The mechanisms of growth and metabolism and the interactions of growth promoters

By
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Introduction

As population increases, global food security becomes increasingly important. The pressure on the meat industry to increase productivity whilst aiming to minimise global warming results in ever intensifying animal production systems and yet clearly further measures are required. Whilst genetic manipulation and the use of exogenous growth promoters to enhance growth have proved successful in increasing meat/muscle output, meat quality characteristics such as toughness may be unfavourable as a result.

PSE

Pale, soft, exudative (PSE) meat is characterised by pale appearance (figure 1.) and loss of structure resulting in reduced water holding capacity (high drip loss) and contributes substantial losses for the meat industry.

Figure 1. PSE pork (left) and normal pork (right). Image taken from Aps.uoguelph.ca

Loss of product due to detrimental quality must be addressed and this can only come from an understanding of the processes involved in growth, the meat conditioning processes and effect of both genotype and environmental factors (including growth promoters) upon these mechanisms.

Understanding the metabolic mechanisms of growth is of vital importance not only in meat production but in terms of human metabolic and muscle disorders such as obesity, type 2 diabetes, muscular dystrophies, muscle atrophy or wasting in the aging process (sarcopenia) and cardiac disorders.

PSE is associated with a genetic mutation of the ryanodine receptor 1 variant (RyR1), known as the "Halothane Gene" whereby a mutation in a protein that forms part of the calcium channel in the sarcoplasmic reticulum (SR) results in impaired control of calcium release, leading to accelerated and prolonged leak of calcium. This leads to a hyperbolic state causing rapid muscle contraction, an increase in the rate of muscle metabolism and rapid pH decline.

It is supposedly eradicated from the UK pig populace and therefore it may be surmised that occurrence is due to other factors such as nutrition, treatment regimens or other environmental stressors.

AMP-activated protein kinase (AMPK)

AMPK is a molecular stress response pathway or metabolic fuel gauge (Hardie, 2003) activated in response to an intracellular increase of adenosine monophosphate (AMP). The AMPK complex is a heterodimer composed of 3 distinct protein

subunits. When ATP (adenosine tri-phosphate) is consumed by the cell, the level of AMP increases.

The system is activated by the increase in cellular ATP to ADP ratio causing a switch from an anabolic to a catabolic state. AMP binds to the gamma (γ) regulatory subunit, promoting a conformational change rendering AMPK a weaker substrate for dephosphorylation. These changes lead to AMP allosterically activating the complex allowing phosphorylation of the catalytic alpha (α) subunit at the critical threonine 172 residue (figure 2.).

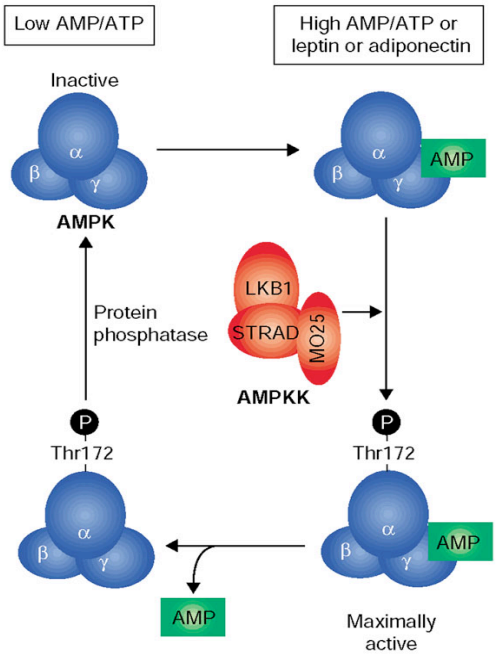

Figure 2. The AMPK complex (*Journal of Biology*, 2003).

Upstream kinases such as AMPK kinase, the tumour suppressor LKB1 and calmodulin-dependent kinase kinase beta (CAMKKβ) further activate the complex by phosphorylation.

During the slaughter process, changing to a catabolic state, AMPK promotes an increase in synthesis and storage of glucose, glycogen, fatty acids, cholesterol and triglyceride in order to preserve and provide energy for the cell.

At the same time, the complex acts to inhibit energy consuming processes such as fatty acid (FA) synthesis, cell hypertrophy and cell proliferation thereby inhibiting growth (figure 3).

