

4.6.2 Effects of GPs on PCK2 Protein Expression

The expression of PCK2 protein were not affected by either growth promoter in SS, LD or liver (Figure 4-29 A and B).

A



B

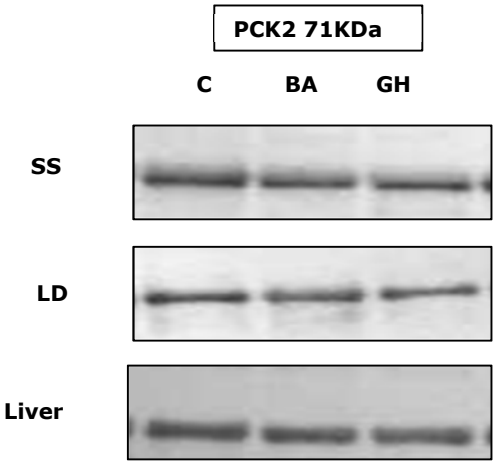


Figure 4-29: Protein expression of PCK2 by western blot analysis in SS, LD muscles and liver.

Western blot PCK2 specific band intensity (A) and representative western blot bands (B) are shown. Total protein loaded in each well is the same (samples normalised for protein concentration). Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA p values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

4.6.3 Effects of GPs on mRNA Asparagine Synthetase (ASNS)

Asparagine synthetase (ASNS) was measured in this project because of its critical role in growth and biosynthesis of the nonessential amino acid asparagine. The enzyme ASNS catalyse the glutamine and ATP dependent conversion of aspartic acid to asparagine. The transcription factor ATF4, which is stimulated by the ER stress response, stimulates ASNS expression, which results in glutamine-dependent asparagine synthesis from aspartate (Ye et

al., 2010). Asparagine plays a vital role in glutamine regulation (Zhang et al., 2014). This is important for growth because a great amount of glutamine is consumed in many tumour cells to preserve TCA cycle anaplerosis and to support the production of nucleotides and nonessential amino acids for cell growth (Zhang et al., 2014).

In the present study, there was a significant effect of treatment on ASNS mRNA expression in the SS and LD muscles ($P<0.001$) and in both cases lambs treated with BA had a significantly higher expression when compared to controls ($P<0.001$, and $P<0.001$). There was no effect on the liver (Figure 4-30).

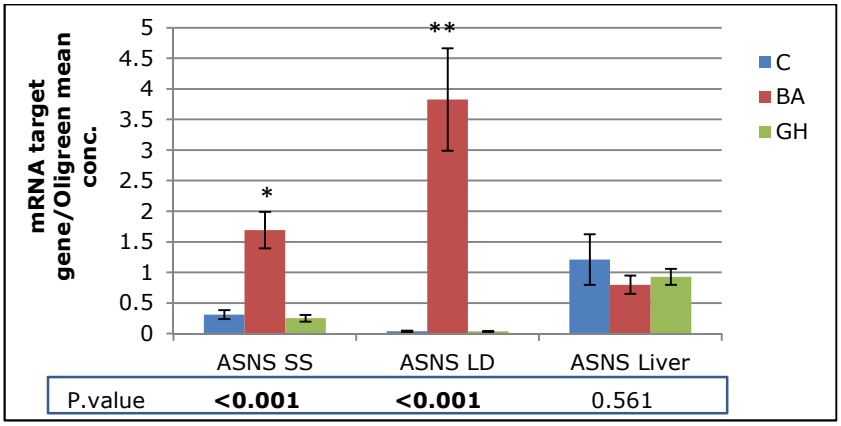


Figure 4-30: Effects of GH and BA on SS, LD muscles and liver asparagine synthetase (ASNS) mRNA expression.

RNA expression normalised to oligreen concentrations Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One way ANOVA p. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test. * $P<0.05$, ** $P<0.001$.

4.7 Effects of BA and GH on Gene Expression Associated with Urea Cycle

A characteristic of some genes examined above is that their transcription is regulated by the transcription factor ATF4, particularly ASNS and enzymes involved with the serine synthesis pathway (Adams, 2007). Recent work from our lab has examined

the effect of GH and BA on pigs over a 28 day time course and identified changes in similar genes (serine synthesis, PCK2 and ASNS) as described in this thesis (unpublished observations). In addition, the work in pigs has shown a significant up-regulation in expression of genes in the urea cycle in skeletal muscle. The urea cycle is strongly connected to the citric acid (TCA) cycle for many reasons. Firstly the urea cycle utilises aspartate, which is derived from oxaloacetate by transamination, to generate argino-succinate which is converted to arginine and fumarate, the latter can then enter the TCA cycle (Shambaugh, 1977). Second, the energy required for biosynthesis of urea is derived from the TCA cycle (Shambaugh, 1977). Third, the CO_2 required for biosynthesis of urea comes mainly from the TCA cycle. Finally, ammonia (NH_3) that is used for production of carbamoyl phosphate comes from glutamate via glutamate dehydrogenase enzyme, which is also related to the TCA cycle. The third step in the urea cycle, in which argininosuccinate is synthesized from citrulline and aspartate, is considered the most important link between the urea and TCA cycles (Shambaugh, 1977).

All five urea cycle enzymes are present in the liver, gut, and kidney, but, except for liver, they are not present at levels that can support urea production (ureagenesis) (Jones et al., 1961).

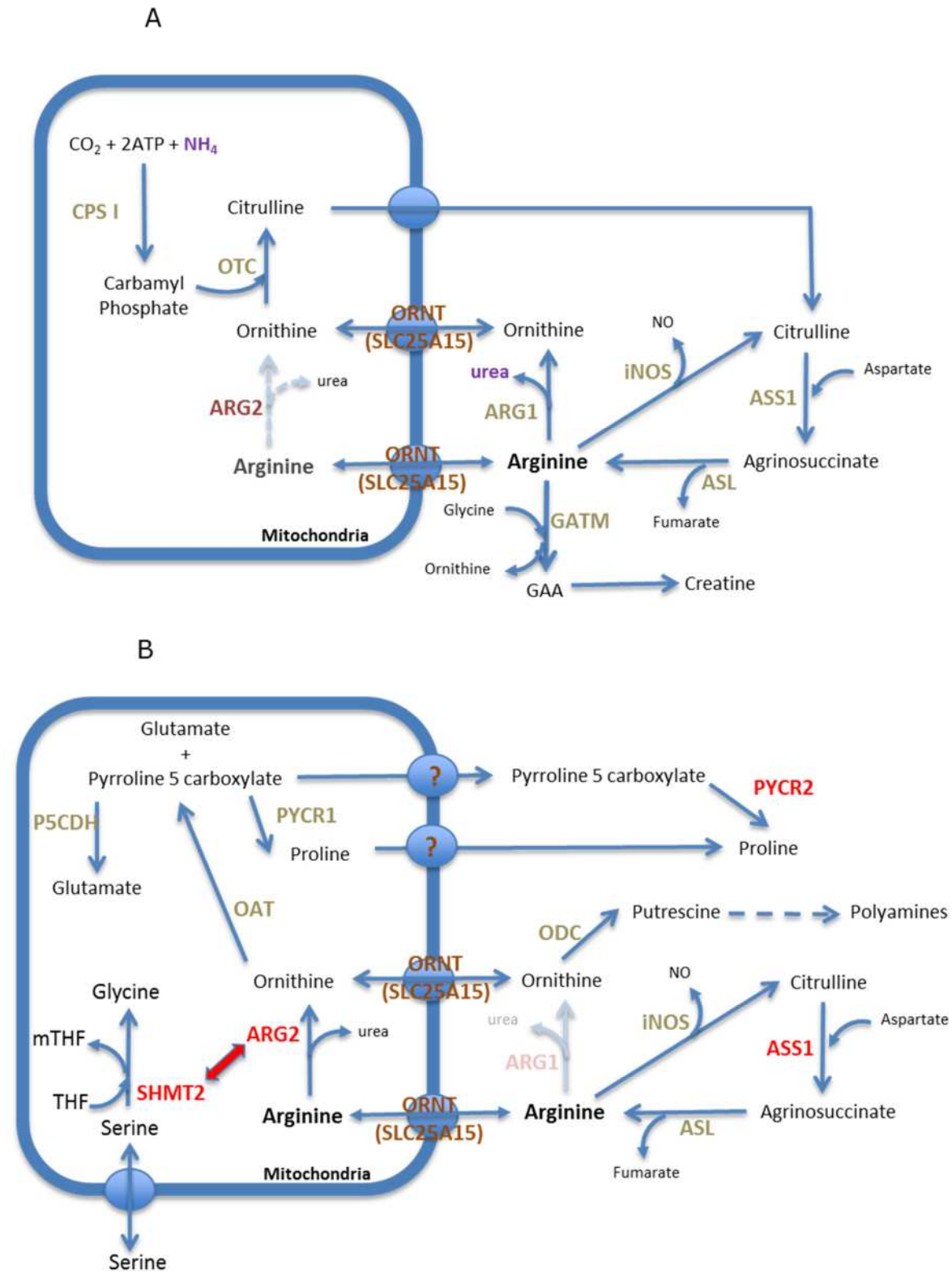


Figure 4-31: Urea cycle and associated intermediates to arginine synthesis indicating the role of ARG1 and ARG2.

The urea cycle and their enzymes to synthesis arginine, indicating the role of arginase 1 (ARG1) (A) and arginase 2 (ARG2) (B). Ornithine aminotransferase (OAT), argininosuccinate synthase 1 (ASS1), argininosuccinate lyase (ASL). The red arrow indicates to amino acids transfer.

4.7.1 Effects of GPs on mRNA expression

The expression of ornithine aminotransferase (OAT) converts proline to ornithine via glutamate formation. There were no significant effects of treatment on LD or liver, but there was a trend for an effect in SS ($P=0.054$), with BA and GH both being lower than control (Figure 4-32).

Ornithine decarboxylase is a major enzyme for cell growth, producing the polyamines, which are required to stabilize newly synthesized DNA. The mRNA expression of ornithine decarboxylase 1 (ODC1) was unaffected by treatment (Figure 4-32 A, B and C).

There was no effect of GH or BA treatment on argininosuccinate synthase 1 (ASS1) expression in SS, LD and liver (Figure 4-32).

There was no significant effect of treatment on argininosuccinate lyase (ASL) mRNA expression in LD and liver, but there was a significant effect of treatment on the expression of ASL ($P=0.049$) in SS muscle, with BA and GH being lower than controls (Figure 4-32).

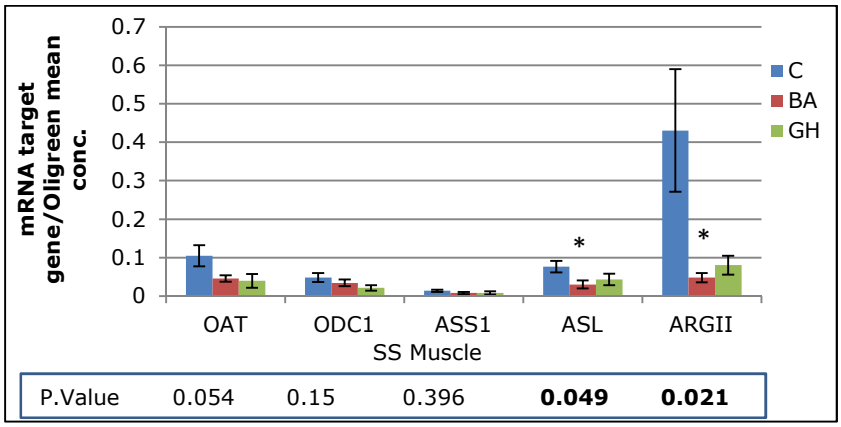
Arginase (ARG) is the only urea cycle enzyme that consists of two isoforms; arginase type I (ARGI) is specific to liver and arginase type II (ARGII) is found in kidney and some other tissues such as muscle. They are encoded by different genes (Jenkinson et al., 1996). ARGII expression is detected in the liver because metabolic studies in the liver suggested that ARGII participates in glutamine synthesis in hepatocytes along with ornithine aminotransferase (O'Sullivan et al., 1998), whilst ARGI is highly expressed in the liver, as it is required for the urea cycle which predominantly takes place in this tissue.

There was a significant effect of treatment on the expression of ARGI mRNA ($P<0.001$), with a decrease in both BA and GH

treatments compared to control (Figure 4-32). No expression of ARGI was detected in muscles, because this gene is specific for liver.

There was a significant effect of treatment on the mRNA expression of ARGII in SS (P=0.021), with GH and BA being lower than control, whilst the expression of ARGII was unaffected by growth promoting agents in the LD and liver (Figure 4-32).

A



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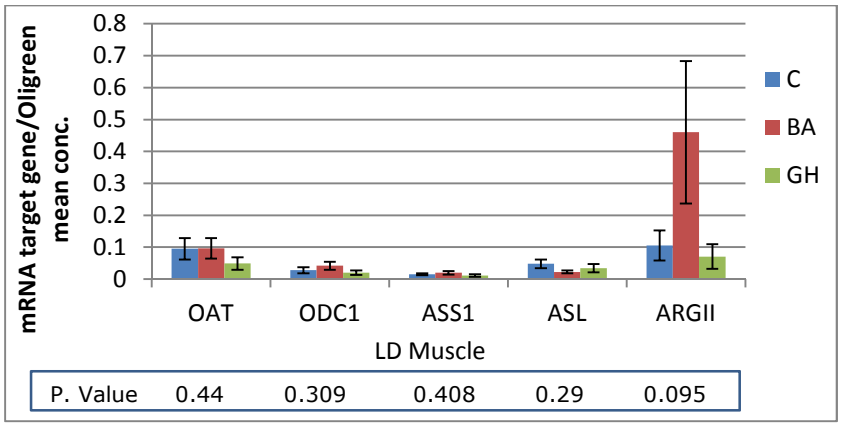

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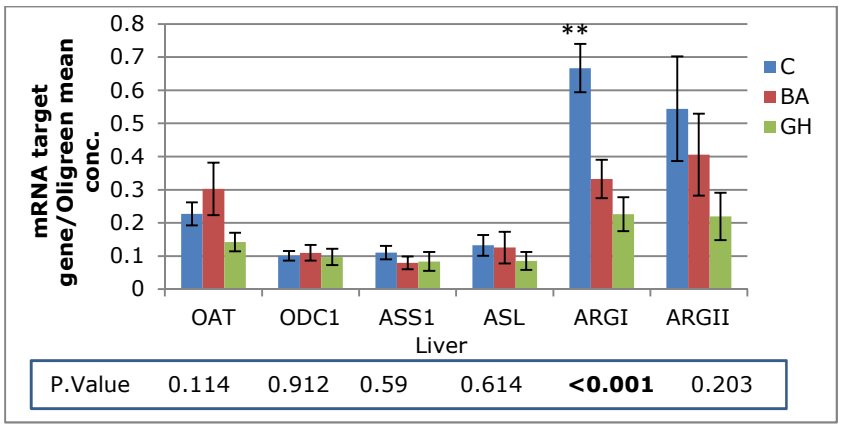


Figure 4-32: Effects of GH and BA on SS (A) and LD (B) muscle and liver (C) gene expression associated with the urea cycle.

RNA expression normalised to oligreen concentrations. Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One way ANOVA p values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test. * P<0.05, ** P<0.001.

4.7.2 Effects of GPs on Arginase II Protein Expression

In lambs treated with BA and GH, the expression of ARGII protein was not affected in SS and LD muscles compared to control, and there were no significant effects of either agent on ARGII protein expression in the liver (Figure 4-33 A and B).

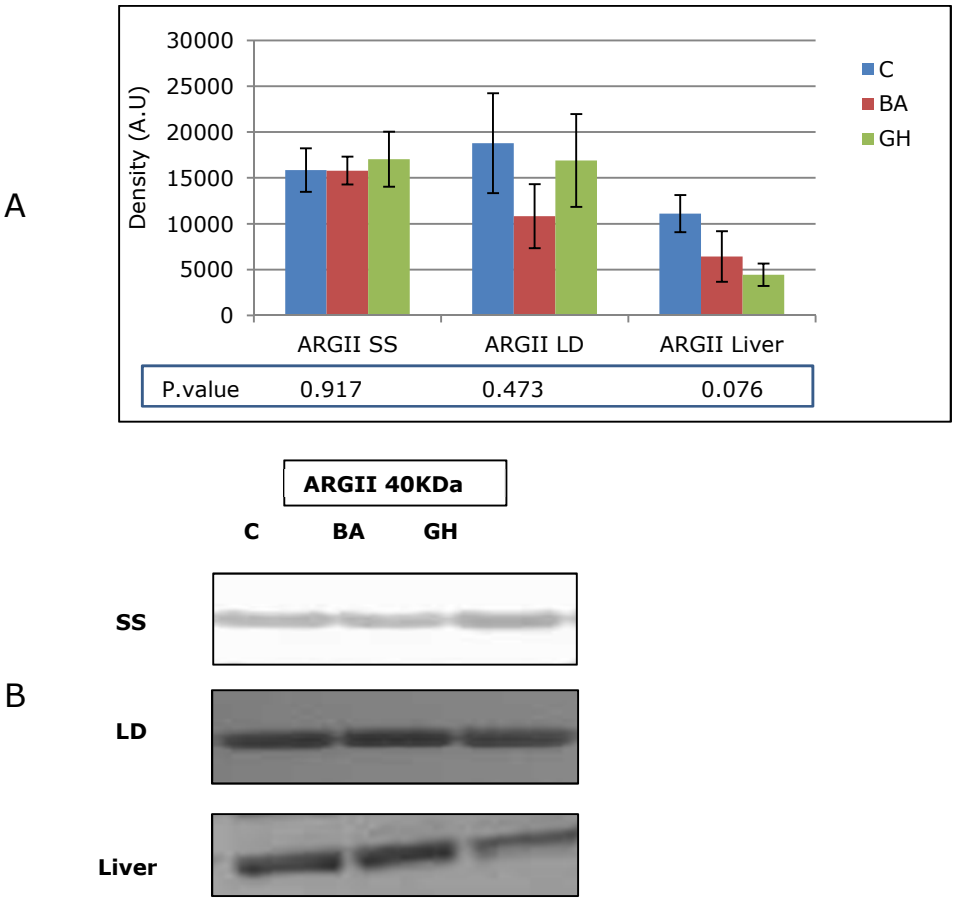


Figure 4-33: Protein expression of ARGII by western blot analysis in SS, LD muscles and liver.

Western blot ARGII specific band intensity (A) and representative western blot bands (B) are shown. Total protein loaded in each well is the same (samples normalised for protein concentration). Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One way ANOVA p. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

4.8 Discussion

Effects of growth promoters, BA and GH, on muscle mass are associated with changes in metabolism in liver and muscles. The BA effect on muscle was much stronger than that of GH. BA increases muscle mass as a result of hypertrophy, associated with an increase in muscle protein synthesis, a decrease in muscle protein degradation, or a combination of both (Kim and Sainz, 1992, Mersmann, 1995, Yang and McElligott, 1989, Beermann et al., 1987). Previous studies have shown BA induced muscle hypertrophy was associated with a switch to a faster fibre type (Hemmings et al., 2009). In the sheep study described in this thesis, the increased muscle weight in BA treated animals was associated with hypertrophy of type II fibres (Hemmings et al., 2009). A similar observation had been previously described (Kim et al., 1987). Also, it has been reported that BA treatment has a greater effect on muscle growth and associated fibre type switching than GH (Enright et al., 1990, Buttery and Dawson, 1990).

Using microarray analysis, the up- and down-regulation of expression of genes associated with serine synthesis, glycolysis, TCA and urea cycles were examined. Analysis of the LD transcriptome identified 477 and 316 transcripts that were significantly altered ($P<0.05$ and 1.5 fold change) by BA and GH respectively, relative to controls. Gene ontology analysis (Genespring) of these transcripts indicated more pathways affected by BA than GH. In this study gene expression was also measured by qRT-PCR in SS and liver, in addition to LD. The high expression of the three enzymes involved in serine synthetic pathway (PHGDH, PSAT1 and PSPH) in malignant tumours, indicates that the serine synthetic pathway may play a critical role in tumorigenesis (Pollari et al., 2011). Based on microarray analysis,

expression of a larger number of genes in the muscle changed in response to BA compared to GH. In the current study glycolysis associated gene expression was upregulated and those associated with the tricarboxylic acid (TCA) cycle were down regulated in BA treated animals. A novel observation was that the enzyme phosphoserine amino transferase (PSAT), which is part of the serine synthesis pathway, was increased in muscle by BA treatment. The general observation from qRT-PCR was in agreement with the microarray data. For example, mRNA for ICDH2, which plays a key role in TCA cycle regulation in mammalian tissues (Comte et al., 2002), was significantly decreased in the SS and LD muscles of BA treated lambs compared to controls. These combined observations have led to the conclusion that the whole process of TCA cycle and oxidative phosphorylation in mitochondria had been decreased by BA. This implied that in muscle the glucose released from glycogen was not being fully oxidized. The observations in this study that expression of enzymes responsible for serine synthesis are increased, suggest that the glycolytic intermediates were being used for synthesis of non-essential amino acids. Indeed in cancer cells a large amount of glycolytic carbon is converted into serine and glycine metabolism via PHGDH, and the changes in glycolysis and serine synthesis required are a characteristic of cancer cells (Locasale et al., 2011).

The skeletal muscle dedicates a significant part of its functions to expressing the enzymes required for energy metabolism. The energy production in the muscle is very important, as it is the largest contributor to basal metabolic rate (when considered as a single tissue) and can increase its metabolic rate dramatically when energy is required for muscle contraction. It appears that during periods of rapid growth, at least that stimulated by BA, the muscle utilizes its energy pathways to generate intermediates that

can be used to synthesize nonessential amino acids, particularly serine. In order for serine to be synthesised by its biosynthetic pathway, adequate provision of substrates are required. Intermediates of the glycolytic pathway are essential for this process, particularly 3-phosphoglycerate. PGK catalyses transfer of a phosphate from 1, 3 bisphosphoglycerate to ADP with production of 3-phosphoglycerate and ATP (Daly et al., 2004). In the current study, BA, but not GH significantly increased the expression of PGK1 mRNA in the SS muscle compared to control, but there was no effect on PGK1 mRNA in the LD muscle. This may be related to differences in fibre type composition within the different muscles.

Enolase is a glycolytic enzyme involved in the dehydration of 2-PG to PEP (Donoghue et al., 2005). Enolase3 and ICDH2 expression are correlated with the expression of type IIB and type I MyHC mRNA isoforms *in vivo*, respectively. The strong correlation between Enolase3 and MyHC IIB mRNA expression, as well as between ICDH2 and type I MyHC mRNA, suggests these genes may be essential candidates for controlling and monitoring metabolic changes in skeletal muscle associated with contractile activity (Brown et al., 2014). Previous studies from our lab have reported that expression of Enolase3 and ICDH2 mRNA were changed (up and down, respectively) in pigs treated with ractopamine for 27 days (Brown et al., 2014), indicating that transitions towards increasing MyHC IIB isoform expression in response to BA, with a shift from an oxidative to a more glycolytic muscle metabolism *in vivo*. But there were no significant effects of treatment on enolase3 mRNA expression in any tissue used in this study. Some other studies reported that BA led to a decrease in oxidative, and increase in glycolytic gene expression and enzyme activity in muscle, suggesting a shift towards a more glycolytic metabolism (Vestergaard et al., 1994, Baker et al., 2006,

Burniston et al., 2007). These differences in observation of the study described in this thesis and other published studies may be due to differences in period of treatment and age of animals. Pyruvate kinase (PK) catalyses the last step in glycolysis to produce ATP and pyruvate, and has four isozymes in mammals called M1, M2, L and R types (Imamura and Tanaka, 1972). PKM1 and PKM2 isozymes are produced by the same gene but are distinguished by differentially RNA splicing of one exon (Noguchi et al., 1986, Jung et al., 2013). PKM1 protein is expressed in tissues such as muscle and brain (Marie et al., 1976, Guguen-Guillouzo et al., 1977), whilst PKM2 protein is expressed in rapidly proliferating tissues, such as embryonic, adult stem cells, and all tumour cells (Christofk et al., 2008). The expression of PKM2 protein is necessary for aerobic glycolysis and metabolism in tumour cells (Christofk et al., 2008). PKM1 protein has only one form which is a tetramer with high affinity for PEP, whereas PKM2 protein exists as two forms, tetramers (high affinity, low Km for PEP) and dimers (low affinity, high Km for PEP) (van Berkel et al., 1974). PKM2 expression has been described as having an essential regulatory contribution to the serine synthetic pathway (Ye et al., 2012). In the current sheep study, BA significantly increased the expression of total PKM, PKM1 and PKM2 in both SS and LD muscles compared to control.

The activity of PKM is related to the concentration of fructose-1, 6-bisphosphate (FBP) (Eigenbrodt et al., 1983). This glycolytic intermediate is required for the formation of the active PKM2 tetramer, but not the PKM1 dimer (Eigenbrodt and Schoner, 1977). In addition to FBP, serine is another activator of PKM2 (Eigenbrodt et al., 1983).

The high expression of the three enzymes involved in the serine synthetic pathway, PHGDH, PSAT1, and PSPH, is often found in

cancer tumour tissue, indicating that serine synthetic pathway may play a critical role in tumorigenesis (Pollari et al., 2011). In cancer, there is the selective up-regulation of specific genes such as phosphoserine amino transferase (PSAT) (Ojala et al., 2002).

Although serine is classified as a nutritionally non-essential amino acid, metabolically serine is essential and plays many important metabolic functions such as supplying precursors for amino acid synthesis, nucleotide synthesis, neurotransmitter synthesis, derived lipids and plays a major role in several cellular processes (Tabatabaie et al., 2010, Kalhan and Hanson, 2012). The phosphorylated intermediates in the serine pathway involve three sequential reactions that are catalyzed by the enzymes PHGDH, PSAT and PSPH (Jaeken et al., 1996).

In the current study, BA, but not GH, treatment for six days in sheep significantly increased the expression of PHGDH and PSAT1 mRNA in both SS and LD muscles, whereas no effects were observed in liver. Previous studies have shown PSAT is an important gene in serine biosynthesis pathway, as over expression in cells in culture is associated with growth and cell proliferation (Baek et al., 2003, Vi et al., 2008).

There is some evidence that serine synthesis is associated with mTORC1 activation (Nicklin et al., 2009, Cohen and Hall, 2009). The activation of mTORC1 has been previously reported as regulating the protein expression of the transcription factor ATF4 (Adams, 2007). In the study carried out by Adams (2007), ATF4 was described as involved in mediating insulin's anabolic functions, regulating amino acid and protein metabolism, as well as having a role in regulating the expression of mRNA. It was also described as influencing translation by generating charged tRNA, through the regulation of mRNA expression for various aminoacyl-tRNA

synthetases. This would then allow mTORC1 to stimulate amino acid and protein anabolism. Amongst ATF4-dependent transcripts are seven enzymes (ASNS, PSAT1, PHGDH, PSPH, SHMT2, methylenetetrahydrofolate dehydrogenase (MTHFD2), and Pyrroline-5-carboxylate reductase 1 (PYCR1)) required for the synthesis of nonessential amino acids such as asparagine and serine. Adams (2007) indicates that insulin and IGF-I increased ATF4 and thereby ATF4-dependent mRNAs, such as those encoding ASNS and PSAT1, resulting in an increased capacity to synthesise asparagine and serine respectively. Therefore, it appears that ATF4 is a factor that may be necessary for the expression of a range of mRNAs encoding amino acid transporters, amino acid biosynthetic enzymes, and aminoacyl-tRNA synthetases, which may then enable increased mTORC1 activity to stimulate protein synthesis. In this study, the enzyme PSPH which catalyses the final step of serine synthesis showed no difference between treatment groups. Also, the mRNA expression of SHMT1 was unaffected by the growth promoting agents used in this study. The role of SHMT in serine biosynthesis is to convert L-serine to glycine.

Asparagine synthetase (ASNS) catalyses the glutamine and ATP dependent conversion of aspartic acid to asparagine, with the transcription factor ATF4 being required for the synthesis of asparagine (Adams, 2007). A study by Siu et al. (2002) provided evidence both *in vitro* and *in vivo* that ATF4 had an important role for the activation of the ASNS gene in response to either amino acid or glucose deprivation.

In present study, BA, but not GH, dramatically increased the mRNA expression of ASNS in both LD and SS muscle, whereas it was not affected in liver.

ATF4 regulates the expression of genes involved in amino acid and protein metabolism, redox homeostasis and ER stress responses, and it is up-regulated in tumours, suggesting that it has a critical role in tumour growth (Ye et al., 2010). Asparagine plays a vital role in glutamine regulation (Zhang et al., 2014), and a large amount of glutamine is consumed in many tumour cells to preserve TCA cycle anaplerosis and to support the production of nucleotides and nonessential amino acids for cell growth (Zhang et al., 2014). ATF4 stimulates ASNS, which results in glutamine-dependent asparagine synthesis from aspartate (Ye et al., 2010). A study by Zhang et al. (2014) reported that suppression of citrate synthase (CS) resulted in redirection of oxaloacetate (OAA) into aspartate and asparagine biosynthesis. It has been concluded that amino acid synthesis and uptake induced via ATF4 expression is associated with two specific pathways, insulin and the general amino acid control (GAAC). They also concluded that expression of ATF4 was stimulated to increase the synthesis of nonessential amino acids, as well as the cell's capacity to take up both nonessential and essential amino acids (Malmberg and Adams, 2008).

PCK is considered the key enzyme in glucose synthesis (gluconeogenesis) in the liver and kidney, as well as for glyceride-glycerol synthesis in white adipose tissue and the small intestine (Hanson and Reshef, 1997). There are two isoforms of PCK, a cytosolic (PCK1) and a mitochondrial (PCK2) form (Nordlie and Lardy, 1963). Relative PCK1 and PCK2 isoform activities are equal in human hepatic tissue, whilst the PCK1 is the predominant isoform in rat hepatic tissue (Modaressi et al., 1996). An earlier study by Nordlie and Lardy (1963) and another by Nolte et al. (1972) concluded that the activity of PCK varies widely depending on the species. PCK is expressed in various tissues of humans,

mainly in liver, kidney, pancreas, intestine and fibroblasts. The PCK1 gene is located on chromosome 20, while the PCK2 gene is located on chromosome 14 (Modaressi et al., 1998). Gluconeogenesis is important in ruminants because a very small amount of glucose is absorbed from the digestive tract; therefore ruminants are dependent on hepatic gluconeogenesis. The volatile fatty acid propionate, is utilized as a major substrate for gluconeogenesis in ruminants (Bergman et al., 1966).

Previous studies demonstrated that glucose-derived pyruvate via glycolysis enters the TCA cycle. The oxidation of pyruvate by pyruvate dehydrogenase (PDH) produces acetyl-CoA. Alternatively, the pyruvate carboxylase (PC) reaction leads to pyruvate being converted to OAA through the assimilation of CO_2 and consumption of ATP. This reaction is important for glucose to stimulate insulin secretion (MacDonald, 1995a, MacDonald, 1995b). In the current project, the expression of PCK1 mRNA tended to be lower in LD and SS muscle and higher in liver in BA-treated lambs, but this was not significant. Whilst there was a significant increase of PCK2 expression in LD muscle of BA-treated lambs and there was a trend for it to be increased in SS muscle, it was not affected in the liver. This means that glucose may be used for OAA synthesis, and then subsequently used to form PEP directly via PCK2 in mitochondria or indirectly via cytoplasmic PCK1.

Mendez-Lucas et al. (2013) demonstrated that PCK2 has a role in gluconeogenesis and TCA cycle function, regulating the metabolism of glucose and lipids in the human liver. PCK2 uses mitochondrial GTP (mtGTP) to convert OAA into mitochondrial PEP (Stark et al., 2009). In this study, increased PSAT in muscle may lead to a potential increase in alpha-ketoglutarate, the TCA cycle intermediate. A study by Lambeth et al. (2004) proposed a cycle in which PCK2 and PCK1 collaborate to provide mtGDP for the

succinyl-CoA synthetase step while transferring a high energy phosphate to the cytosol. In this mechanism PCK2 consumes mtGTP from succinyl-CoA synthetase (SCS-GTP) to make PEP. PEP is transported out of the mitochondria where it serves as a high-energy phosphate donor to generate GTP in the cytosol via PCK1. Cytosolic malate dehydrogenase (cytMDH) then reduces OAA to malate that can return to the matrix to be oxidized back to OAA by the mitochondrial isoform completing the cycle. Such a PEP cycle would only be possible in tissues that contain PCK1, an enzyme that is less widely expressed compared to PCK2.

During the metabolic pathway of glyceroneogenesis, 3-phosphoglycerate can be generated and used for the synthesis of serine in the livers of starved animals via utilization of carbon that is derived from PEP via PCK (Yang et al., 2009). Drahota et al. (1983) indicated specific activities of PCK2 in brown adipose tissue mitochondria of hamster and rat. They concluded that high rates of PEP were exported from mitochondria to the cytosol in cells provided with malate and alpha-ketoglutarate.

Despite some understanding of the role of PCK2 in gluconeogenic organs (e.g. liver and kidney), the role in non-gluconeogenic organs (e.g. brain and muscle) remains unclear. It has been reported that PCK1 may favour conversion of pyruvate and amino acids to glucose during gluconeogenesis, while PCK2 may best serve gluconeogenesis from lactate (the Cori cycle) (Watford et al., 1981). A study by Mendez-Lucas et al. (2013) demonstrated that PCK2 plays its role in gluconeogenesis and the TCA cycle better in the presence of PCK1. This supported the direct relationship between PCK2 function and TCA cycle flux in non-gluconeogenic tissue. PCK1 has a cataplerotic role in skeletal muscle. The concentrations of intermediates of the TCA cycle (citrate, malate, fumarate) increase dramatically in the mitochondria during

energetic exercise and are then removed after exercise (Spencer et al., 1991). It has been suggested that muscle PCK has an important metabolic role in providing pyruvate from PEP via the oxidation of some amino acids to acetyl-CoA or conversion to alanine in mammalian muscle (Newsholme and Williams, 1978, Snell and Duff, 1977, Chin et al., 1998, Snell and Duff, 1979).

In mammals, CCAAT/enhancer binding protein (C/EBP), beta and other transcription factors have been reported to participate in the transcriptional regulation of the PCK1 gene (Eubank et al., 2001). PCK1 can be regulated by a variety of dietary and hormonal signals, such as glucagon, cAMP, glucocorticoids, and thyroid hormone that all stimulate PCK1 expression, whereas expression is suppressed by insulin. It is not known whether PCK2 is under such hormonal control (Hanson and Reshef, 1997). PCK2 was recently identified as a gene that responds to endoplasmic reticulum (ER) stress, which is perhaps mediated by ATF4 (Mendez-Lucas et al., 2014). But PCK2 also provides glycolytic intermediates through the conversion of mitochondrial OAA into PEP (Mendez-Lucas et al., 2014). Glutamine is an essential element in the metabolism and growth of tumours and plays a vital role in protein synthesis, as well as providing ATP and metabolic intermediates that can be used for the synthesis of nucleotides and antioxidant capacity (DeBerardinis and Cheng, 2010). Thus glutamine can be converted to OAA via the TCA cycle, and then to PEP via PCK2.

The urea cycle is consists of five enzymes and takes place mainly in the liver. The efficient functioning of the pathway also requires other enzymes and mitochondrial amino acid transporters. The expression of genes involved in this cycle has been examined because the recent work from our lab has identified that pigs treated with GH and BA over 28 days have changes in similar genes (serine synthesis, PCK2 and ASNS) as those described in

this thesis (unpublished observations). In addition, the work in pigs has shown a significant up-regulation in the genes of the urea cycle in skeletal muscle. ARGII has been shown to be regulated by ATF4 and, in the pig study, there were changes in amino acids and transamination (unpublished observations).

Arginine is a nonessential amino acid synthesised from glutamine, glutamate and proline via the intestinal-renal axis in most mammals, including sheep (Wu et al., 2009). It has also been classified as a semi-essential or conditionally essential amino acid (Morris, 2004), because in healthy adult animals the synthesis of arginine is sufficient to meet metabolic demands. In cases of catabolic stress such as infection or inflammation or conditions such as the kidney or small intestine dysfunction, endogenous synthesis of arginine may not be sufficient to meet metabolic requirements. Arginase (ARG) consists of two isoforms; ARGI specific for liver and ARGII which tends to be found in the kidney (Jenkinson et al., 1996). This does not mean that ARGII it is not expressed in the liver, because metabolic studies in the liver suggest that ARGII participates in glutamine synthesis, as it is co-expressed with ornithine aminotransferase in hepatocytes (O'Sullivan et al., 1998).

An earlier study by Greengard et al. (1970) estimated that low concentrations of arginase present in extrahepatic tissues may participate in protein synthesis via citrulline to form arginine, rather than participate in biosynthesis of urea. In this project, the expression of ARGII significantly decreased in SS muscle of lambs treated with BA, this may be associated with its participation in protein synthesis.

It has been found that citrulline stimulates protein synthesis in the muscle (Ventura et al., 2013, Cynober et al., 2010). The citrulline

effect is thought to be when protein intake is low, with its effect on protein synthesis being associated with the mTOR pathway (Cynober et al., 2010). In this study, no significant effects of growth promoting agents were observed on OAT, ODC, ASS and ASL in liver nor in both SS and LD, but there was a trend for lower ASL in the SS of the BA treated group. From the results obtained of urea cycle mRNA gene expression, it appears that ARGII, but not ARGI, may have a specific role, rather than generating urea.

The interactions among urea cycle enzymes in the regulation of arginine are complex and make it difficult to interpret the effect of arginine on metabolism. This is because arginine is a substrate for various enzymes, but also ornithine and citrulline are key products of arginine metabolism also regenerated by the urea cycle. Some of these key enzymes are expressed as different isoforms that make this metabolic pathway more complex to understand (Morris, 2004). However, in the current study, ARGI in lamb's liver was significantly higher in the control group compared to the 2 treatment groups (GH and BA), but this isoform was hardly detected in muscles as it is specific to liver. There was a significant decrease of ARG II in SS muscle of lambs treated with BA. In liver there was no effect of BA, suggesting that ornithine and citrulline are produced from other amino acids rather than arginine (Morris, 2004).

4.9 Conclusion

This study shows for the first time that up-regulation of the serine biosynthetic pathway is associated with BA stimulated muscle hypertrophic growth. BA had a stronger muscle hypertrophic effect than GH over the short time frame of a 6 day treatment. This appears to be associated with a decrease in ICDH gene expression,

suggesting a decrease in TCA cycle activity. BA increased the muscle's glycolytic potential, generally decreased TCA cycle enzyme expression and this was associated with an apparent increased capacity for serine synthesis and presumably other related metabolites required for growth. It appears that GH and BA have differential effects on metabolism that lead to differential effects on muscle mass over this short time frame. The transcription factor ATF4 may regulate many/ some of the genes associated with a shift to increase the capacity to enhance the concentration of glycolytic intermediates and synthesise the nonessential amino acids, asparagine and serine. Unlike GH, BA does not appear to have a major effect upon the systemic mobilization of nutrients, but instead seems to target muscle, activating muscle biosynthetic pathways that potentially provide the substrates required for growth. Changes in gene expression stimulated by growth promoters described in this chapter were associated with many substances and intermediates that are related to the metabolism of energy and protein sources. Therefore, as described in the next chapter, metabolomics was utilised to determine the plasma concentrations of metabolites, to try to strengthen and support the interpretation of the gene expression data.

5 Metabolomics Analysis of Sheep Plasma Treated with Growth Promoting Agents

5.1 Introduction

Based on the results of the previous chapter, the microarray study demonstrated general changes in the gene expression of various metabolic pathways. For example, BA decreased the expression of genes associated with the TCA cycle, whereas glycolysis and the serine synthesis pathway gene expression were increased. The consequence of the change in the expression of specific genes, and presumably their respective proteins, would be an expected change in associated metabolites. Changes in the expression of the serine synthesis pathway would be expected to influence the amino acid product of the pathway, but also associated amino acids such as glycine, glutamate, glutamine, as well as methionine and cysteine. Given the changes in the expression of genes associated with energy metabolism pathways, changes in some carbohydrates and their metabolic intermediates might be expected, for example glucose, lactate, pyruvate and alpha-ketoglutarate. In addition to these energy metabolism changes, the previously reported liopolytic effects of GH and BA, would suggest that these agents would alter the non-esterified fatty acid (NEFA) and glycerol concentrations. Growth promoters stimulate increases in protein accretion, therefore the associated changes in protein and amino acid metabolism would be expected to influence a range of amino acids and their metabolites particularly, because of the changes in gene expression of enzymes in the urea cycle. Examination of blood metabolites in the sheep treated with growth promoters could be informative of changes in the activity of metabolic pathways. Assessment of protein expression by western blot was

difficult because antibodies often don’t cross react with sheep protein and determination of enzyme activities is difficult to do for all targets. Therefore the approach which was taken was to try to strengthen our interpretation of the gene expression data described in the previous chapter by examining the concentrations of plasma metabolites by a metabolomics technique.

5.2 Results

5.2.1 Effects of BA and GH on Plasma Carbohydrates and Non-carbohydrate Concentrations in Sheep

GH, but not BA, significantly increased (P<0.001) plasma glucose concentration compared to control (Figure 5-1), while BA treatment tended to increase plasma lactate concentrations (P<0.058).

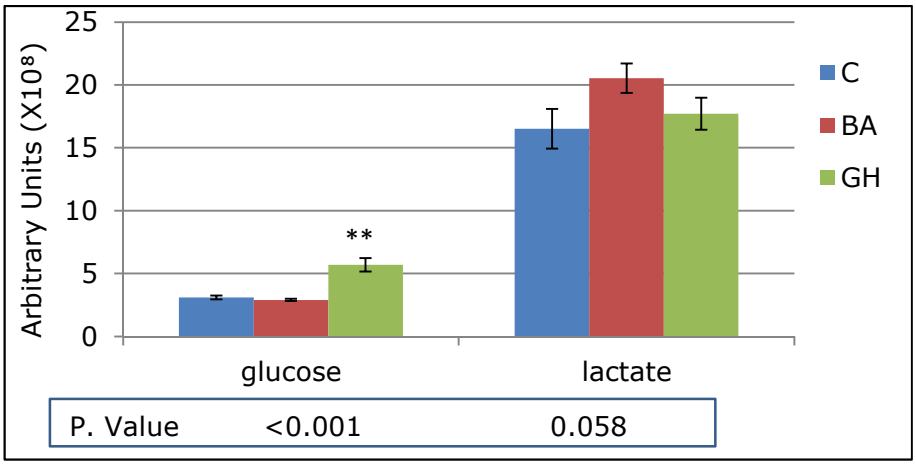
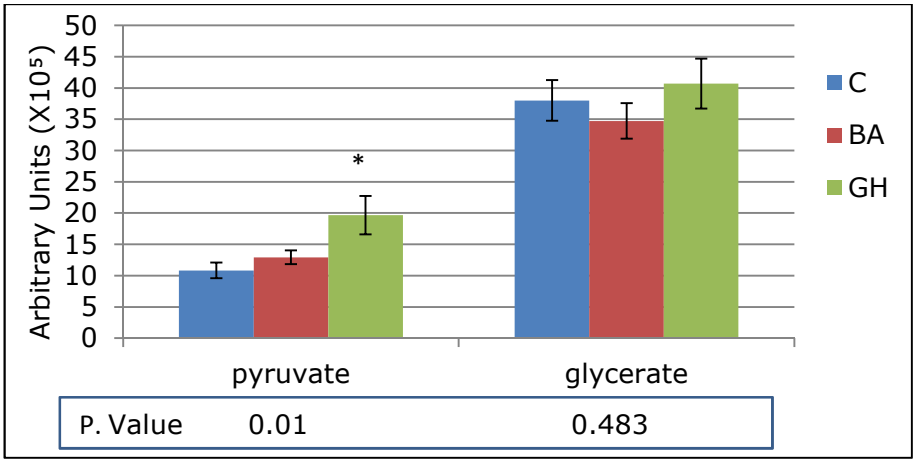


Figure 5-1: Effects of GH and BA on plasma glucose and lactate concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test. ** P<0.001.

Treatment with growth promoters had a significant effect on plasma pyruvate concentrations, with GH significantly increasing (P<0.01) the concentrations compared to control (Figure 5-2 A). There was no effect of BA on plasma pyruvate concentrations. Plasma glycerate concentrations were unaffected (P<0.483) by growth promoter treatment. There was a significant effect of growth promoter treatment on plasma fructose, with GH treatment significantly increasing plasma fructose concentrations (P<0.003) compared to control (Figure 5-2 B).

A



B

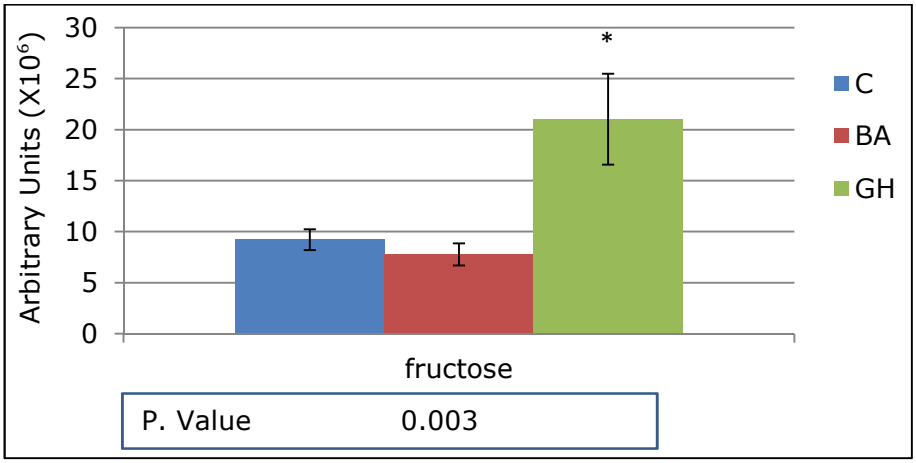


Figure 5-2: Effects of GH and BA on (A) plasma pyruvate and glycerate concentrations and (B) fructose concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test. * $P<0.05$.

There were no significant effects of growth promoter treatment on the plasma concentrations ($P>0.05$) of pentose monosaccharides (Figure 5-3).

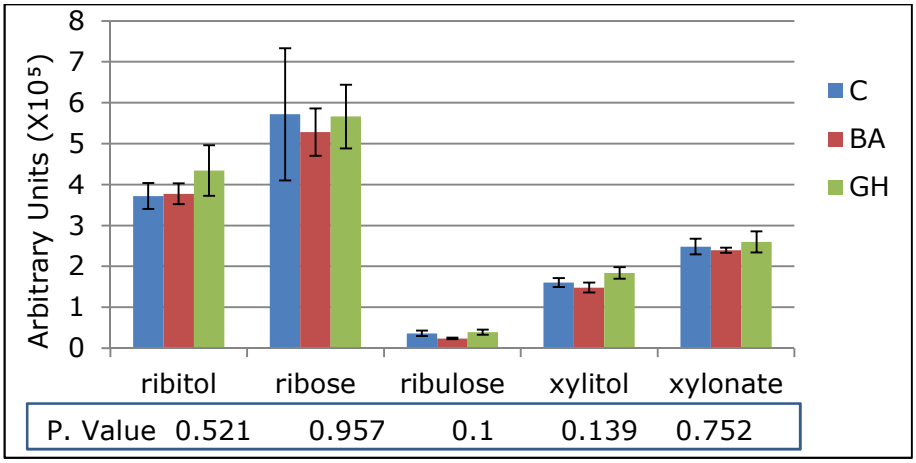


Figure 5-3: Effects of GH and BA on plasma concentrations of pentose monosaccharides.

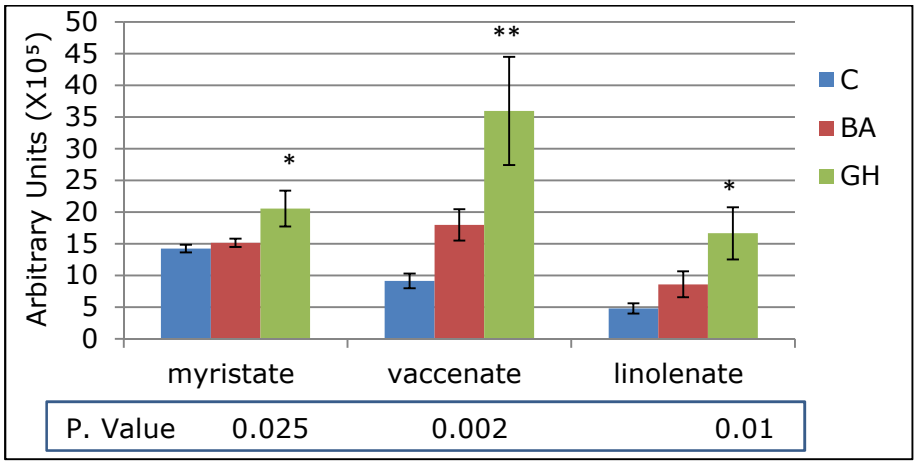
Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

5.2.2 Effects of BA and GH Treatment of Sheep on Plasma Non-esterified fatty acid (NEFA) Concentrations

One of the reported effects of treatment of livestock with GH and BA is an increase in lipolysis. There was a significant effect of treatment on various NEFA plasma concentrations. Plasma myristate concentrations were significantly ($P<0.025$) increased in lambs treated with GH compared to control, while no significant effects were observed in lambs treated with BA (Figure 5-4 A). GH, but not BA, significantly increased the plasma concentrations of other saturated NEFAs, palmitate and stearate, ($P<0.004$, $P<.001$, respectively), whilst the same was the case for oleate ($P<0.008$). The concentrations of specific polyunsaturated fatty acids were also affected, with the plasma concentrations of vaccenate ($P<0.002$), linoleate ($P<0.024$) and linolenate ($P<0.01$) all significantly increased in lambs treated with GH compared to control (Figure 5-4 A and B). In this study, GH stimulated an increase in circulating fatty acids after 6 days of treatment. Whilst there were

no significant effects of BA on plasma NEFA concentrations compared to control (Figure 5-4).

A



B

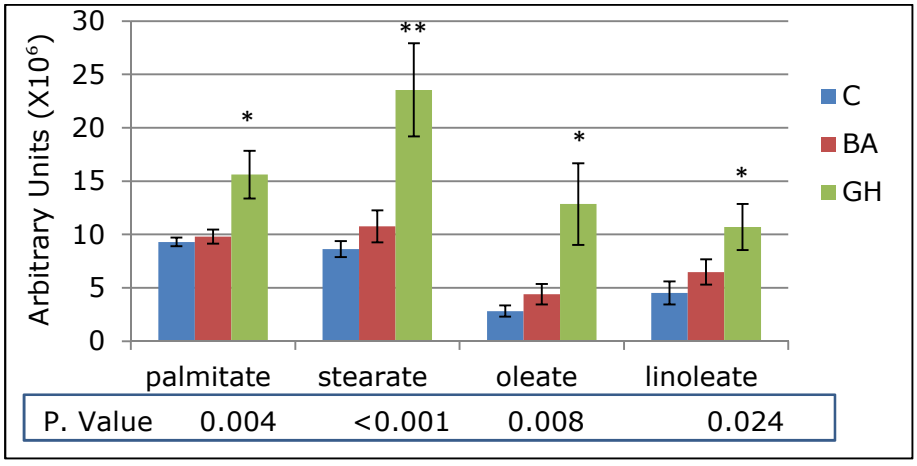


Figure 5-4: Effects of GH and BA on plasma nonesterified fatty acids concentrations (A) myristate, vaccenate and linolenate (B) palmitate, stearate, oleate and linoleate.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test. *P<0.05, ** P<0.001.

5.2.3 Effects of BA and GH Treatment of Sheep on Plasma Amino Acids Concentrations

The plasma concentrations of the nonessential amino acids, serine and asparagine, were not affected by treatment (P<0.081,

P<0.199 respectively); likewise the conditionally essential amino acid tyrosine (P<0.765) (Figure 5-5).

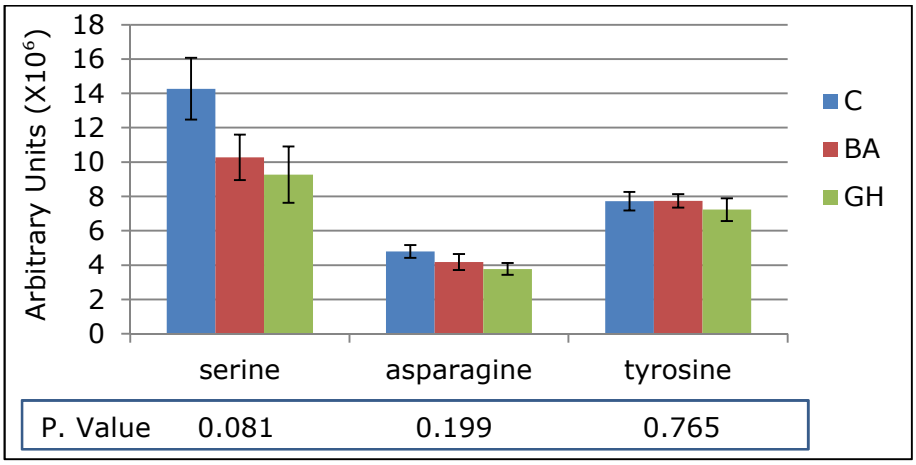


Figure 5-5: Effects of GH and BA on plasma serine, asparagine and tyrosine concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

There was an effect of growth promoter treatment on plasma glycine concentrations, with GH significantly decreasing (P<0.03) the concentrations compared to control. Both growth promoters had no effect (P<0.506) on plasma alanine concentrations in lambs (Figure 5-6).

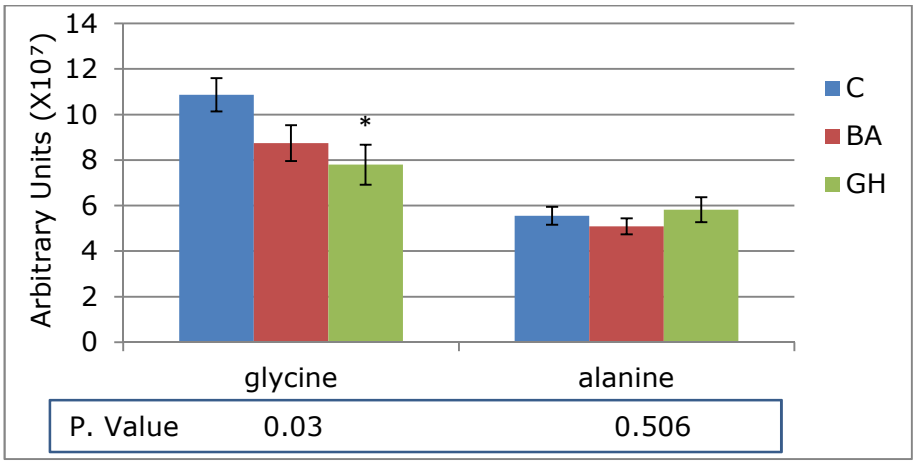


Figure 5-6: Effects of GH and BA on plasma glycine and alanine concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test. * P<0.05.

There was a significant effect of treatment on plasma histidine and 3-methylhistidine concentrations. BA, but not GH, significantly decreased (P<0.01) plasma histidine compared to control, whilst GH significantly decreased (P<0.015) plasma 3-methylhistidine concentrations compared to control (Figure 5-7).

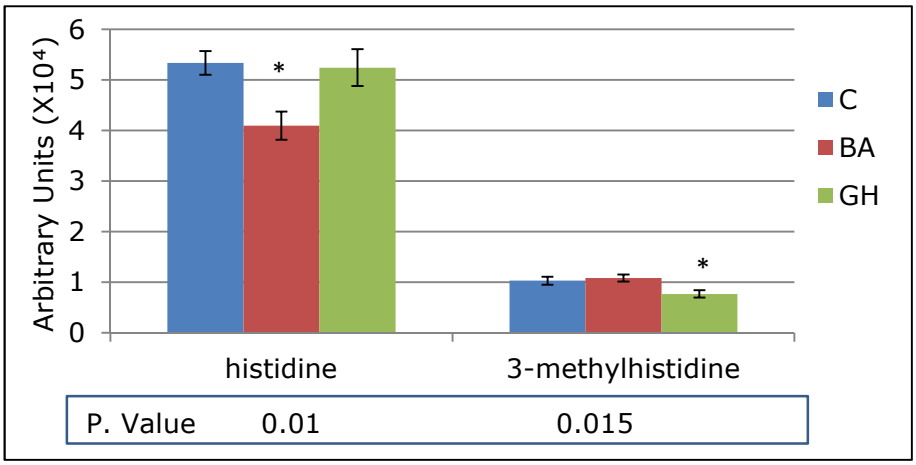


Figure 5-7: Effects of GH and BA on plasma histidine and 3-methlhistidine concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test. * P<0.05.

There were no significant effects of BA or GH on plasma glutamate concentrations in lambs administrated growth promoters for six days, but there was a significant effect on plasma glutamine concentrations, with GH significantly decreasing (P<0.007) concentrations compared to control (Figure 5-8).

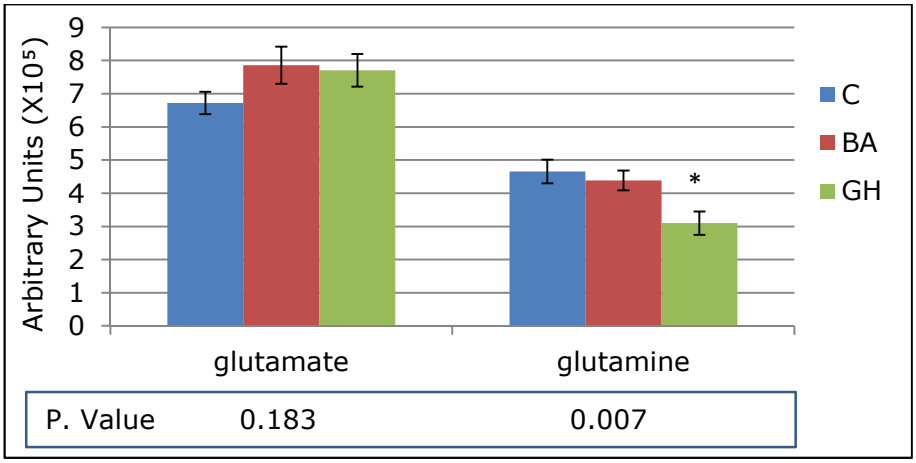


Figure 5-8: Effects of BA and GH on plasma glutamate and glutamine concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test. * P<0.05.

There was a significant effect of treatment on plasma methionine concentrations, with BA significantly decreasing (P<0.001) concentrations compared to control. There were no significant effects of BA or GH on plasma concentrations of the essential amino acids, threonine (P<0.382) and lysine (P<0.274), as shown in Figure 5-9.

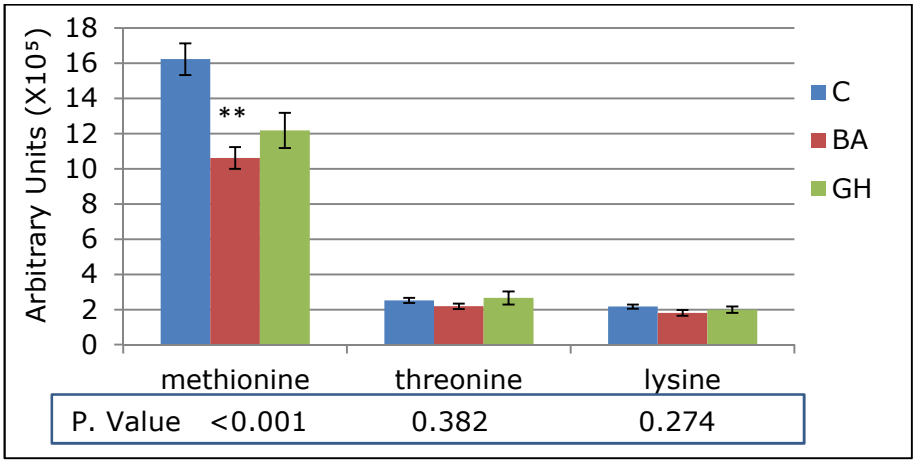


Figure 5-9: Effects of GH and BA on plasma methionine, threonine and lysine concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for

BA and GH). Star means there was a significant effect of treatment relative to control measured by Dunnett's test. ** P<0.001.

There were no significant effects of treatment observed on plasma concentrations of tryptophan (P<0.128) and phenylalanine (P<0.185). However, although not significant, BA treatment was associated with a numerical decrease in the plasma concentrations of the branched chain amino acids, valine (P<0.263), leucine (P<0.191) and isoleucine (P<0.226), compared to control (Figure 5-10).

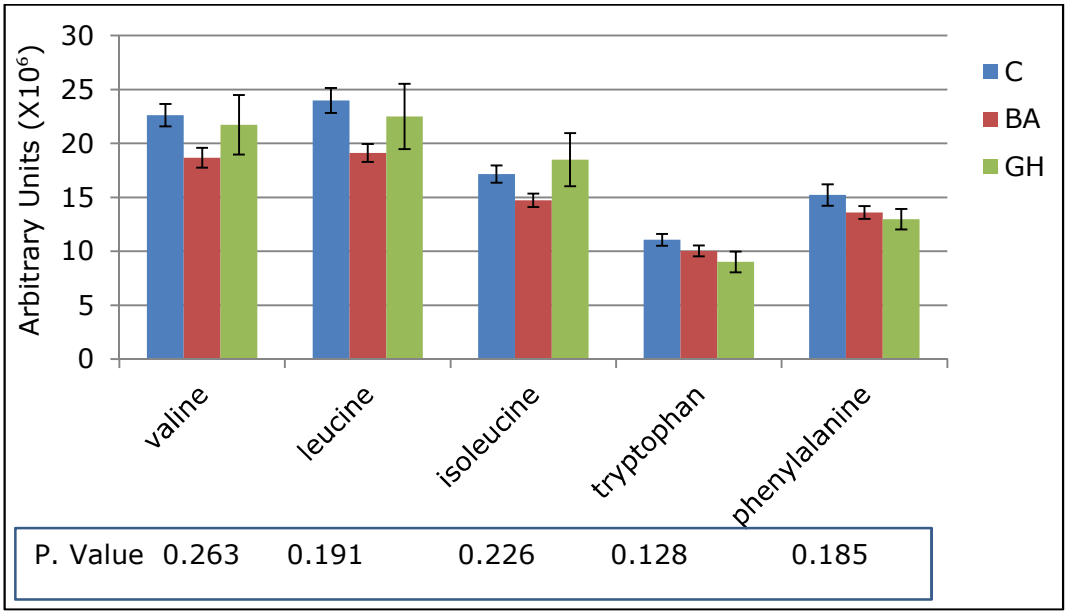


Figure 5-10: Effects of GH and BA on plasma essential amino acids concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

5.2.4 Effects of BA and GH Treatment of Sheep on Plasma TCA Cycle Intermediates Concentrations

In lambs treated with BA and GH for 6 days, there were no significant effects of treatment on plasma concentrations of the

TCA cycle intermediates, alpha-ketoglutarate (P<0.401), fumarate (P<0.628) and malate (P< 0.414) (Figure 5-11).

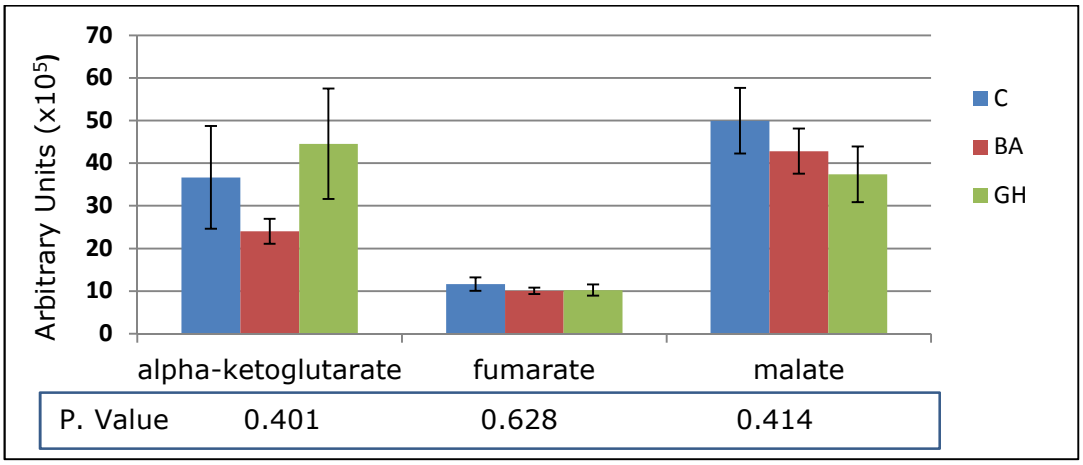


Figure 5-11: Effects of GH and BA on plasma TCA cycle intermediates concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

The treatments also had no effect (P<0.641) on plasma citrate concentrations (Figure 5-12).

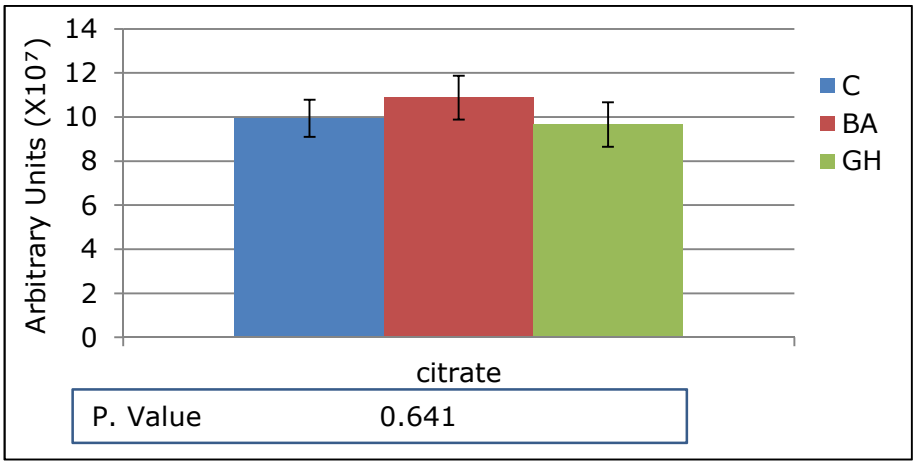


Figure 5-12: Effects of GH and BA on plasma citrate concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

5.2.5 Effects of BA and GH Treatment of Sheep on Plasma Urea Cycle Intermediate Concentrations

Neither treatment had any significant effects on plasma concentrations of urea cycle intermediates such as dimethyl arginine (P<0.243), arginine (P<0.169), homocitrulline (P<0.188), N-methyl proline (P<0.223), and hydroxyproline (P<0.278). However for the majority of these urea cycle intermediates, their concentrations were numerically reduced in plasma when comparing BA treatment to control (Figure 5-13 A and B). GH tended to increase (P< 0.087) plasma citrulline concentrations compared to BA and control (Figure 5-13 A).

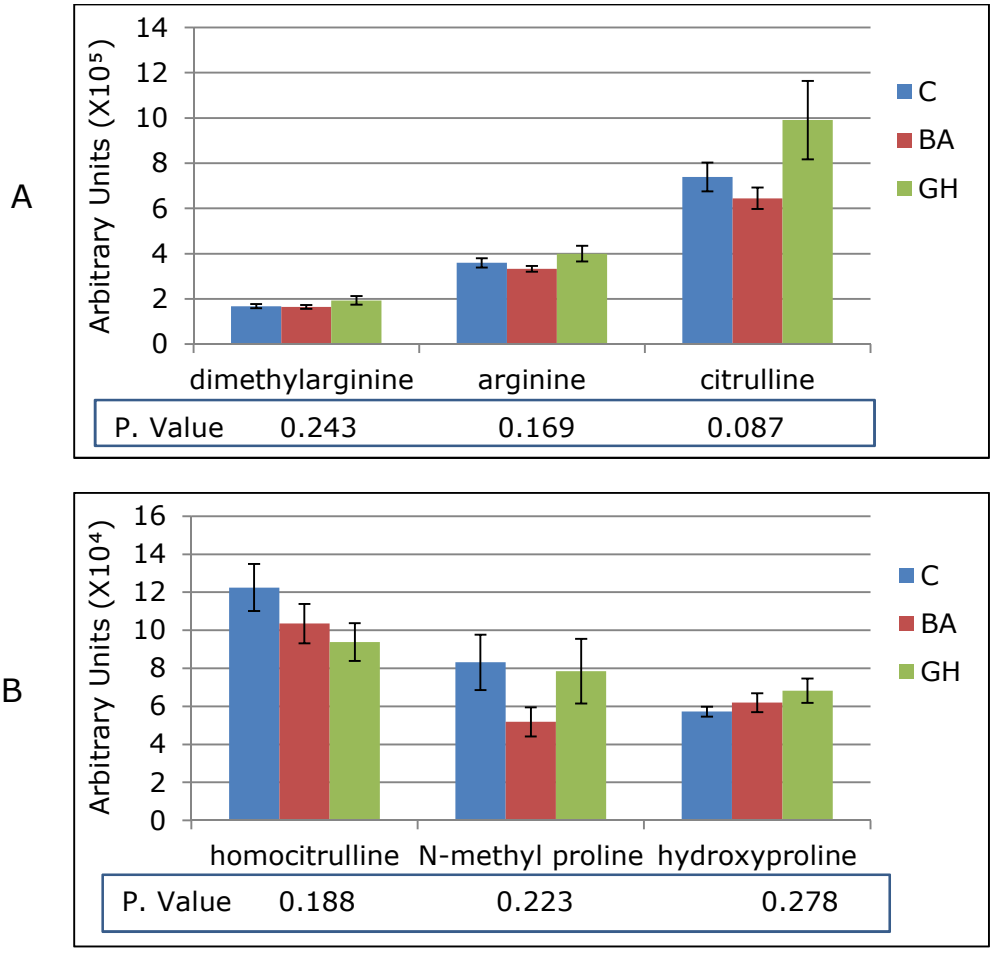
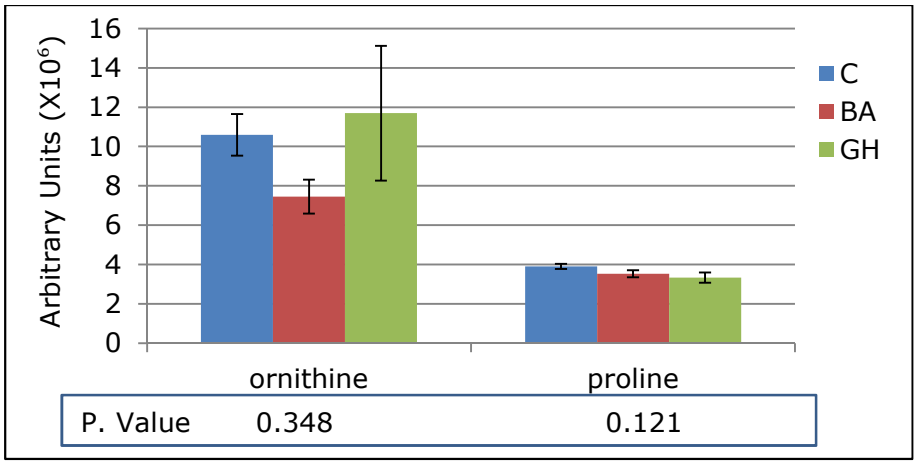


Figure 5-13: Effects of GH and BA on plasma urea cycle intermediates concentrations (A) dimethylarginine, arginine and citrulline; (B) homocitrulline, N-methyl prolline and hydroxyproline.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

There were no significant effects of treatment on plasma concentrations of urea cycle intermediates, ornithine (P<0.348), proline (P<0.121), and urea (P<0.176) (Figure 5-14 A and B).

A



B

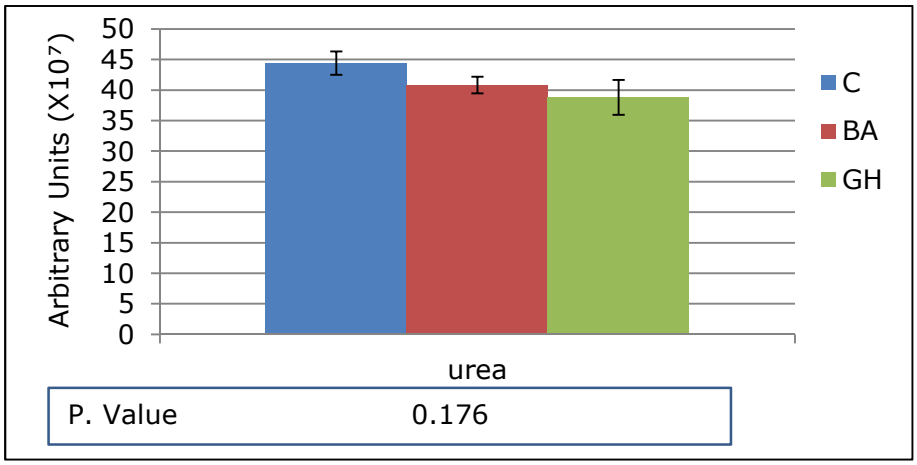


Figure 5-14: Effects of GH and BA on plasma (A) ornithine and proline concentrations and (B) urea concentrations.

Blue C (control), Red BA (beta agonist) and green GH (growth hormone) treatments. One-way ANOVA P. values are indicated; error bars are ± SEM (n=11 for C and n=10 for BA and GH).

5.3 Discussion

Changes in circulating metabolites were observed in the plasma of sheep treated with BA and GH. Previous studies reported that plasma insulin, glucose, glycerol and non-esterified fatty acids (NEFA) concentrations were increased two hours after treating lambs with BA (cimaterol), suggesting immediate stimulation of glycogenolysis and lipolysis by cimaterol (O'Connor et al., 1991, Byrem et al., 1998). A similar observation was seen in calves (Blum and Flueckiger, 1988), and steers (Chikhou et al., 1991, Eisemann et al., 1988). In cows treated with GH, Plasma GH, IGF-I, insulin, and glucose concentrations all increased (Shingu et al., 2009).

In the current study, glucose and other carbohydrates plus metabolites derived from glucose, such as pyruvate, were all increased with GH, but not BA treatment, relative to control. Previous studies have described that glycolysis, lactate concentrations and oxygen consumption are increased in BA-treated animals, while plasma insulin concentrations are decreased and adipocytes become less sensitive to insulin (Fiems, 1987). During the "acute" stage of BA-treated lambs (within 6 to 12 hours of treatment), the significant rise in both insulin and glucose levels suggests an increased peripheral tissue resistance to insulin (O'Connor et al., 1991). In cows treated with GH it was reported that insulin resistance developed (Dominici et al., 1999), and this was also seen in rabbits treated with GH (Costa et al., 1998). A study by Peterla and Scanes (1990) reported that pig incubated adipocytes with BA (cimaterol) exhibited a decrease in glucose incorporation into triglycerides and increased release of glycerol. In BA-treated calves, serum glucose, lactate, non-esterified fatty acids and insulin were all temporarily raised within 2-4 h of treatment, in a dose-dependent manner (Blum and Flueckiger,

1988). Acute administration of BA (2 and 4 hours) decreased the activity of insulin and reduced peripheral utilization of glucose in both humans and ruminants (Fryburg et al., 1995, McDowell, 1983). In contrast, chronic administration of BA increased insulin activity and stimulated the utilization of glucose (Beermann et al., 1987). A study by Chikhou et al. (1991) reported that BA acutely increased plasma insulin, glucose and NEFA and decreased GH and urea concentrations, whereas chronically it increased GH and NEFA and decreased IGF-I concentrations in steers.

During administration of stress hormones, consumption of oxygen increases. This indicates an increase in metabolic activity in skeletal muscle such as glycogenolysis and glycolysis, leading to increased pyruvate production and an excess pyruvate is transaminated to form alanine. There was a significant decrease in the arterial plasma concentrations of all amino acids except alanine (Wernerman et al., 1985), which is transported from the periphery to the splanchnic tissues for glucose production (Wernerman et al., 1985, Del Prato et al., 1990). In the study described in this thesis, both growth promoting agents stimulated a decrease in plasma amino acids which presumably were being utilized for muscle tissue anabolism. In general this study found amino acids were decreased with GH and BA treatment, but the effect of BA was bigger than GH. Moreover, in the current study, GH significantly decreased 3-methylhistidine, a non-metabolizable amino acid that is a marker of muscle catabolism, suggesting an increase in muscle weight, which is usually seen if GH is given for longer than 6 days, may be partly associated with decreased muscle breakdown.

The suggested effect of BA in reducing mitochondrial pyruvate oxidative capacity might be expected to increase lactate production. In the present study, although BA did not significantly increase plasma lactate concentrations, there was an indication

that it was elevated, which presumably would be an effect of increasing glycolysis and decreased oxidative respiration. Increased plasma lactate concentrations have previously been shown in BA-treated animals (Vestergaard et al., 1994), and the associated decrease in oxidative respiration capacity may cause a lower acetyl-coA formation, which could reduce exercise time to muscle exhaustion (Saltin and Rowell, 1980). BA has been reported to stimulate increased anaerobic glycolysis and this was correlated with high activity of lactate dehydrogenase (LDH) (Vestergaard et al., 1994).

In addition to the effects on carbohydrate metabolism, BA and GH treated animals decrease fat deposition via two mechanisms, namely increased fatty acid oxidation and decreased lipid deposition. The oxidation of fatty acids can occur by production of acetyl-CoA which can then be further metabolized to produce energy. In this study, BA and GH treatments appeared to increase plasma free fatty acids compared to control, indicating increased hydrolysis of triacylglycerol (lipolysis) in adipose tissue. The effect was greater with GH than BA, because there was a significant increase in fatty acids in GH-treated lambs.

The urea cycle is more important in ruminants than non-ruminants because ammonia is produced in the rumen from dietary protein via rumen bacteria and protozoa and this ammonia may be absorbed by the portal system and metabolised in the liver. In non-ruminants, ammonia used in the urea cycle is derived mainly from amino acids by deamination (McDonald, 1948). No significant change was observed in urea levels in BA-treated calves (Blum and Flueckiger, 1988). While there was a decreased in plasma urea observed following treatment with cimaterol (Chikhou et al., 1991). GH treatment induced a decrease in plasma urea N concentrations in cows (Shingu et al., 2009). In the present study, BA, but not GH

induced a decrease in plasma metabolic intermediates of the urea cycle. A study by Fiems (1987) reported that BA increased nitrogen retention. It has been found that citrulline stimulates muscle protein synthesis and can be transported efficiently into enterocytes thereby escaping hepatic uptake (Ventura et al., 2013, Cynober et al., 2010).

5.4 Conclusion

Changes in circulating metabolites were observed in the plasma from sheep treated with BA and GH. GH, but not BA treatment, increased plasma glucose and other carbohydrates as well as metabolites derived from glucose, such as pyruvate. BA tended to increase plasma lactate concentrations. GH significantly increased the plasma concentrations of more plasma fatty acids than BA. Both growth promoting agents significantly decreased plasma concentrations of certain amino acids, such as histidine, methionine and glutamine, but the effect of BA was bigger than GH. BA stimulated a decrease in metabolic intermediates of the urea cycle in plasma.

6 The Response of Genes Associated with Serine Biosynthesis in Myogenic Cells

6.1 Introduction

Rapidly proliferating cancer cells have a large requirement for macromolecular biosynthesis in order to support rapid cell growth. This appears to include elevated activity of the serine metabolic pathway, as serine has a critical role for this growth (Possemato et al., 201 1). As described in this thesis BA stimulated muscle growth *in vivo* is associated with co-ordinate increases in the expression of genes encoding serine synthesis enzymes. *In vivo* this growth is associated with hypertrophy rather than hyperplasia. During hypertrophy muscle fibres increase their cross sectional area. As described previously one of the mechanisms by which hypertrophy is reported to occur is via recruitment of satellite cells and associated fibre enlargement. However in the sheep trial described in this thesis, it was not possible to determine whether there were any effects of the growth promoters on satellite or other precursor muscle cells associated with muscle fibres, particularly with respect to effects on the serine synthesis pathway.

In vitro studies testing the effects of growth promoters on differentiating muscle cells in culture have proven inconclusive, but none have measured the effects of these agents on the serine synthesis pathway. In those studies which have been carried out some indicate increased cell fusion in response to growth promoting agents, whilst others have shown no effect of these agents. Therefore, an *in vitro* study was carried out on mouse C2C12 myoblasts treated with Des (1-3) IGF-I or dibutyryl cyclic adenosine monophosphate (dbcAMP) during myogenesis to

investigate the effects of these potentially anabolic agents on genes associated with serine biosynthesis.

6.2 Materials and Methods

6.2.1 Cell Treatments

6.2.1.1 Cells without Treatment

To investigate the time-dependent effects on cell proliferation and differentiation, C2C12 cells were grown in DMEM growth medium containing 10% (v/v) foetal bovine serum, 1% (v/v) P/S. When cells had reached 60-70% confluence, they were passaged using trypsin digest and the recovered cells seeded at 100,000 cells per well, in 2 ml of culture medium. After one day of incubation the growth medium was replaced with differentiation medium (DMEM, 2% horse serum (Invitrogen, Paisley, UK) and 1% P/S), with this day identified as day 0. Differentiating cells were harvested on day -1, day 2 and day 4.

6.2.1.2 Cells Treated with Des (1-3) IGF-I

To investigate the time-dependent effects of differentiation as well as the effect of treatment with IGF-I, cells were incubated with recombinant human insulin like growth factor-I Des (1-3) (Des1-3 IGF-I) (Autogen Bioclear UK Ltd, T/A Source BioScience UK Limited). This was dissolved in 0.1 M acetic acid to a concentration of 100ng/ml, and then diluted in phosphate buffered saline (PBS) to 50ng/ml. The Des (1-3) IGF-I was then added to differentiation medium at a final concentration of 20ng/ml. Proliferating C2C12 cells were seeded at 100,000 cells per well in 6-well plates (3 treatments per plate), as described above. Differentiation media,

with or without Des (1-3) IGF-I present, was added to cells at day 0. Cells were harvested on day 0, day 2 and day 4.

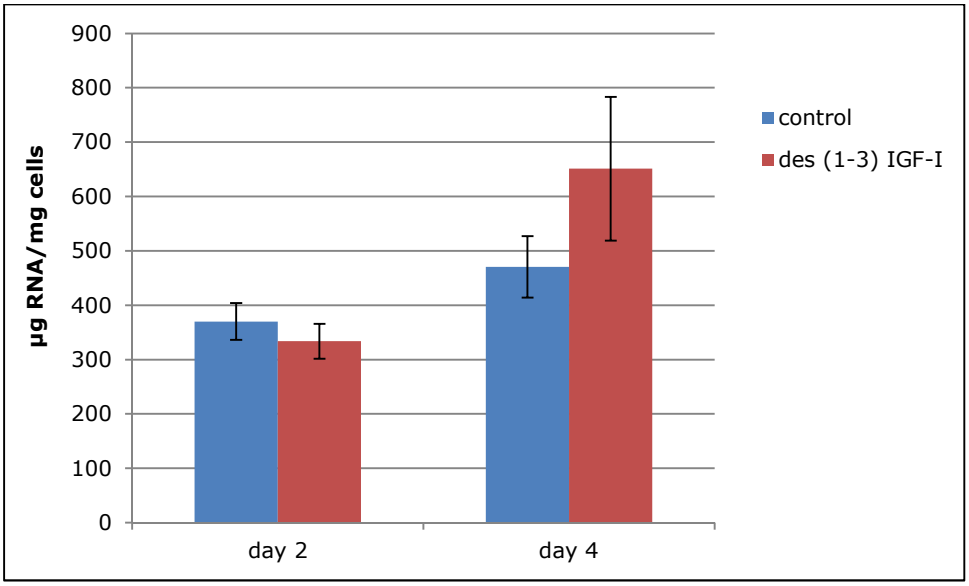
6.2.1.3 Cells Treated with dbcAMP

In addition to examining the effects of IGF-I on differentiating C2C12 cells, differentiating C2C12s were treated with dibutyryl cyclic adenosine monophosphate (dbcAMP) (Sigma-Aldrich). The dbcAMP was dissolved in differentiation medium at a final concentration of 1mM. Proliferating C2C12 cells were seeded at 100,000 cells per well in 6-well plates, as described above. Differentiation media, with or without dbcAMP present, was added to cells at day 0. Cells were harvested on day -1, day 3, day 6 and day 7.

6.2.2 Effect of Des (1-3) IGF-I on RNA Quantity and Quality

Total RNA was extracted from C2C12 myoblasts treated with Des (1-3) IGF-I on following 2 and 4 days of treatment using the methods as described in the materials and methods section. There were no significant differences ($P< 0.354$) in RNA concentration per well in treated cells compared to control, nor was there a significant interaction ($P<0.17$) between time and treatment (Figure 6-1 A). However there was a significant ($P<0.014$) effect of time on RNA concentration compared to control. A 260nm/280nm ratio reading of greater than 1.8-2.0 was deemed acceptable for RNA quality. There was no significant effect of treatment, time or the interaction between treatment and time on the 260/280 ratio ($P<0.195$, $P<0.57$, $P<0.704$, respectively) (Figure 6-1 B).

A



B

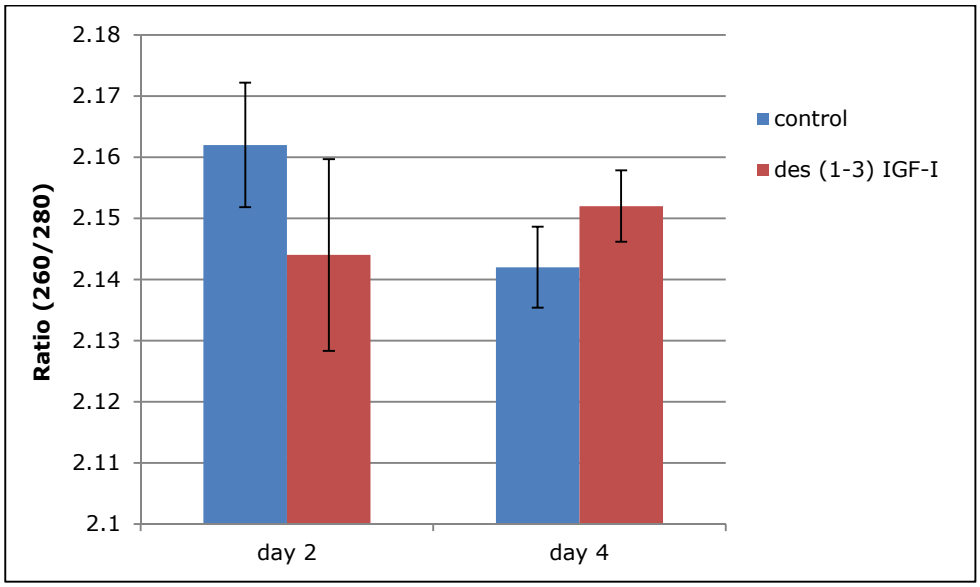


Figure 6-1: Effects of Des (1-3) IGF-I on (A) total RNA concentrations and (B) 260/280 ratio during differentiation of C2C12 myoblastst.

Blue C (control), Red des (1-3) IGF-I treatments. The error bars are ± SEM (n=5 for C and n=5 for des (1-3) IGF-I.

The total RNA integrity was examined by electrophoresis using a non-denaturing agarose gel and an image was captured using the Gel Doc system and MultiAnalyst (Bio-Rad, UK). An example of total RNA run on agarose gel is shown in the representative images for the cells treated with IGF-I at days 0, 2 and 4 (Figure 6-2).

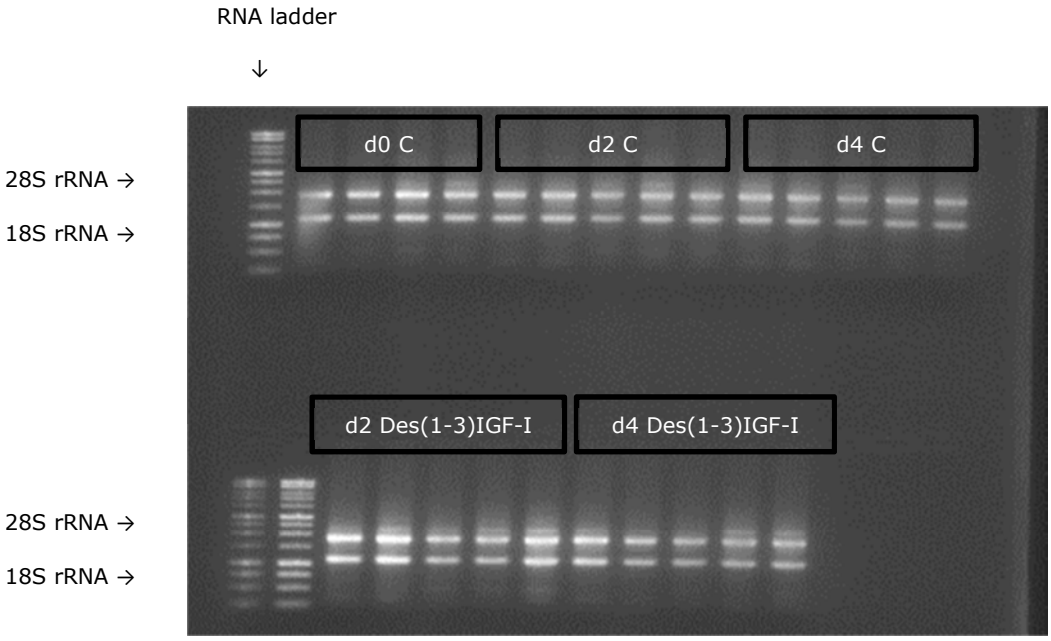


Figure 6-2: Total RNA integrity from-mouse C2C12 muscle cells determined by non-denaturing agarose gel.

Top line represents control treatments (C) from left to right, the first four wells are day 0 replicates proceeded by five wells for day 2 then day 4, Des (1-3). Bottom line represents IGF-I treated cells from left to right day 2 (first 5 wells) then day 4. RNA fragment size ladder is shown and is used to calibrate the gel also indicated is the position of the ribosomal RNA 28S and 18S bands. S =Svedberg unit (sedimentation coefficient).

6.2.3 Mouse Tissues (Animals and Sampling)

Five mice were killed by rising CO_2 concentration and then the whole muscles from thigh and shoulder were collected, placed in plastic bags and frozen in liquid nitrogen. Mouse muscles were crushed in liquid nitrogen using a pestle and mortar. Crushed samples were used for RNA extraction followed by cDNA synthesis utilising the same procedures as described for sheep tissues (chapter 2). Mouse PCR primers used in this study are shown in Appendix 5.

6.2.4 Statistics

Data were analysed by one-way ANOVA (Genstat, edition 15), followed by a Post Hoc Dunnett's test to examine the effects on

gene expression in differentiating C2C12 cells, as well as in mouse tissues. For cells treated with Des (1-3) IGF-I or dbcAMP, a two way ANOVA was used to determine effects of time, treatment and time x treatment interaction. All data are displayed as mean ± standard error of the mean (SEM). Significance was accepted at $P<0.05$.

6.3 Results

6.3.1 The Effect of C2C12 Myoblast Differentiation on the Expression of the Serine Synthesis Pathway Genes

Representative images of the mouse C2C12 cells without treatments at different stages of proliferation and differentiation are shown in Figure 6-3. Cells from day -1 were proliferating myoblasts (60-70% confluence) and continued to proliferate to reach approximately 90% confluence at day 0. On day 2 myotubes started to form and myotubes were clearly observed on day 4.

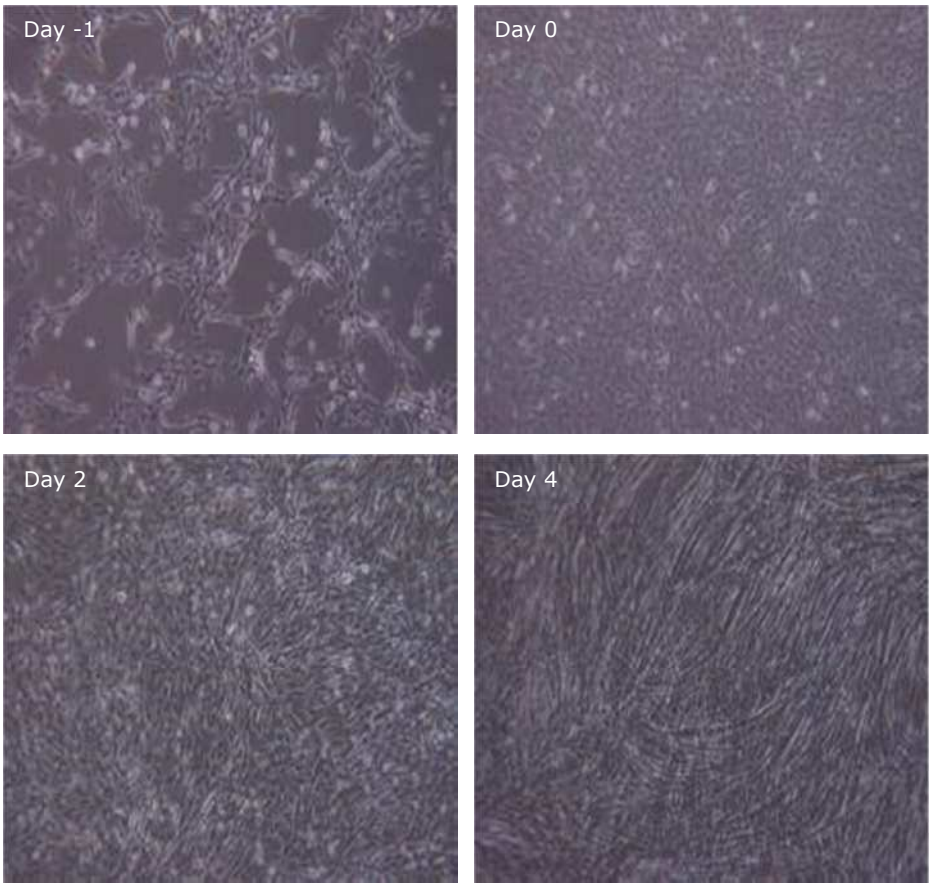


Figure 6-3: Changes in the phenotype of C2C12 cells at days -1, 0, 2 and 4 during differentiation.

Representative microscopy images of C2C12 myoblast cells during differentiation. Differentiation media (2% horse serum) was applied to cells at day 0. Magnification was 63x.

In differentiating C2C12 cells there was no effect of the time of incubation on oligreen quantities (P= 0.36) (Figure 6-4).

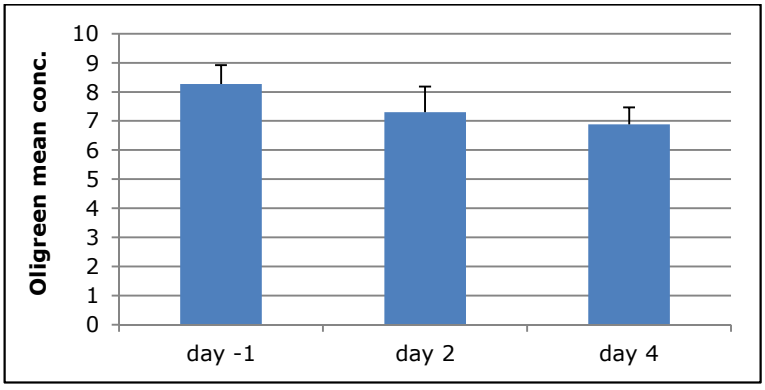


Figure 6-4: Effects of incubation time on oligreen concentrations (cDNA quantity) in differentiating C2C12. (Day 0 is the time at which media was changed to differentiation media, n=6).

There was a significant change in the expression of PHGDH and PSPH mRNA during differentiation (P<0.001 and P=001, respectively), whilst there was no change in PSAT mRNA (P=0.373). Expression of PHGDH mRNA was higher on day -1 compared to day 2 and day 4 and a similar pattern of gene expression was seen for PSPH (P=0.001) (Figure 6-5).

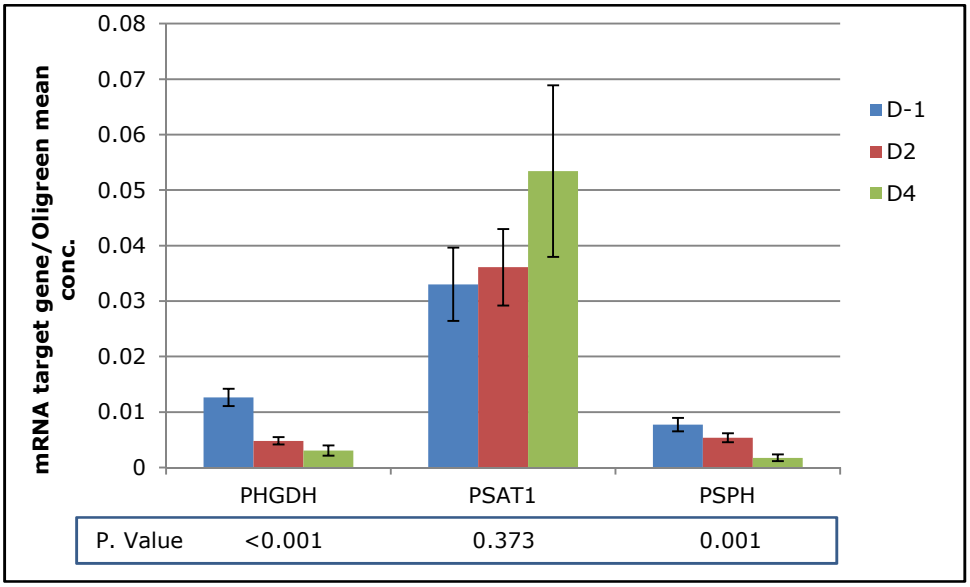


Figure 6-5: The effect of C2C12 myoblast differentiation on gene expression of the enzymes associated with serine synthesis pathway.

Day-1 (D-1) blue, day 2 (D2) red, day 4 (D4) green, n=6. One-way ANOVA P. values are indicated; error bars are ± SEM. Significance was accepted if P<0.05.

Previous studies in our lab have shown there was a change in gene expression of myogenic regulatory factors (MRFs) during differentiation of C2C12 myoblasts, with myogenin increasing early during differentiation (day 2 of differentiation) then decreasing (day 6); whilst Myf5 expression was high in proliferating myoblasts but decreased on day 2 in differentiation media (Brown et al., 2012). Likewise in this study there was a significant change in

expression of myogenin across the time course (P<0.001), with relatively low expression in myoblasts on day -1, which was increased on day 2, then declining again on day 4 (Figure 6-6). There were also significant changes in Myf5 mRNA during differentiation (P<0.001), with expression decreasing over the time course (Figure 6-6).

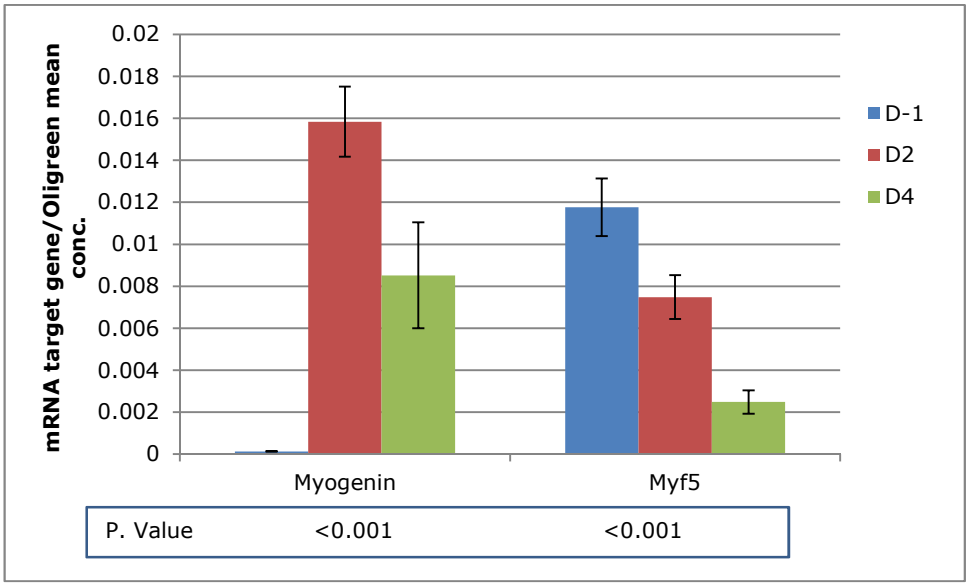


Figure 6-6: The effect of C2C12 myoblast differentiation on gene expression of Myogenin and Myf5.

Day-1 (D-1) blue, day 2 (D2) red, day 4 (D4) green, n=6. One-way ANOVA P. values are indicated; error bars are ± SEM. Significance was accepted if P<0.05.

6.3.2 The Effect of Des (1-3) IGF-1 on C2C12 Myoblast Differentiation and the Expression of the Serine Synthesis Pathway Genes

The *in vivo* study in sheep described in this thesis examined the effects of GH and BA treatment for 6 days which resulted in an increase in the expression of genes for the serine synthesis pathway enzymes in response to BA stimulation. Therefore the current *in vitro* study was carried out to determine whether the agent Des (1-3) IGF-I altered expression of genes associated with

the serine synthesis pathway during mouse C2C12 differentiation. Representative images of the mouse C2C12 cells at different stages of proliferation and differentiation treated with Des (1-3) IGF-I are shown in Figure 6-7. Cells from day -1 were proliferating myoblasts (60-70% confluence) and continued to proliferate to reach approximately 90% confluence at day 0. On day 2 in both control and Des (1-3) IGF-I treatment myotubes started to form and phenotypic differences were observed between the treatments as incubation time was extended.

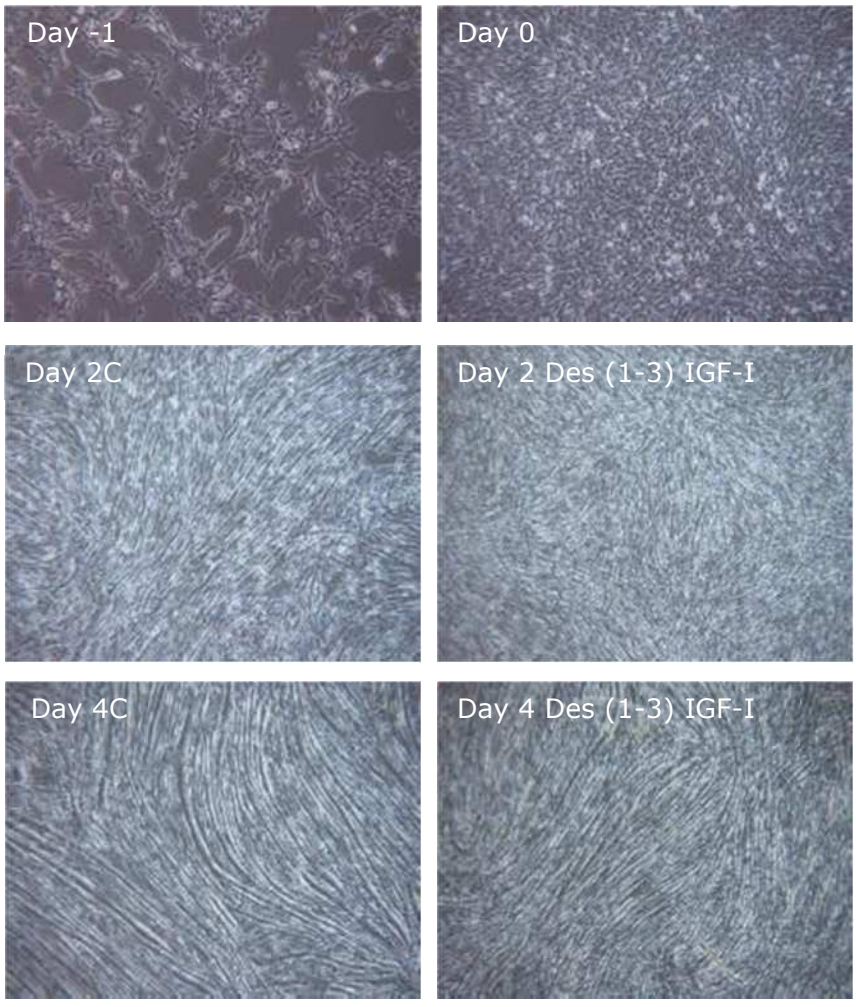


Figure 6-7: Changes in the phenotype of C2C12 cells treated with Des (1-3) IGF-I during differentiation.

Representative microscopy images of C2C12 myoblast cells during differentiation. Differentiation media (2% horse serum) containing Des (1-3) IGF-I (20ng/ml) or control (C) was applied to cells at day 0. Magnification was 63X.

6.3.3 Oligreen in C2C12 Treated with Des (1-3) IGF-I

There were no significant effects of day (P=0.497) or treatment (P=0.706), as well as no significant interaction between day and treatment (P= 0.146) on oligreen observed across the time course in C2C12 treated with Des (1-3) IGF-I (Figure 6-8).

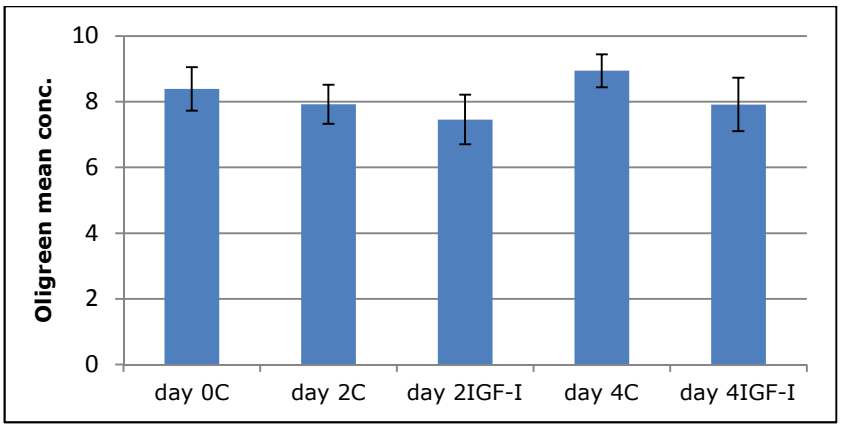
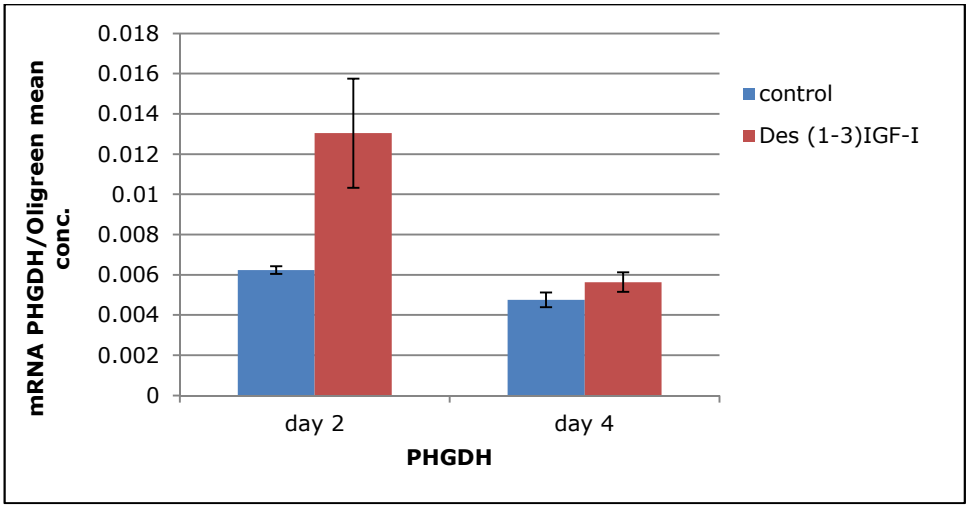


Figure 6-8: Effects of Des (1-3) IGF-I on oligreen concentration (cDNA quantity) during C2C12 myogenesis. Error bars are ±SEM, n=5.

The effect of treatment with Des (1-3) IGF-I on gene expression of the enzymes in the serine synthesis pathway in C2C12 during differentiation (time) indicated an interaction between time and treatment for PHGDH (P=0.032) and PSPH (P=0.004). For PHGDH, gene expression was the highest in Des (1-3) IGF-I treated cells on day 2, but a similar difference was not seen on day 4 (Figure 6-9). For PSPH the gene expression for Des (1-3) IGF-I treated cells was higher than controls at day 2, but this was reversed on day 4 (Figure 6-9). There was no significant interaction between time and treatment (P=0.433) for PSAT1 mRNA expression, however there was effect of treatment (P=0.047), with the expression of PSAT1 being lower in C2C12 treated with Des (1-3) IGF-I compared to controls (Figure 6-9 and Table 6-1).

A



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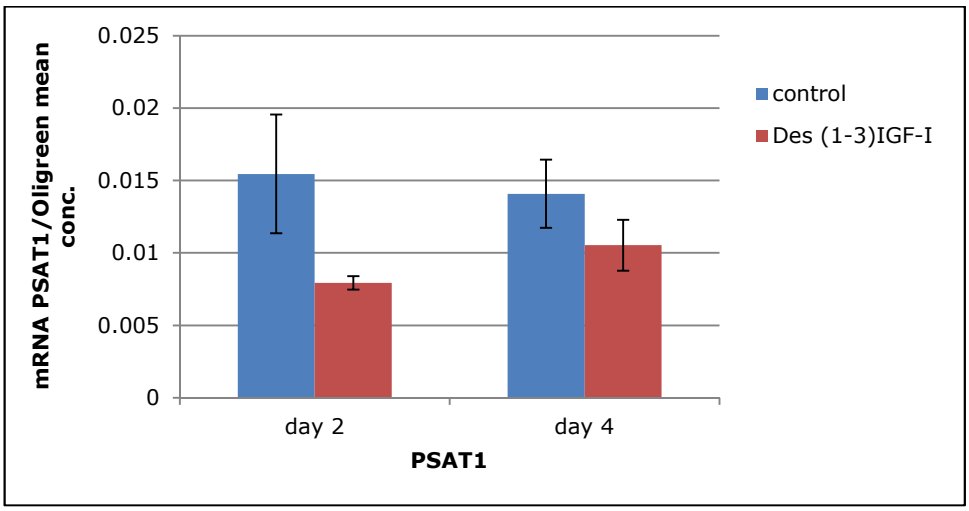

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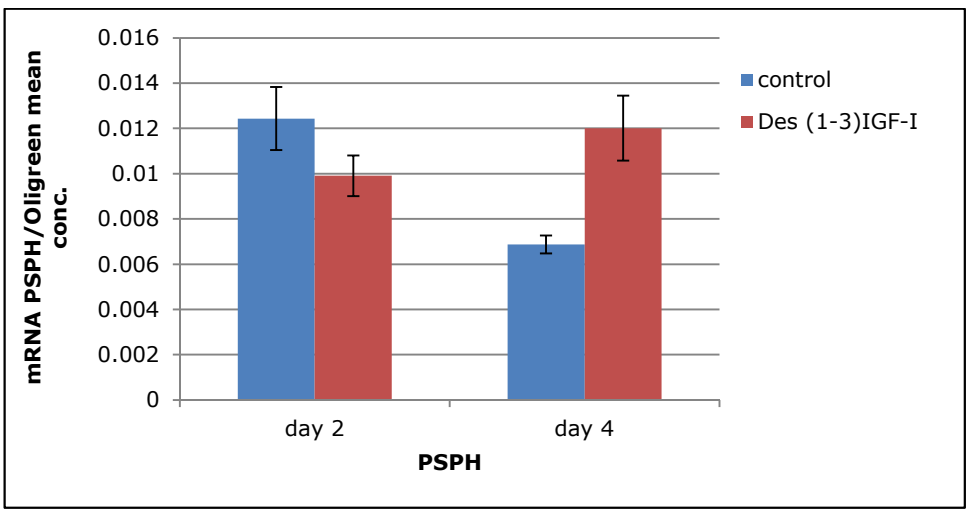
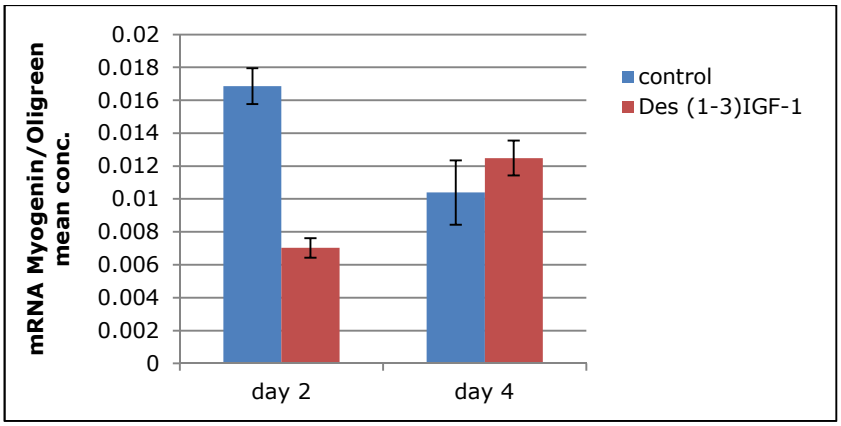


Figure 6-9: The effect of Des (1-3) IGF-I treatment on gene expression of the enzymes associated with serine synthesis pathway in differentiating C2C12 myoblasts.

Graph A, PHGD; graph B, PSAT1, graph c, PSPH. Day refer to days in differentiation media. Control, blue, Des (1-3) IGF-I, red, n=5. The error bars are ± SEM.

The effect of Des (1-3) IGF-I on the expression of the MRFs myogenin and MYf5 in differentiating (time) C2C12 myoblasts was examined. There was a significant interaction between time and treatment ($P<0.001$) for Myogenin mRNA expression. On day 2 Myogenin gene expression was low in C2C12 treated with Des (1-3) IGF-I compared to control, whilst on day 4 mRNA Myogenin was higher in treated compared to untreated myoblasts (Figure 6-10). Likewise for Myf5 mRNA expression, there was a significant interaction between time and treatment ($P=0.008$). Myf5 mRNA expression was higher on day 2 than on day 4 in differentiating C2C12 cells treated with Des (1-3) IGF-I compared to control (Figure 6-10 and Table 6-1).

A



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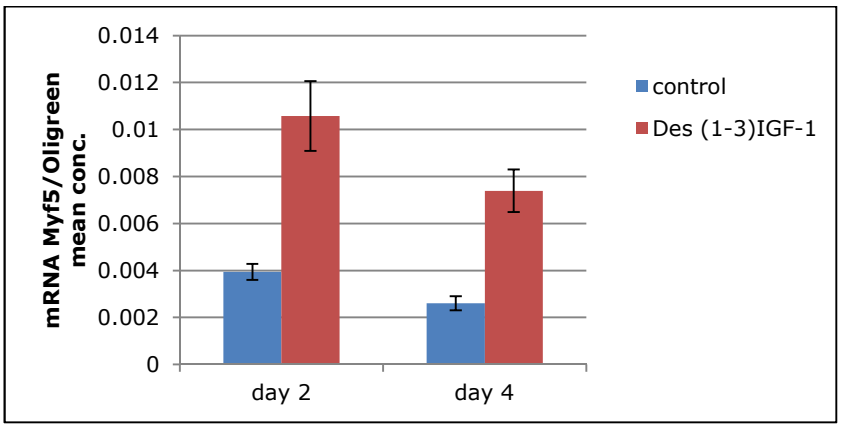


Figure 6-10: Effects of Des (1-3) IGF-I on Myogenin and Myf5 mRNA expression during C2C12 differentiation.

Graph A, Myogenon and graph B Myf5. Day refer to days in differentiation media. Control, blue, Des (1-3) IGF-I, red, n=5. The error bars are ± SEM.

Table 6-1: P. values for two way ANOVA analysis of gene expression of differentiating C2C12 myoblast treated with Des (1-3) IGF-I.

IGF-I	**P.values**				
Source of variation	**PHGDH**	**PSAT1**	**PSPH**	**Myogenin**	**Myf5**
Day[1]	0.003	0.806	0.143	<.001	<.001
Treatment[2]	0.013	0.047	0.141	0.04	<.001
Day.Treatment	0.032	0.433	0.004	<.001	0.008

[1] effect of day of differentiation on gene expression

[2] effect of treatment with Des (1-3) IGF-I

6.3.4 The Effect of Dibutyryl Cyclic Adenosine Monophosphate (dbcAMP) on C2C12 Myoblast Differentiation and the Expression of the Serine Synthesis Pathway Genes

In vivo BA treatment of sheep for 6 days stimulated muscle growth and this was associated with an increase in the expression of genes associated with serine synthesis pathway. Therefore the current *in vitro* study was carried out to determine whether the agent dbcAMP effected the expression of genes associated with the serine synthesis pathway during mouse C2C12 differentiation.

Representative images of the mouse C2C12 cells treated with dibutyryl cyclic adenosine monophosphate (dbcAMP) at different stages of differentiation are shown in Figure 6-11. Cells from day -1 were proliferating myoblasts (60-70% confluence) and continued to proliferate to reach approximately 90% confluence before differentiation media was added (data not shown). On day 3 there were differences in the myotubes observed in control compared to dbcAMP treated cells and the difference became greater on day 6 and day 7 with dbcAMP treated cells being lost at day 6 and 7. This cell loss maybe due to more myotubes forming as extensive

myotube formation can lead to them contracting and becoming detached from the plate.

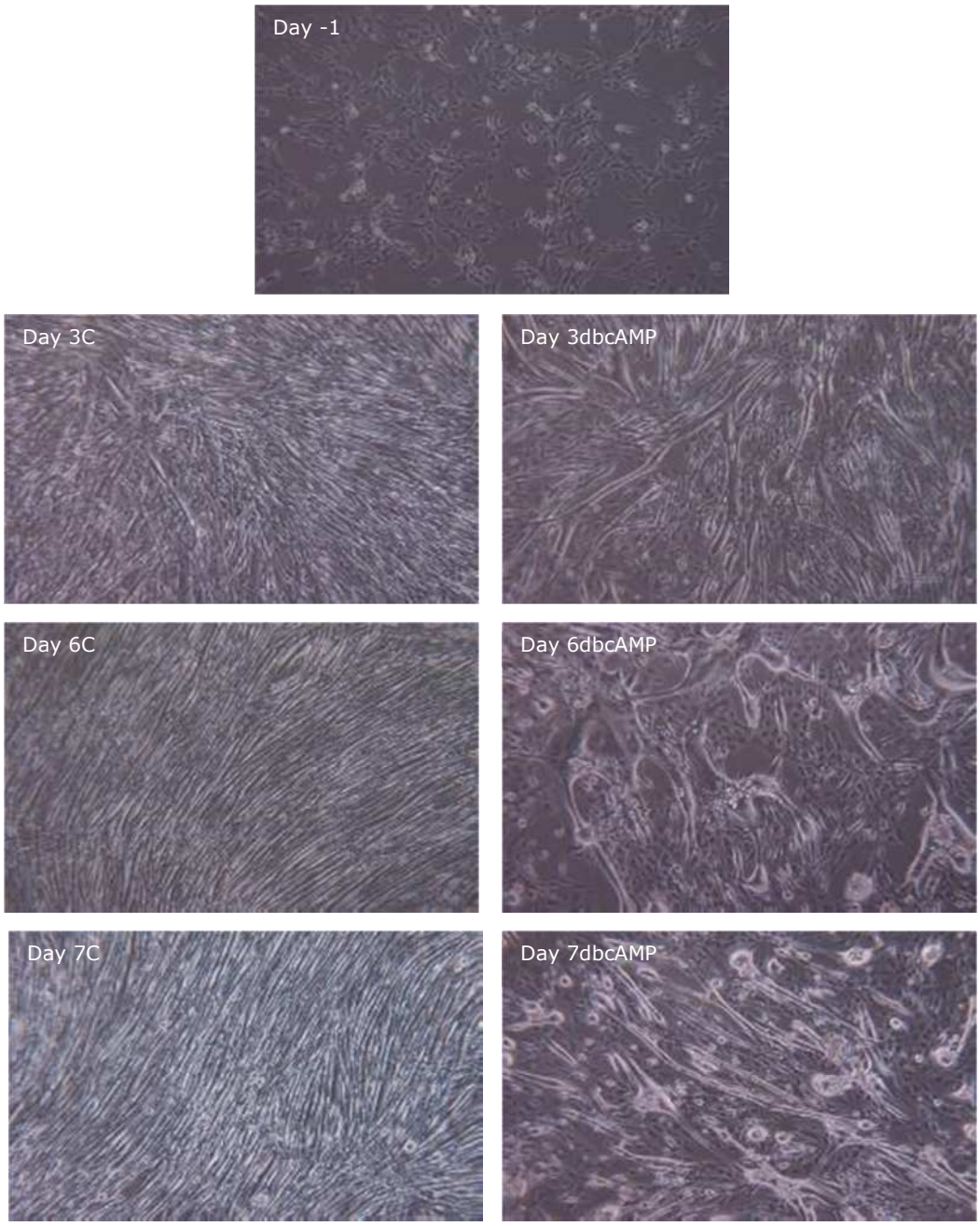


Figure 6-11: Changes in the phenotype of C2C12 cells treated with dbcAMP during differentiation.

Representative microscopy images of C2C12 myoblast cells during differentiation. Differentiation media (2% horse serum) containing dibutyryl cyclic adenosine monophosphate (dbcAMP) or control was applied to cells at day 0. dibutyryl cyclic adenosine monophosphate (dbcAMP), control (c). Magnification was 63X.

6.3.5 Oligreen in C2C12 Treated with dbcAMP

There were no significant effects of time or treatment (P=0.487, P=0.41 respectively) observed in cDNA (oligreen mean concentration) across the time course in C2C12 myoblasts treated with dbcAMP during proliferation and differentiation. There was also no interaction effects (P=0.722) of time and treatment observed (Figure 6-12).

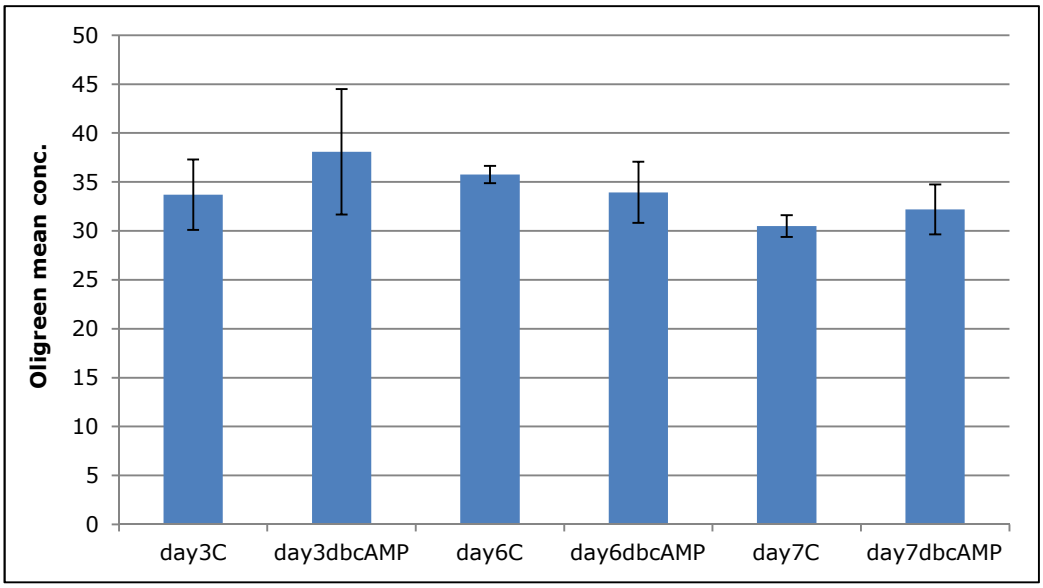


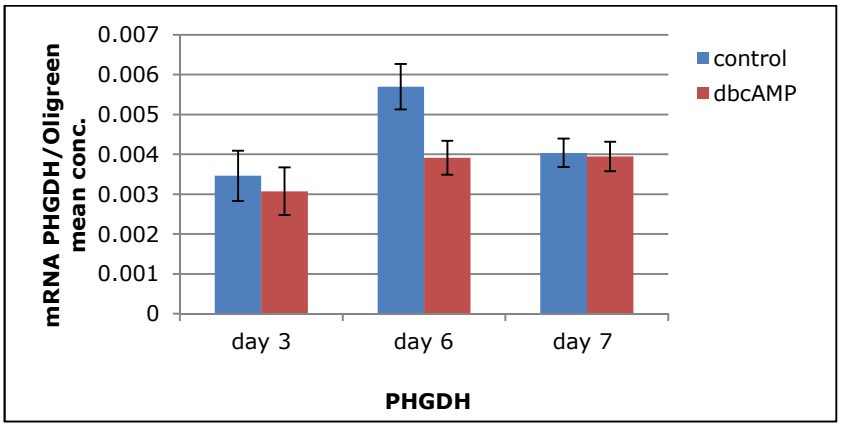
Figure 6-12: Effects of dbcAMP on oligreen concentration (cDNA quantity) during C2C12 myogenesis.

Control (C), dibutyryl cyclic adenosine monophosphate means (dbcAMP), n=4. The error bars are ± SEM.

Examination of the effects of treatment C2C12 with dbcAMP during differentiation (time) on gene expression of the enzymes in the serine synthesis pathway indicated an interaction between time and treatment for PSAT ($P<0.05$) and PSPH ($P<0.05$). The effect of dbcAMP on PSAT was to increase the expression of this gene relative to control on day 7. For PSPH gene expression was lower in dbcAMP treated cells than control early in differentiation. There was no interaction between treatment and time on the expression

of PHGDH mRNA, however there was a trend for an effect of treatment (P=0.084) with dbcAMP treated cells being higher than control. The expression of mRNA PHGDH significantly changed over the time course (P=0.023), increasing from day 3 to day 7 (Figure 6-13 and Table 6-2).

A



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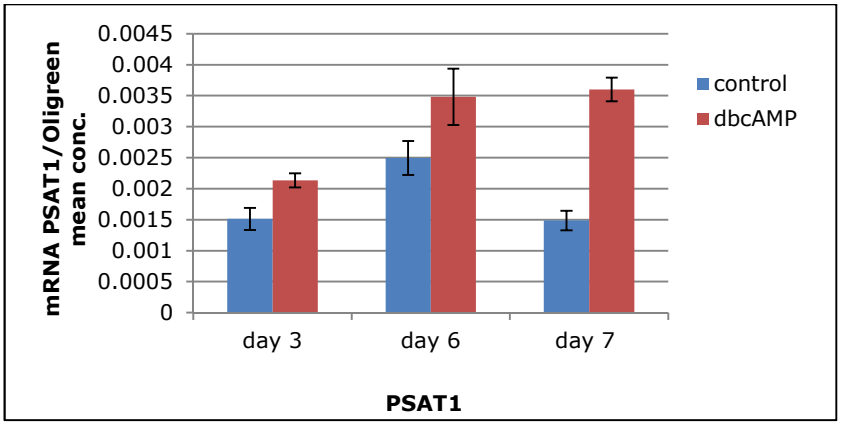

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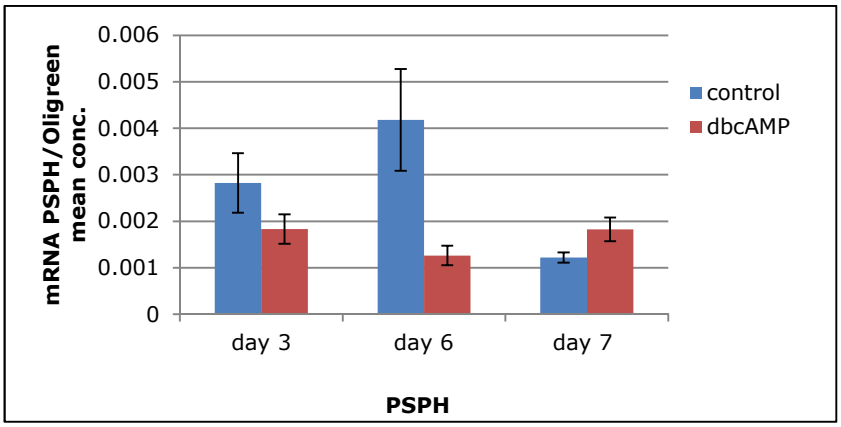
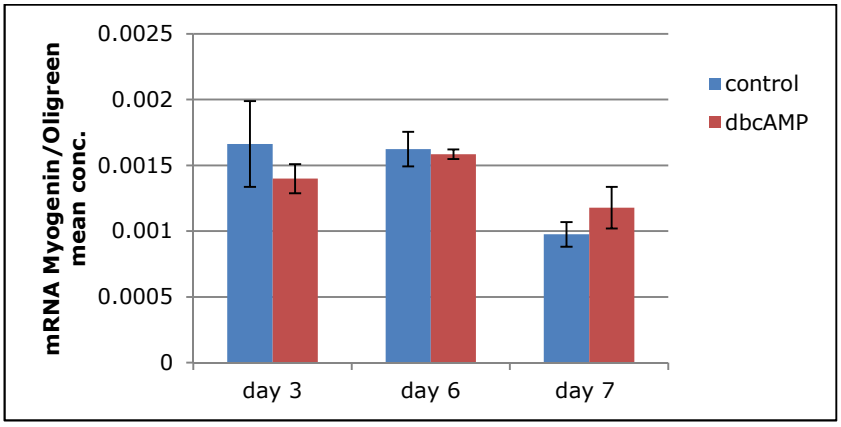


Figure 6-13: The effect of dbcAMP treatment on gene expression of the enzymes associated with serine synthesis pathway in differentiating C2C12 myoblast.

Graph A, PHGDH; graph B, PSAT; graph C, PSPH, n=4. Day refer to days in differentiation media. Control, blue, dbcAMP, red. The error bars are ± SEM.

There was no interaction between time and treatment for myogenin mRNA expression, but there was a significant effect of day (P=0.012). Myogenin expression declined over the time course (Figure 6-14 and Table 6-2). In contrast there was a time and dbcAMP treatment interaction in Myf5 mRNA expression (P<0.001) being higher in dbcAMP treated cells on day 3 then reversed on day 6 and 7 (Figure 6-14 and Table 6-2).

A



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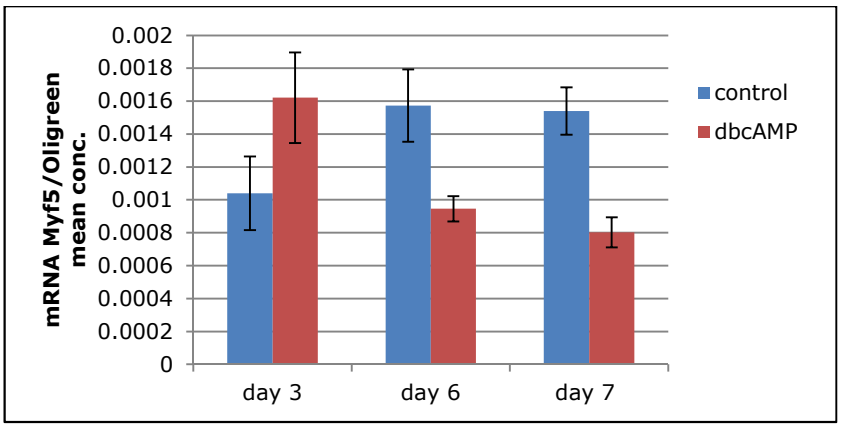

Figure 6-14: Effects of dbcAMP on Myogenin and Myf5 mRNA expression during C2C12 differentiation.

Graph A, Myogenin and graph B, Myf5. Day refer to days in differentiation media. Control, blue, dbcAMP, red, n=4. The error bars are ± SEM.

Table 6-2: P. values for two way ANOVA analysis of gene expression C2C12 myoblast treated with dbcAMP.

dbcAMP	P.value				
Source of variation	PHGDH	PSAT1	PSPH	Myogenin	Myf5
Day[1]	0.023	<.001	0.114	0.012	0.698
Treatment[2]	0.084	<.001	0.025	0.812	0.103
Day.Treatment	0.227	0.023	0.018	0.401	0.004

[1] effect of day of differentiation on gene expression

[2] effect of treatment with dbcAMP

6.3.6 Gene Expression of Serine Synthesis Pathway Enzymes in Mouse Muscle

Expression of mRNA for all three enzymes of the serine synthesis pathway was not significantly different in the mouse shoulder and thigh muscles (Figure 6-15).

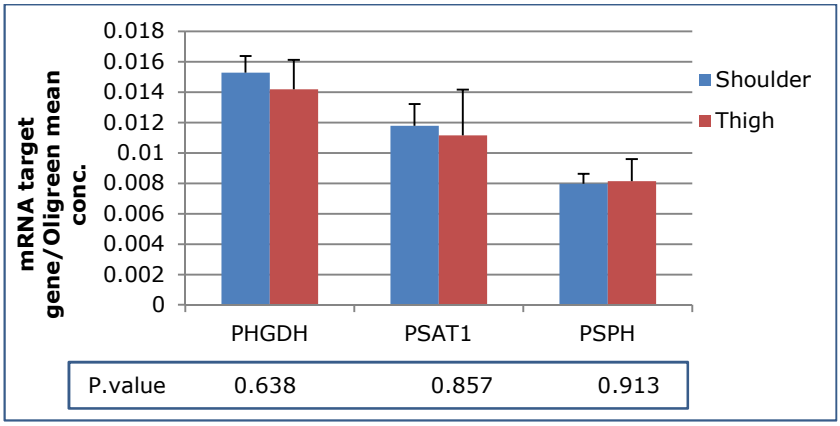

Figure 6-15: Expression of serine synthesis pathway enzyme mRNA in mouse muscle from shoulder and thigh.

Blue (shoulder muscles), red (thigh muscles), genes (PHGDH, PSAT1 and PSPH). One-way ANOVA P. values are indicated; error bars are ± SEM, n=5.

6.4 Discussion

In cancer cells, which are highly proliferative, there appears to be a drive for macromolecular biosynthesis which is required for rapid cell growth. As part of this biosynthetic requirement there is an increase in the activity of the serine metabolic pathway, as serine appears to have a critical role for growth (Possemato et al., 2011). In cancer cells, associated with an increase in biosynthesis, 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). The study described in this thesis examined the effects of GH and BA on sheep over a 6 day time course. The predominant finding was BA stimulated muscle growth there was an increase in the expression of genes for the enzymes responsible for serine synthesis. Therefore the cell-based study described in this chapter sought to determine whether agents associated with a BA or GH response affected the expression of genes associated with serine synthesis pathway during mouse C2C12 differentiation. These myoblast cultures are utilised as a cell-based model of hypertrophy and atrophy in skeletal muscle *in vivo,* allowing the key mechanical patterns of the signalling and molecular pathways to be identified (Sharples and Stewart, 2011).

During the process of differentiation of C2C12 myoblasts the expression of two genes associated with the serine synthesis pathway enzymes, PHGDH and PSPH were significantly decreased. This is not very surprising as during differentiation myoblasts switch from a proliferating phenotype to terminally differentiated myotubes. As C2C12 is a cell line the proliferative rate of the single cell myoblasts is relatively high, therefore there is likely to be a similar requirement for a biosynthetic pathway as seen in cancer

cells. Indeed the activity of the serine synthesis pathway enzymes is high in a number of cancer cell lines (Pollari et al., 2011).

The process of myogenesis is regulated by four myogenic regulatory factors (MRFs), consisting of MyoD, Myf-5, myogenin, and MRF4 (Bentzinger et al., 2012, Sabourin and Rudnicki, 2000). Myogenesis regulation is well characterized and comprehensively understood (Bentzinger et al., 2012).

The myogenic regulatory factors (MRFs) can activate skeletal muscle differentiation when overexpressed in non-muscular cells. These transformed cells are then able to undertake myoblast fusion and subsequent expression of muscle specific genes (Weintraub et al., 1989, Lattanzi et al., 1998). The myogenic regulatory factors MyoD and Myf5, are expressed in the early stages of myoblast differentiation, whilst myogenin exhibits effects in the differentiation of myoblasts into myotubes (Rudnicki et al., 1993). In differentiating myoblasts, Myf5 mRNA expression was high on day-1, then declined over the 4 days after the differentiation media was added, this agrees with the observations of Brown et al.(2012). This supports the idea that Myf5 is the most important proliferative transcription factor during myogenesis (Kitzmann et al., 1998, Gayraud-Morel et al., 2007, Ustanina et al., 2007). Myogenin is considered to be a key signalling transcription factor required for muscle cell differentiation (Nabeshima et al., 1993, Hasty et al., 1993). In the current study in C2C12 mouse muscle cells the expression of myogenin mRNA was not seen in myoblasts (day-1), before differentiation media was added. It increased as cells began to differentiate, then began to decrease as cells became further differentiated. This was similar to the observations previously reported in our laboratory (Brown et al., 2012).

Phosphorylation of Serine 318 in the insulin receptor substrate-1 (IRS-1) is mediated by factors such as insulin or IGF-I binding the insulin receptor. The phosphorylated IRS-1 can then induce activation of PI 3-kinase/mTOR pathway. However, the activation of this pathway can also be carried out by other unknown kinases, which have yet to be identified (Mussig et al., 2005). Modifications in mTOR signalling not only increase protein synthesis, but also increase ribosome biogenesis, which would be expected to increase the capacity of the tissue to synthesize protein (Kimball et al., 2002). The proliferation and differentiation of skeletal muscle cells in culture are generally regulated by serum compounds, and the differentiation can be induced by a decrease in the serum concentration (Yoshiko et al., 1996). Therefore increasing IGF-I, a serum factor, might be expected to increase cell proliferation.

IGF-I and its more potent myogenic inducer, Des (1-3) IGF-I, induced myogenin mRNA expression in C2C12 cells (Kaliman et al., 1998, Florini et al., 1991, Vinals and Ventura, 2004) and myogenin protein at 4 and 8h post differentiation (Vinals and Ventura, 2004). Treatment of C2C12 with Des (1-3) IGF-I in the experiment described within this thesis had no significant effect on myogenin expression. For Myf5 mRNA expression there was a day and treatment interaction. Myf5 mRNA expression was higher on day 2 than on day 4 in the differentiating C2C12 treated with Des (1-3) IGF-I compared to control.

In vitro studies testing the effects of BA on muscle cell differentiation have proven inconclusive. Some studies indicate increased cell fusion in response to BA (Grant et al., 1990, Shappell et al., 2000), whilst others have shown no effect (McMillan et al., 1992, McFarland et al., 1995).

It has been shown that BA (ractopamine) caused a 30% increase in cell number, protein, and DNA concentrations in mouse C2C12 myoblasts after treatment for 48h, but there were no differences found in myotubes derived from the C2C12 myoblasts (Shappell et al., 2000). This might be due to the high doses of ractopamine (10μM) applied or the source of the cells used, because there was no effect of ractopamine on the cells derived from later cell culture passages (Miller et al., 2012). A study by Shappell et al. (2000) also concluded that in turkey muscle cells ractopamine may work indirectly through extramuscular changes, such as increased blood flow and uptake of amino acids by muscles, but not directly through the stimulation of the β-adrenergic receptor (β-AR) on muscle cells. Their study suggested that hypertrophy was a characteristic associated with muscle fibres rather than myoblasts. Shappell et al. (2000) pointed out that *in vitro* studies had indicated there is no strong evidence in support of BA stimulation of β-AR mediating muscle hypertrophy through proliferation and fusion of satellite cells. However it has been shown that BA induces significant anabolic effects on skeletal muscle during the early stages of C2C12 differentiation (Ryall et al., 2004, Ryall et al., 2010). In the current study, the expression of mRNA PHGDH was not affected by dbcAMP compared to control. The expression of mRNA PHGDH changed during the time course from day 3 to day 7. For PSAT1 expression there was a significant interaction between time and treatment in C2C12 myoblasts treated with dbcAMP. On day 6 and 7 the expression of PSAT1 in treated cells was higher whilst this was reversed at day 3. For PSPH mRNA expression there was also a significant interaction between time and treatment, with gene expression being lower than control in day 3 and 6 but not on day 7. These observations maybe associated with phenotype shown by the cells in response to treatment.

Myogenin mRNA was not affected by dbcAMP treatment, but expression was affected by time in differentiating C2C12s. Myogenin expression was significantly increased on day 3 and 6, but then declined on day 7. Myogenin mRNA expression has been utilized as a marker of muscle cell differentiation in C2C12 cells. This is because it is not expressed in myoblasts but increases early during differentiation, reaching a peak at around day 3, then declines as cells become fully differentiated into myotubes (Brown et al., 2012, Andres and Walsh, 1996). In contrast, there was a significant interaction between time and treatments on Myf5 mRNA in mouse C2C12 myoblast treated with dbcAMP. As expected expression of Myf5 mRNA was higher on day 3 in dbcAMP treated cells relative to controls, but this was reversed on day 6 and 7.

6.5 Conclusion

Gene expression of enzymes involved in the serine biosynthesis pathway (PHGDH and PSPH) displayed a greater level of expression in proliferating mouse C2C12 myoblasts compared to differentiated myotubes, whilst PSAT1 was higher in differentiated myotubes than myoblasts. There was no significant change in the gene expression of enzymes associated with serine synthesis pathway in C2C12 myoblasts treated with Des (1-3) IGF-I or dbcAMP during differentiation.

7 General Discussion

7.1 Main Finding

The hypothesis investigated in this thesis was that growth promoting agents, such as beta-adrenergic agonists (BA) and growth hormone (GH), enhance muscle growth through the modification of metabolism which then impacts on protein accretion. Changes in energy metabolism in the liver and muscle in ruminants are key to the effectiveness of these growth promoters.

This thesis examined and compared how the anabolic agents BA or GH effect lamb growth and found that BA had greater effects on muscle growth than GH, whereas GH had a wider effect on whole body metabolism than BA. Muscle hypertrophy stimulated by BA or GH, but especially by BA, was associated with changes in the gene expression of distinct metabolic pathways in muscle and liver. The changes in expression of metabolic pathway genes were partially supported by changes in circulating metabolite concentrations.

The *in vitro* study in this thesis, utilizing the myogenic C2C12 cell line, sought to determine if the genes involved in the serine synthesis pathway associated with the sheep growth promoter response were similarly affected during myogenesis or when treated with agents such as Des (1-3) IGF-I or dbcAMP. There were no major effects of either des (1-3) IGF-I or dbcAMP in serine synthesis pathway gene expression. The lack of effect of des (1-3) IGF-I in serine synthesis in these cells might be expected as there had been no effect of GH in the *in vivo* study in lambs. Des (1-3) IGF-I tends to be "Mitogenic" leading to enhanced proliferation. *In vivo* BA treatment resulted in changes in the expression of serine synthesis pathway enzyme genes, which was associated with hypertrophy. There were no major changes observed with dbcAMP treatment of C2C12 cells, suggesting that the effect of BA *in vivo* is

associated with the mature fibres rather than being via effects on differentiating myoblasts.

7.2 Effects of Growth Promoters on Sheep

Using growth promoters such as BA and GH to improve the efficiency of animal production is very important for the world animal production sector, especially in countries which have struggled to meet their increasing meat consumption due to insufficient availability of feed (Sillence, 2004). Both GH and BA have been reported to have a positive effect on growth rate when given over extended periods (Owens et al., 1999, Byrem et al., 1998, Beermann et al., 1987, Kim et al., 1989). In the current study, there were no significant effects of BA and GH on body weight observed, due to the short time of administration of the growth promoters (6 days). However BA, but not GH, significantly increased the weights of both SS and ST muscles, with a trend for an increase in VL weights compared to control. This increase was associated with hypertrophy of type II fibres (Hemmings et al., 2009).

Experimental evidence indicates cimaterol has direct effects on protein accretion in skeletal muscle (Byrem et al., 1998), but the effect varies between muscles. For example, BA treatment caused a significant increase in LD and VL muscle mass, but no effect was observed on ST muscle mass (Dawson et al., 1991). This is thought to be due to differences in muscle fibre type content of different muscles. This may be the reason that in the current study only certain muscles were positively affected in terms of weight gain.

Muscle glycogen decreased in BA-treated animals; this was associated with increased size and proportion of fast glycolytic

muscle fibres, which would be expected to have an increased capacity to metabolize glycogen (Maltin et al., 1990), utilize glucose and produce lactate (Hamby et al., 1986). Previous studies have indicated that acute administration of BA decreased activity of insulin and reduced peripheral utilization of glucose in humans and ruminants (Fryburg et al., 1995, McDowell, 1983), whereas chronic administration of BA increased insulin activity and stimulated the utilization of glucose (Beermann et al., 1987). In the sheep trial, GH appeared to induce insulin resistance as plasma glucose concentrations were increased.

Previous studies have indicated that higher muscle weights in cimaterol-fed lambs were the result of an increased cross sectional area of the muscle. However there was not an increase in carcass weights, suggesting a repartitioning effect (Beermann et al., 1986). Cross-sectional area of type II fibres increased approximately 50% in SS and LD muscles in lambs fed cimaterol, suggesting that the increase in muscle mass was due to hypertrophy of type II fibres only (Kim et al., 1987, Hemmings et al., 2014). Another effect of BA that contributes to enhanced muscle growth is an increase in blood flow to the muscle. This increases the availability of substrates and energy sources necessary for protein synthesis. In addition there is enhanced lipid degradation, which results in non-esterified fatty acids being taken away from adipose tissue, because BA causes an increase in heart rate that leads to increased blood flow to many organs (Mersmann, 1987). Thus, BA play a critical role in skeletal muscle growth and development through these various indirect mechanisms that then impact on protein synthesis and degradation (Ryall et al., 2010).

Numerous studies confirmed that administration of GH to growing animals increases weight gain, muscle growth and decreases fat deposition (Etherton, 2004, Campbell et al., 1988, Caperna et al.,

1990, Chung et al., 1985, Sève et al., 1993). Both BA and GH stimulate decreased fat deposition utilising two mechanisms- namely increased fatty acid oxidation and decreased lipid deposition (Blum and Flueckiger, 1988). In the present study, growth promoting agents, particularly GH, increased the plasma concentrations of non-esterified fatty acids, indicating an increase in the hydrolysis of fat. Although a limited number of NEFA were increased in BA after 6 day treatment, previous studies have shown that glycerol and non-esterified fatty acid (NEFA) concentrations were increased two hours after cimaterol ingestion in lambs (O'Connor et al., 1991, Byrem et al., 1998).

In contrast to the effects of BA, GH has wider effects on whole body metabolism, causing considerable hypertrophy of many organs, whilst BA has a more specific effect on skeletal muscle hypertrophy (Reeds et al., 1986, Reeds and Mersmann, 1991, Beermann et al., 1987). GH may work by having effects at different sites compared to BA. The changes in liver weight seen with GH may be due to increased absorption rate of nutrients, as the liver is considered the central site of metabolic regulation (e.g. liver receives most if not all dietary nutrients via the portal vein), and is the main site of GH binding. Therefore, GH could impact on liver metabolism leading to increased liver size or changes in the composition of nutrients within it, which could lead to a change in the nutrients available to target tissues such as muscle. In addition, GH may change the endocrine status in the circulation which would impact on other tissues (Pell et al., 1990). The liver is the major source of IGF-I production, which plays an important role in growth and development (Yakar et al., 1999).

Total protein in whole sheep livers were decreased with BA compared to GH treatment, possibly due to protein being shifted towards muscle in BA treated sheep. In agreement with this

suggestion, (Reeds et al., 1986) reported that BA increased metabolic rate of protein and shifted amino acids from other tissues towards skeletal muscle. Also in the current study, GH significantly increased total liver DNA content. Unfortunately muscle DNA content was not measured in this project, but previous studies demonstrated that BA decreased muscle DNA content as a result of hypertrophy, which resulted in an increase in both protein accretion and RNA concentration, suggesting an increase in the size of cells without incorporation of satellite cell nuclei into the growing muscle (Reeds et al., 1986, Beermann et al., 1987).

Based on microarray analysis, expressions of a larger number of genes were changed in the muscle by BA compared to GH. This reflected that BA had a stronger effect on muscle than GH, indicating specificity of BA effects on muscle. In BA treated animals in the current study, gene expression associated with glycolysis and the serine synthesis pathway were increased, whilst those associated with the TCA cycle were down regulated. This effect was not seen in the GH treated sheep. This has led to the conclusion that the TCA cycle and oxidative phosphorylation had been decreased by BA, suggesting that glucose released from glycogen was not being fully oxidized. The increase in the expression of genes associated with serine synthesis suggested that glycolytic intermediates were instead being used for serine synthesis. This lead to the hypothesis that the increase in the synthesis of the nonessential amino acid, serine, was involved in the BA-stimulated increase in muscle protein synthesis, which subsequently influenced muscle growth. This situation was similar to metabolism in cancer cells where a large amount of glycolytic carbon is converted into serine and glycine metabolism via PHGDH (Locasale et al., 2011).

The energy production in the muscle is very important, as it is the largest contributor to basal metabolic rate, and can increase metabolic rate dramatically when energy is required for muscle contraction. The experimental observations in this thesis suggest that during periods of growth (at least that stimulated by BA); the muscle utilizes glycolysis to generate intermediates that can be used to synthesize nonessential amino acids, particularly serine. The reduced expression of critical enzymes in the TCA cycle, such as ICDH, suggests that the activity of this pathway is reduced. This is counter intuitive in regard to the hypothesis above, as the TCA cycle might be expected to be retained so that biosynthetic pathway intermediates can leave the TCA cycle and be converted to products such as glucose (in the liver), nucleotides, lipids, or non-essential amino acids, a process called cataplerosis. In order to maintain function, intermediates removed from the TCA cycle must be replaced, this process is called anaplerosis (Owen et al., 2002). The observations in this thesis suggest that the TCA cycle reduced its activity. However this reduced activity may be associated with energy production, but with cataplerosis and associated anaplerosis being retained, so biosynthetic molecules could be generated for muscle hypertrophy. Examination of the concentration of TCA cycle intermediates in the circulation, by metabolomics, found that there were no significant differences. However the source of these in the plasma is not clear so care must be taken when interpreting this data.

Clearly in this study BA had the strongest effect on growth and the predominant effect on gene expression was the co-ordinate increase in the serine synthesis pathway. The high expression of enzymes involved in serine synthetic pathway, PHGDH, PSAT1, and PSPH, is often found in cancer tumours, indicating that the serine synthetic pathway may play a critical role in tumorigenesis (Pollari

et al., 2011). In cancer, there are changes in vital cellular functions such as transcription, protein synthesis and mitochondrial metabolism, which are essential for increased cell proliferation and growth and these changes are associated with the upregulation of genes such as those associated with serine synthesis (Ojala et al., 2002). In the current study, BA, but not GH, significantly increased the expression of PHGDH and PSAT1 mRNA in both SS and LD muscles, whereas no effects were observed on liver. There was a trend for plasma serine concentrations to be decreased, but this was also seen in GH treated lambs. As with all metabolomics data based on single "tissue" measurements it is difficult to make an interpretation without information on other tissues which might give an indication where the metabolite is going to.

Studies have shown that over expression of PSAT in cells in culture is associated with growth and cell proliferation (Baek et al., 2003, Vi et al., 2008). In some types of tumour and breast cancers, increased serine pathway flux is induced by PHGDH, which leads to transfer of a large amount of glycolytic carbon into serine and glycine metabolism (Locasale et al., 2011), presumably, because this is a biosynthetic requirement for growth. It might be expected that this increased serine production would not be released into the plasma.

Serine synthesis is associated with mTORC1 activation. The activation of mTORC1, which stimulates cell growth, is presumably required for the increased protein deposition seen in BA induced muscle hypertrophy. Activation of mTORC1 by amino acids such as leucine and glutamine has been described (Nicklin et al., 2009, Cohen and Hall, 2009), but serine has also been described as directly or indirectly contributing to mTORC1 activation (Ye et al., 2012).

Co-ordinate regulation of the gene expression of enzymes in the serine synthesis pathway would be expected to involve a common factor, such as a transcription factor, or specific signalling pathway. The transcription factor, activating transcription factor 4 (ATF4), which is part of transcription factor family cAMP regulated element binding proteins (CREB), which regulate the transcription of genes involved in redox homeostasis and endoplasmic reticulum (ER) stress responses (Adams, 2007). The ER is a membrane-bound and structurally complex set of cellular organelles that is present in all eukaryotic cells. The ER is the major site for internal calcium storage, lipid biosynthesis, and is important in the folding and assembly of proteins which can be secreted (Park and Ozcan, 2013).

The ER is also up-regulated in tumours, suggesting that it has a critical role in tumour growth (Ye et al., 2010), regulating lipid metabolism in the liver (Xiao et al., 2013), and glucose metabolism in mice (Yoshizawa et al., 2009). It has also been described as being involved in mediating insulin's anabolic functions, by regulating amino acid and protein metabolism, as well as having a role in regulating asparagine and serine synthesis (Adams, 2007).

As the serine synthesis pathway utilizes intermediates of glycolysis to synthesise serine, it would be expected that glycolytic intermediates are essential for this process. Therefore it might be expected that, in order to enable (or stimulate) an increased generation of serine, there would be an increase in the glycolytic intermediate, 3-phosphoglycerate, the substrate of this pathway (Daly et al., 2004). In the current study, BA, but not GH significantly increased the expression of PGK1 mRNA in the SS muscle, but there was no effect found on PGK1 mRNA in the LD muscle or in the liver. These differences may be associated with differences in fibre type composition within different muscles. An

increase in 3-phosphoglycerate could also be produced by a decrease in the activity of enzymes further down glycolysis. Recent studies have suggested that changes in pyruvate kinase play a critical role in promoting the increase in serine synthesis, particularly in cancer cells (Ye et al., 2012). As described in chapter 4, BA significantly increased the expression of total PKM, PKM1 and PKM2 in both SS and LD muscles compared to control. The lack of a differential increase in PKM2 suggested that the regulation of muscle PK in BA treated animals was not critical to the potential increase in serine synthesis capacity. No clear change in PKM isoform gene expression suggested this was not the mechanism by which the serine synthesis pathway was stimulated; however changes in the expression of other genes gave the indication that the increase in this pathway was being co-ordinately regulated. Asparagine synthetase (ASNS), which catalyses the glutamine and ATP-dependent conversion of aspartic acid to asparagine was increased, although no change in plasma asparagine was observed. This gene is a well characterised marker of ATF4 mediated increases in gene expression (Adams, 2007). In the present study, BA, but not GH, dramatically increased the mRNA expression of ASNS in both LD and SS muscles, whereas liver was not affected. This observation suggests the increase in the expression of the serine synthesis genes was co-ordinately regulated with other genes responsible for changes in metabolism. Increases in asparagine are associated with increased requirements for this amino acid in protein synthesis, as asparagine's only function is as a structural amino acid (Zhang et al., 2014). Asparagine plays a vital role in glutamine regulation, and a great amount of glutamine is consumed in many tumour cells to preserve TCA cycle anaplerosis and to support the production of nucleotides and nonessential amino acids for cell growth (Zhang et al., 2014). However, in the serine synthesis

pathway, high expression of PHGDH contributes approximately 50% of the total anaplerotic flux of glutamine to alpha-ketoglutarate into the TCA cycle (Possemato et al., 2011). Suppression of both PHGDH and PSAT1 expression caused a significant decrease in serine pathway flux and alpha-ketoglutarate levels (Possemato et al., 2011). From the above results it is clear that both ASNS and PSAT utilise glutamate for NH_3, and this generates alpha-ketoglutarate. This is then an intermediate for the TCA cycle. Therefore these two pathways, serine synthesis or asparagine synthesis, will have effects on glutamate and alpha-ketoglutarate metabolism, which are associated with metabolism of nonessential amino acids.

For serine synthesis to take place, glycolytic intermediates have to be provided. A potential pathway by which this can be achieved is via the utilisation of gluconeogenesis. Phosphoenolpyruvate carboxykinase (PCK) is considered the key regulator in gluconeogenesis. This process has been well characterised in liver and kidney for glucose synthesis, and for glyceride-glycerol generation in white adipose tissue and the small intestine (Hanson and Reshef, 1997). In the current study, BA-treatment significantly increased of PCK2 mRNA expression in LD muscle and there was a trend to be increased in SS muscle, but not liver. There was no effect of BA on PCK1.

PCK2 uses mitochondrial GTP (mtGTP) to convert oxaloacetate (OAA) into mitochondrial phosphoenolpyruvate (PEP), and then this can escape from the mitochondria to the cytosol to enter glycolysis (Stark et al., 2009). Although PCK has long been considered a determinant of gluconeogenesis, it appears to play a major role in the integration of various metabolic pathways of energy metabolism (She et al., 2000). It has been proposed that PCK plays a key role in four important metabolic pathways;

gluconeogenesis, glyceroneogenesis, serine synthesis, and the conversion of the carbon skeletons of amino acids, such as glutamine and glutamate, to PEP (via PCK) and then to pyruvate (via PK) for net oxidation in the TCA cycle as acetyl-CoA (Yang et al., 2009). PCK2 has recently been identified as a gene whose transcription is regulated by ATF4 (Mendez-Lucas et al., 2014). More recently the unfolded protein response (UPR) transcription factor, ATF4, was identified as being necessary for the up-regulation of glycolytic enzymes and LDH activity, suggesting that ATF4 plays a role in shifting metabolism from one based on oxidative phosphorylation to one more reliant on glycolysis, which is seen in cancer and cell proliferation (Lee et al., 2015). Limitation of essential amino acids can increase the expression of ATF4. But the shortage of essential amino acids can also regulate many different steps of gene expression, which begin at DNA and end with protein production, such as changes in chromatin structure, transcription rates, RNA splicing, RNA export and translation (Kilberg et al., 2005). Amino acid limitation can cause the activation of eIF2a kinase on serine 51 through the action of different factors such as the general control non-derepressible 2 (GCN2) kinase. However other factors associated with ER stress can also activate this kinase, such as double-stranded RNA-activated protein kinase (PKR), double-stranded RNA activated protein kinase-like ER kinase (PERK) and heme regulated inhibitor kinase (HRI). Activation of these factors leads to inhibition of global translation but at the same time increased mRNA expression for ATF4 (Kilberg et al., 2009). In the case of response to ER stress, the unfolded protein response (UPR) is carried out to re-organize cellular homeostasis in the ER. Increased expression of proteins that contribute in the ER stress is considered to be the early stages of UPR signalling. Subsequently this process leads to improved protein folding as well as to reduced translation of

general proteins, which consequently leads to reduced load in the ER (Lee and Ozcan, 2014). However, when ER homeostasis is not quite recovered in the long-term, UPR signalling pathways of cell death would start (Lee and Ozcan, 2014). Phosphorylation of PERK and eIF2α mediates the translational control of certain transcription factors, such as ATF4, which activates the transcription of UPR genes, and also increases transcription of genes involved in amino acid metabolism (Wek and Cavener, 2007, Teske et al., 2011).

The greatest response to the growth promoters used in this study was to BA. As described above, the effects of these agents were to change gene expression of energy metabolism and metabolism of non-essential amino acids. The potential changes in amino acid metabolism would be expected to influence nitrogen metabolism, which might be expected to be mediated through changes in the urea cycle and associated enzymes. Examination of the gene expression of enzymes involved in these pathways showed no changes; likewise the associated metabolites in the plasma did not change to any great degree.

7.3 Effects of GPs on C2C12 Myoblasts Response to Genes Associated with Serine Biosynthesis

Serine has a critical role in cell growth and proliferation (Possemato et al., 2011). In fact, proliferating cancer cells are associated with the glycolytic phenotype (Gatenby and Gillies, 2004). During differentiation, C2C12 myoblasts change from a proliferating phenotype to terminally differentiated myotubes, therefore it might be expected that the biosynthetic capacity of cells declines. Indeed, in the study described in this thesis there was a general reduction in gene expression in myotubes compared to myoblasts for enzymes involved in serine biosynthesis. Clearly

from the effect of 6 day treatment of lambs, BA induced serine synthesis pathway expression, which suggests this pathway can be induced from an apparent low level of expression in terminally differentiated cells in mature muscle tissue. It is not clear whether this elevation was an increase within satellite cells or myofibres. Muscle hypertrophy is thought to involve an increased proliferation then fusion of satellite cells with muscle myofibres and therefore could be the source of gene expression changes in the serine synthesis pathway. The insulin-like growth factor (IGF) system has been characterized as essential in the proliferation, differentiation and hypertrophy of primary skeletal muscle cells in human and mouse (Stewart et al., 1993).

In this study, there was no clear, consistent and co-ordinated effect of Des (1-3) IGF-I treatment on the expression of genes associated with the serine synthesis pathway during differentiation. This was not surprising as GH, which would be expected to mediate its effect through IGF-I, did not have an effect on the expression of these genes in the sheep trial. When the expression of the MRFs myogenin and Myf5, were examined, myogenin appeared to be down regulated by Des (1-3) IGF-I early in differentiation, whilst Myf5 was increased during differentiation. This probably reflects a mitogenic effect of Des (1-3) IGF-I which has been previously reported in C2C12 cells (Milasincic et al., 1996), although in their experiment the authors described it as leading to differentiation of the cells. In the current study the myoblasts did also appear to differentiate.

In the differentiating cells exposed to dbcAMP there was, as for Des (1-3) IGF-I, no clear effect on the expression of the serine synthesis pathway genes, however it did appear that at day 7, PSAT was increased, although for the other genes of the serine synthesis pathway a similar effect was not apparent. At this stage

myotubes would be expected to be formed. However as can be seen from the images of the cells at day 7, there was a distinct phenotype in the treated cells, which appeared to indicate that the rate of differentiation had been increased and cells were being lost from the plate at day 7, which makes it difficult to interpret the gene expression data. This was also reflected in the expression of the MRFs.

Des (1-3) IGF-I and dbcAMP treatment of C2C12 cells would be expected to mediate their effects through pathways which would be stimulated by BA and GH *in vivo*. The data from the examination of the effects of these agents on C2C12 cells indicate a lack of clear effects on differentiating C2C12 cells in culture. This may be due to the effects of GH and especially BA being mediated via mechanisms that are not stimulated by dbcAMP, or the effects of BA are not at the level of differentiation but are directed at mature muscle fibres. It appears the metabolic switch toward a faster muscle phenotype is an intrinsic part of the response to BA and may be essential to enable the proposed changes in non-essential amino acid metabolism which were observed in this thesis.

7.4 Conclusion

This study shows for the first time that up-regulation of the serine biosynthetic pathway is associated with muscle hypertrophic growth in BA stimulated lambs. BAs increase muscle's glycolytic potential and this is associated with a capacity to increase the synthesis of serine and presumably other related metabolites required for growth.

BA had a stronger muscle hypertrophic effect than GH over the short time frame of a 6 day treatment in lambs. Treatment with

BA, but not GH, increased mRNA expression of genes involved in glycolysis and the serine synthesis pathway in muscle, but decreased expression of genes in the TCA cycle. Whilst GH had more effects on whole body metabolism, administration of GH to both sheep and pigs, increased liver weights. In pigs, this appeared to be due to an increase in glycogen and possibly associated water content, rather than an increase in cell number or protein content. Whilst in sheep, this was associated with an increase in the whole liver content of glycogen, protein, DNA and lipid contents.

GH, but not BA, treatment increased circulating glucose and other carbohydrates derived from glucose, such as pyruvate. BA stimulated an increase in plasma lactate. Both growth promoting agents significantly decreased certain plasma amino acids, but the effect of BA was bigger than GH.

The *in vitro* study in this thesis showed no significant changes in enzymes associated with the serine synthesis pathway in C2C12 myoblasts treated with Des (1-3) IGF-I or dbcAMP during differentiation.

7.5 Future Work

In order to establish the role of the serine synthesis pathway in the hypertrophic growth of different muscles, further investigations in different animal species are required, incorporating both metabolic pathways and molecular experiments. Moreover, experiments are necessary in order to investigate the effects of BA on genes associated with lipid metabolism that may have direct or indirect impact on serine synthesis in both liver and muscle. This will help improve understanding of the mechanisms behind BA effects on muscle growth. BAs increase muscle's glycolytic potential and this is associated with a capacity to increase the synthesis of serine and

presumably other related metabolites required for growth. Therefore measurements of the non-essential amino acids, especially serine, in muscle will indicate whether increased muscle growth is generally related to an increased capacity of the serine synthesis pathway. However the muscle hypertrophy response to BA is also associated with metabolic changes in muscle fibres in different muscle. This is thought to be mediated by the rate of protein accretion. Therefore it is important to examine the factors involved in the regulation of protein synthesis and degradation in different fibre types, as well as the changes in serine synthesis.

8 Appendices

Appendix 1: Feed composition for lambs (A) and pig (B) growth promoter trials

A

Lamb creep

Diet Composition	**%**
Barley	48.48
Soya High Protein	27
Straw	15
Molassed Meal	5
Int Lamb 21	2.5
Minsal g 109 E50	0.02
Limestone	1
Salt	0.5
Vegetable Oil	0.5
Sum	**100%**

Lamb fattener

Diet Composition	**%**
Barley	55
Oat	35
Extracted Soybean Meal	2.5
Molassed Feed Meal	5
Vitamins+Minerals	2.5
Sum	**100%**

B

Composition of pig diet

Ingredient	**g/kg**
Wheat	600.0
Barley	185.5
Soyabean meal	125.0
Rapeseed meal	35.0
Lysine	4.0
Methionine	0.9
Threonine	0.9
Soya Oil	20.2
Dicalcium phosphate	12.5
Limestone	10.0
Salt	3.5
Premix (vitamins)	2.5

Calculated chemical composition

DE	14MJ/kg
Crude Protein	16.7%
Crude Fibre	3.4%
Ash	5.2%

Appendix 2: Quantitative PCR primers for sheep transcripts

Target	Name and Accession Number	Primer Sequence (5' to 3')	Length (bp)
Cyclophilin	(NM_001308578.1)	**F** CATACAGGTCCTGGCATCTTGTC **R** TGCCATCCAACCACTCAGTCT	23 21
ACTB	Actin, beta (NM_001009784.1)	**F** CTGGCACCACACCTTCTACAAC **R** GTCTCAAACATGATCTGGGTCATC	22 24
RPLP0	Ribosomal protein, large, P0 (XM_004017413.1)	**F** CAACCCTGAAGTGCTTGACAT **R** AGGCAGATGGATCAGCCA	21 18
PHGDH	Phosphoglycerate Dehydrogenase 1 (XM_004002389.1)	**F** TGACTTCGTCACTGTGCACACA **R** CCTTCTTGCACTGGGCAAA	22 19
PSAT1	Pho.serine Aminotransferase 1 (XM_004004296.2)	**F** CAAAGTGCAGGCTGGAAATAACT **R** CCCCGCCGTTGTTCTTAA	23 18
PSPH	Phosphoserine Phosphatase (NC_019481.1)	**F** GGGTGGGCATGTAAACTTTGA **R** GGATCAGAGCTAAGCGCTGTGT	21 22
SHMT1	Serine Hydroxymethyltransferase 1 (NM_001009469.1)	**F** TGAATCTATGCCTTACAAGGTGAATC **R** ATCAGCCTCGGGTGGAAGA	26 19
PGK 1	Phosphoglycerate Kinase 1 (NM_001142516.1)	**F** TGCTTCTGGGAACAAGGTTAAAG **R** CGGTGAGCAGTGCCAAAAG	23 19
ENO3	Enolase3 (XM_012122780.1)	**F** TTACCGACACATTGCAGATCTTG **R** CATGGCCAGCTTGTTTCCA	23 19
TPKM	Total Pyruvate Kinase Muscle (XM_012129071.1)	**F** GGATATGGTGTTTGCGTCTTTCA **R** TTCTTTCCTTTCTCTCCCAGGAT	23 23
PKM1	Pyruvate Kinase Muscle type I (XM_012129070.1)	**F** ACCGCAAGCTGTTTGAAGAA **R** TCCATGAGGTCTGTGGAGTG	20 20
PKM2	Pyruvate Kinase Muscle type II (XM_012129071.1)	**F** GAGGCCTCCTTCAAGTGCT **R** CCAGACTTGGTGAGGACGAT	19 20
ICDH2	Isocitrate Dehydrogenase 2 (AY208679.1)	**F** CCCGTATTATCTGGCAGTTCATC **R** GGGAGCCCCAAGTCAAAATACT	23 22
PCK1	Phosphoenolpyruvate carboxykinase 1 (soluble) (XM_004014441.1)	**F** GTGAGGGAGTTCGTGGAGAGTAG **R** TCGGACCCATCACAGATGTG	23 20
PCK2	Phosphoenolpyruvate carboxykinase2 (mitoch.) (XM_004010899.1)	**F** TGGGAGGGCATCGATCAG **R** GGGCTCCTTGTCACCAGGTT	18 20
ASNS	Asparagine Synthetase (XM_004007747.2)	**F** CTGCCCATGGCCTTGAAT **R** CATATCTGGTGGCAGAGACAAGTAG	18 25
OAT	Ornithineaminotransferase (XM_004023273.1)	**F** CCAACCCATGGCGACATC **R** TCTAGAATCTCATCCTCCTTGATCAC	18 26
ODC1	Ornithine decarboxylase 1 (XM_004005681.1)	**F** ACGTGGGAAGTGGCTGTACTG **R** CTCAGCGCCCATGTCAAAG	21 19
ASS1	Argininosuccinate synthase 1 (XM_004005582.1)	**F** TGGAGGATGCCCGAGTTCTA **R** GCTTCGCGTACTCCATCAGAT	20 21
ASL	Argininosuccinate lyase (XM_004022777.1)	**F** GGATGCGGCAGAATTGCT **R** GATCCACCATGGTTCTGATGAGT	18 23
ARGI	Arginase I, liver, type I (XM_004011323.1)	**F** TTGGCGATTGGTAGCATCTTT **R** GCATCCACCCAAATGACACA	21 20
ARGII	Arginase type II (XM_004010737.1)	**F** CCTTGGTGTGATCTGGGTTGA **R** CCGTGTAGATTTCCAGATGAAGTG	21 24

Appendix 3: The antibodies and their immunoprobing conditions used for western blots. The table shows the specific conditions used for SS, LD muscles and liver. The SDS-PAGE gel % and the protein loads per well are indicated along with antibodies used and development systems for each antibody.

Antibody **SS** muscle	Gel%	Volume of Sample per lane µl	Dilution	2° Antibody	Detection	Source and Catalogue #
PHDH	4-15%	20(5µg protein/µl)	1:10000	Anti-rabbit HRP 1:40,000	ECL	HPA021241, Sigma-Aldrich
β-Enolase	4-15%	20(5µg protein/µl)	1:4000	Anti-rabbit HRP 1:40,000	ECL	Mouse, 611169, BD, UK
PKM	7.5%	20(5µg protein/µl)	1:5000	Anti-rabbit HRP 1:40,000	ECL	Rabbit, 3190, Cell Signalling
PCK2	4-15%	20(5µg protein/µl)	1:10000	Anti-rabbit HRP 1:40,000	ECL	Antibody, 6924, Cell Signalling
ARGII	4-15%	20(5µg protein/µl)	1:10000	Anti-rabbit HRP 1:40,000	ECL	Rabbit, AV54567, Sigma-Aldrich
AKT	4-15%	20(5µg protein/µl)	1:5000	Anti-rabbit HRP 1:40,000	ECL	Rabbit, 4691, Cell Signalling
P-AKT	4-15%	20(5µg protein/µl)	1:5000	Anti-rabbit HRP 1:40,000	ECL	(Ser473), 9271, Cell Signalling

Antibody **LD** muscle	Gel%	Volume of Sample per lane µl	Dilution	2° Antibody	Detection	Source and Catalogue #
PHDH	4-15%	25(4µg protein/µl)	1:10000	Anti-rabbit HRP 1:40,000	ECL	HPA021241, Sigma-Aldrich
β-Enolase	4-15%	25(4µg protein/µl)	1:4000	Anti-rabbit HRP 1:40,000	ECL	Mouse, 611169, BD, UK
PKM	7.5%	25(4µg protein/µl)	1:5000	Anti-rabbit HRP 1:40,000	ECL	Rabbit, 3190, Cell Signalling
PCK2	4-15%	25(4µg protein/µl)	1:10000	Anti-rabbit HRP 1:40,000	ECL	Antibody, 6924, Cell Signalling
ARGII	4-15%	25(4µg protein/µl)	1:10000	Anti-rabbit HRP 1:40,000	ECL	Rabbit, AV54567, Sigma-Aldrich
AKT	4-15%	25(4µg protein/µl)	1:5000	Anti-rabbit HRP 1:40,000	ECL	Rabbit, 4691, Cell Signalling
P-AKT	4-15%	25(4µg protein/µl)	1:5000	Anti-rabbit HRP 1:40,000	ECL	(Ser473), 9271, Cell Signalling

Antibody **Liver**	Gel%	Volume of Sample per lane µl	Dilution	2° Antibody	Detection	Source and Catalogue #
PHDH	4-15%	15(5µg protein/µl)	1:10000	Anti-rabbit HRP 1:40,000	ECL	HPA021241, Sigma-Aldrich
β-Enolase	4-15%	15(5µg protein/µl)	1:4000	Anti-rabbit HRP 1:40,000	ECL	Mouse, 611169, BD, UK
PKM	4-15%	15(5µg protein/µl)	1:5000	Anti-rabbit HRP 1:40,000	ECL	Rabbit, 3190, Cell Signalling
PCK2	4-15%	15(5µg protein/µl)	1:10000	Anti-rabbit HRP 1:40,000	ECL	Antibody, 6924, Cell Signalling
ARGII	4-15%	15(5µg protein/µl)	1:10000	Anti-rabbit HRP 1:40,000	ECL	Rabbit, AV54567, Sigma-Aldrich
AKT	4-15%	15(5µg protein/µl)	1:5000	Anti-rabbit HRP 1:40,000	ECL	Rabbit, 4691, Cell Signalling
P-AKT	4-15%	15(5µg protein/µl)	1:5000	Anti-rabbit HRP 1:40,000	ECL	(Ser473), 9271, Cell Signalling

Appendix 4A: Sheep microarray data for BA vs Control transcriptome comparisons. Values are expressed as fold change relative to control the average of n=3 per group for probes that demonstrated 1.5 fold and significant change (P<0.05) relative to control. The 31 genes ranked with the highest and the lowest fold changes in transcript of BA relative to control.

Fold Change	ID	Symbol	Entrez Gene Name	Location	Type(s)
6.564	206667_s_at	SCAMP1	secretory carrier membrane protein 1	Cytoplasm	transporter
6.372	44783_s_at	HEY1	hairy/enhancer-of-split related with YRPW motif 1	Nucleus	transcription regulator
5.251	209432_s_at	CREB3	cAMP responsive element binding protein 3	Nucleus	transcription regulator
4.412	1569871_at	LOC650392	hypothetical protein LOC650392	unknown	other
4.359	201825_s_at	SCCPDH	saccharopine dehydrogenase (putative)	Cytoplasm	other
4.122	212857_x_at	SUB1	SUB1 homolog (S. cerevisiae)	Nucleus	transcription regulator
4.06	234865_at	LOC100293705	similar to hCG2043257	unknown	other
4.022	205132_at	ACTC1	actin, alpha, cardiac muscle 1	Cytoplasm	enzyme
3.243	232006_at	STK35	serine/threonine kinase 35	Cytoplasm	kinase
2.887	218140_x_at	SRPRB	signal recognition particle receptor, B subunit	Cytoplasm	other
2.828	211587_x_at	CHRNA3	cholinergic receptor, nicotinic, alpha 3	Plasma Membrane	transmembrane receptor
2.802	233159_at	STARD13	StAR-related lipid transfer (START) domain containing 13	Cytoplasm	other
2.682	1556766_at	ERICH1	glutamate-rich 1	unknown	other
2.678	230487_at	C6ORF99	chromosome 6 open reading frame 99	unknown	other
2.67	1555267_at	GRID1	glutamate receptor, ionotropic, delta 1	Plasma Membrane	ion channel
2.615	218459_at	TOR3A	torsin family 3, member A	unknown	enzyme
2.598	220892_s_at	PSAT1	phosphoserine aminotransferase 1	Cytoplasm	enzyme
2.586	1562743_at	ZNF33B	zinc finger protein 33B	Nucleus	transcription regulator
2.562	206910_x_at	CFHR2	complement factor H-related 2	Extracellular Space	other
2.532	228471_at	ANKRD44	ankyrin repeat domain 44	unknown	other
2.498	238802_at	TYSND1	trypsin domain containing 1	Cytoplasm	peptidase
2.452	208790_s_at	PTRF	polymerase I and transcript release factor	Nucleus	transcription regulator
2.435	224665_at	C10ORF104	chromosome 10 open reading frame 104	unknown	other
2.425	208527_x_at	HIST1H2BE	histone cluster 1, H2be	Nucleus	other
2.414	216525_x_at	PMS2L3	postmeiotic segregation increased 2-like 3	unknown	other
2.412	213393_at	MFSD9	major facilitator superfamily domain containing 9	unknown	transporter
2.392	1562411_at	MYLK3	myosin light chain kinase 3	unknown	kinase
2.324	219267_at	GLTP	glycolipid transfer protein	Cytoplasm	transporter
2.313	221908_at	RNFT2	ring finger protein, transmembrane 2	unknown	other
2.292	208563_x_at	POU3F3	POU class 3 homeobox 3	Nucleus	transcription regulator
2.289	229197_at	ING5	inhibitor of growth family, member 5	unknown	other

Fold Change	ID	Symbol	Entrez Gene Name	Location	Type(s)
-9.872	204173_at	MYL6B	myosin, light chain 6B, alkali, smooth muscle and non-muscle	Cytoplasm	other
-5.024	1552777_a_a	RAET1E	retinoic acid early transcript 1E	Plasma Membrane	other
-3.636	203543_s_at	KLF9	Kruppel-like factor 9	Nucleus	transcription regulator
-3.549	213301_x_at	TRIM24	tripartite motif-containing 24	Nucleus	transcription regulator
-3.523	229060_at	YPEL2	yippee-like 2 (Drosophila)	Nucleus	other
-3.123	224357_s_at	MS4A4A	membrane-spanning 4-domains, subfamily A, member 4	unknown	other
-2.962	223179_at	YPEL3	yippee-like 3 (Drosophila)	unknown	other
-2.938	212977_at	CXCR7	chemokine (C-X-C motif) receptor 7	Plasma Membrane	G-protein coupled receptor
-2.722	202001_s_at	NDUFA6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	Cytoplasm	enzyme
-2.716	235852_at	STON2	stonin 2	Cytoplasm	other
-2.67	224838_at	FOXP1	forkhead box P1	Nucleus	transcription regulator
-2.667	241717_at	LOC285281	hypothetical protein LOC285281	unknown	other
-2.63	218510_x_at	FAM134B	family with sequence similarity 134, member B	unknown	other
-2.624	203327_at	IDE	insulin-degrading enzyme	Extracellular Space	peptidase
-2.614	219284_at	HSPBAP1	HSPB (heat shock 27kDa) associated protein 1	unknown	other
-2.613	211074_at	FOLR1	folate receptor 1 (adult)	Plasma Membrane	transporter
-2.525	235916_at	YPEL4	yippee-like 4 (Drosophila)	Nucleus	other
-2.442	202681_at	USP4	ubiquitin specific peptidase 4 (proto-oncogene)	Nucleus	peptidase
-2.438	212254_s_at	DST	dystonin	Plasma Membrane	other
-2.422	218533_s_at	UCKL1	uridine-cytidine kinase 1-like 1	unknown	kinase
-2.421	226465_s_at	SON	SON DNA binding protein	Nucleus	other
-2.418	240602_at	HBS1L	HBS1-like (S. cerevisiae)	Cytoplasm	translation regulator
-2.416	205491_s_at	GJB3	gap junction protein, beta 3, 31kDa	Plasma Membrane	transporter
-2.415	214671_s_at	ABR	active BCR-related gene	unknown	other
-2.41	220653_at	ZIM2	zinc finger, imprinted 2	Nucleus	other
-2.405	227526_at	CDON	Cdon homolog (mouse)	Plasma Membrane	other
-2.389	210950_s_at	FDFT1	farnesyl-diphosphate farnesyltransferase 1	Cytoplasm	enzyme
-2.298	235252_at	KSR1	kinase suppressor of ras 1	Cytoplasm	kinase
-2.288	205330_at	MN1	meningioma (disrupted in balanced translocation) 1	Nucleus	other
-2.262	215726_s_at	CYB5A	cytochrome b5 type A (microsomal)	Cytoplasm	enzyme
-2.259	209355_s_at	PPAP2B	phosphatidic acid phosphatase type 2B	Plasma Membrane	phosphatase

Appendix 4B: Sheep microarray data for GH vs Control transcriptome comparisons. Values are expressed as fold change relative to control the average of n=3 per group for probes that demonstrated 1.5 fold and significant change (P<0.05) relative to control. The 31 genes ranked with the highest and the lowest fold changes in transcript of GH relative to control.

Fold Change	ID	Symbol	Entrez Gene Name	Location	Type(s)
3.614	204204_at	SLC31A2	solute carrier family 31 (copper transporters), member 2	Plasma Membrane	transporter
3.469	218387_s_at	PGLS	6-phosphogluconolactonase	Cytoplasm	enzyme
3.352	201825_s_at	SCCPDH	saccharopine dehydrogenase (putative)	Cytoplasm	other
2.915	230464_at	S1PR5	sphingosine-1-phosphate receptor 5	Plasma Membrane	G-protein coupled receptor
2.761	210691_s_at	CACYBP	calcyclin binding protein	Nucleus	other
2.718	1562411_at	MYLK3	myosin light chain kinase 3	unknown	kinase
2.632	217316_at	OR7A10	olfactory receptor, family 7, subfamily A, member 10	Plasma Membrane	G-protein coupled receptor
2.598	205509_at	CPB1	carboxypeptidase B1 (tissue)	Extracellular Space	peptidase
2.441	208790_s_at	PTRF	polymerase I and transcript release factor	Nucleus	transcription regulator
2.418	1557987_at	LOC641298	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase pseudogene	unknown	other
2.406	229012_at	C9ORF24	chromosome 9 open reading frame 24	unknown	other
2.401	228177_at	CREBBP	CREB binding protein	Nucleus	transcription regulator
2.365	242284_at	LOC199899	hypothetical protein LOC199899	unknown	other
2.306	235720_at	CRIP3	cysteine-rich protein 3	Cytoplasm	other
2.264	216525_x_at	PMS2L3	postmeiotic segregation increased 2-like 3	unknown	other
2.26	236325_at	KIAA1377	KIAA1377	unknown	other
2.251	1568779_a_at	ECM2	extracellular matrix protein 2, female organ and adipocyte specific	Extracellular Space	other
2.217	220137_at	VSIG10	V-set and immunoglobulin domain containing 10	unknown	other
2.208	212216_at	PREPL	prolyl endopeptidase-like	unknown	peptidase
2.192	229016_s_at	TRERF1	transcriptional regulating factor 1	Nucleus	transcription regulator
2.189	219961_s_at	PLK1S1	polo-like kinase 1 substrate 1	unknown	other
2.159	Hs.434123	TBC1D28	TBC1 domain family, member 28	unknown	other
2.138	1553346_a_at	TNRC6A	trinucleotide repeat containing 6A	Nucleus	other
2.108	228081_at	CCNG2	cyclin G2	Nucleus	other
2.087	208918_s_at	NADK	NAD kinase	unknown	kinase
2.084	206027_at	S100A3	S100 calcium binding protein A3	unknown	transporter
2.068	208483_x_at	KRT33A	keratin 33A	Cytoplasm	other
2.066	220017_x_at	CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9	Cytoplasm	enzyme
2.066	202359_s_at	SNX19	sorting nexin 19	Cytoplasm	transporter
2.053	225202_at	RHOBTB3	Rho-related BTB domain containing 3	unknown	enzyme
2.036	236652_at	LOC149703	hypothetical protein LOC149703	unknown	other

Fold Change	ID	Symbol	Entrez Gene Name	Location	Type(s)
-4.649	1552777_a_at	RAET1E	retinoic acid early transcript 1E	Plasma Membrane	other
-3.376	244684_at	PGGT1B	protein geranylgeranyltransferase type I, beta subunit	Cytoplasm	enzyme
-2.9	212924_s_at	LSM4	LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae)	Nucleus	other
-2.749	218533_s_at	UCKL1	uridine-cytidine kinase 1-like 1	unknown	kinase
-2.73	220093_at	ANTXR1	anthrax toxin receptor 1	Plasma Membrane	other
-2.568	202233_s_at	UQCRH	ubiquinol-cytochrome c reductase hinge protein	Cytoplasm	enzyme
-2.518	235252_at	KSR1	kinase suppressor of ras 1	Cytoplasm	kinase
-2.5	202681_at	USP4	ubiquitin specific peptidase 4 (proto-oncogene)	Nucleus	peptidase
-2.417	226465_s_at	SON	SON DNA binding protein	Nucleus	other
-2.341	213702_x_at	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	Cytoplasm	enzyme
-2.327	226813_at	C1ORF57	chromosome 1 open reading frame 57	unknown	other
-2.16	202037_s_at	SFRP1	secreted frizzled-related protein 1	Plasma Membrane	transmembrane receptor
-2.106	1562641_at	FAM122C	family with sequence similarity 122C	unknown	other
-2.105	217871_s_at	MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	Extracellular Space	cytokine
-2.098	202584_at	NFX1	nuclear transcription factor, X-box binding 1	Nucleus	transcription regulator
-2.093	221354_s_at	MCHR1	melanin-concentrating hormone receptor 1	Plasma Membrane	G-protein coupled receptor
-2.073	215726_s_at	CYB5A	cytochrome b5 type A (microsomal)	Cytoplasm	enzyme
-2.059	211074_at	FOLR1	folate receptor 1 (adult)	Plasma Membrane	transporter
-2.035	222143_s_at	MTMR14	myotubularin related protein 14	unknown	phosphatase
-2.029	219792_at	AGMAT	agmatine ureohydrolase (agmatinase)	Cytoplasm	enzyme
-2.018	225890_at	C20ORF72	chromosome 20 open reading frame 72	unknown	other
-1.999	206129_s_at	ARSB	arylsulfatase B	Cytoplasm	enzyme
-1.989	217106_x_at	DIMT1L	DIM1 dimethyladenosine transferase 1-like (S. cerevisiae)	Cytoplasm	enzyme
-1.976	201227_s_at	NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa	Cytoplasm	enzyme
-1.969	1559705_s_at	PHKA2	phosphorylase kinase, alpha 2 (liver)	Cytoplasm	kinase
-1.956	208501_at	GFI1B	growth factor independent 1B transcription repressor	Nucleus	transcription regulator
-1.945	1569032_at	LOC642852	hypothetical LOC642852	unknown	other
-1.932	233043_at	LOC221814	hypothetical protein LOC221814	unknown	other
-1.932	232879_at	CRTC3	CREB regulated transcription coactivator 3	unknown	other
-1.929	219970_at	GIPC2	GIPC PDZ domain containing family, member 2	Cytoplasm	other
-1.92	235916_at	YPEL4	yippee-like 4 (Drosophila)	Nucleus	other

Appendix 5: Mouse primers used in this study for qPCR

Target	Name and Accession Number	Primer Sequence (5' to 3')	Length bp
MYOG	Myogenin (NM_031189.2)	**F** CCCATGGTGCCCAGTGAA **R** GCAGATTGTGGGCGTCTGTA	18 20
MYF5	Myogenic Factor 5 (NM_008656.5)	**F** CAGCCCCACCTCCAACTG **R** GCAGCACATGCATTTGATACATC	18 23
PHGDH	Phosphoglycerate Dehydrogenase 1 (NM_016966.3)	**F**CGTGAACTTGGTGAACGCTAAG **R** GTGGGAGGTGGTGACATTGAG	22 21
PSAT1	Phosphoserine Aminotransferase 1 (NM_001205339.1)	**F** CGTGCTTCAGCATCTACGTCAT **R** GCCCCGCCGTTGTTCT	22 16
PSPH	Phosphoserine Phosphatase (NM_133900.4)	**F** GGCATAAGGGAGCTGGTAAGC **R** GCCACCAGAGATGAGGAACAC	21 21

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