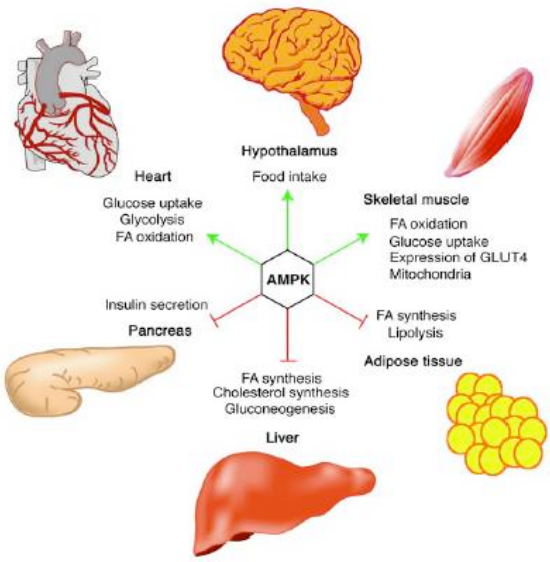

Figure 3. Physiological targets proteins and pathways regulated by the AMPK complex (*Hardie, 2004*).

Growth promoters

Exogenous growth promoters are metabolic modifiers that markedly increase rate of growth by repartitioning nutrients. They improve productivity efficiency by promoting muscle mass and decreasing carcass fat content (Etherton & Smith, 1991). As their name implies, they promote growth but differ in their mode of action and mechanisms.

Beta-adrenergic agonists

β-adrenergic agonists (beta-agonists) improve the efficiency of feed utilization and enhance carcass leanness by inducing skeletal muscle hypertrophy or increase in muscle mass. A physiological response is exerted by the β-agonist binding to a β-adrenergic receptor (β-AR). The normal physiological β-AR agonists are adrenaline and nor-adrenaline. The 3 receptor types (1, 2 and 3) are present on most mammalian cells but the proportion and distribution vary between tissues and even species (Mersmann, 1998). The increase in muscle mass with β-agonistsis attenuated with prolonged administration.

Porcine Growth Hormone

Porcine growth hormone (pGH) administered in maximally effective doses can increase average daily gain (ADG) by as much as 20%, improve feed efficiency by up to 30%, decrease adipose (fat) deposition rates by as much as 80% and increase protein deposition by 50% (Etherton, 2001). GH orchestrates several physiological processes in a variety of tissues (figure 4). The effect of pGH on carcass composition and performance appears to depend on several factors such as dietary levels of

amino acids, dose and gender. Relative improvement however seems to be in inverse relation to the animals' potential for muscle growth (Essen-Gustavsson et al, 1992).

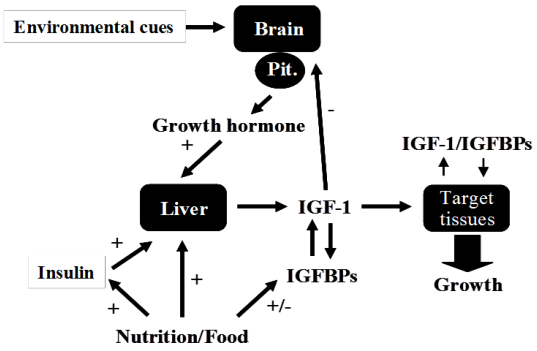

Figure 4. GH-IGF axis. Multiple hormonal and nutritional factors may stimulate (+) the production and/or modify (+/-) the activity of IGF-1. Negative feedback (-) by IGF-1 inhibits growth hormone secretion by the pituitary (Côté et al, 2007).

The purpose of this study was to examine mechanisms involved in the muscle of pigs during a growth trial, particularly those associated with protein deposition and glycolysis and whether the administration of growth promoters in the form of β-agonists (BA) and growth hormone (GH) has a subsequent effect on these mechanisms. Treatments were administered for differing lengths of time to examine any effects in a time-dependent manner. The aims were to determine the glycogen content of muscle and whether growth promoters influence the activity of AMPK. Outcomes were to be examined in relation to pH measurements taken at 45 minutes (pH45) and 24 hours (pH24) post mortem. Pigs were tested to ensure none were of the Halothane genotype.

Hypotheses

The hypotheses are that:

1. Growth will occur with both growth promoters.
2. Growth will occur at later time points (day 14-28) with BA.
3. Expectation is that use of BA will reduce muscle glycogen content and increase AMPK activity.
4. GH will increase liver weight.

Materials and methods

Animals and treatments

63 gilts (White Duroc x (Landrace x Large White)) 5 months of age were sourced from PIC, Cheshire, weighing between 61 and 77 kg. Pigs were randomly assigned to 1 of 3 treatment groups, control receiving no treatment (n=21), β-agonist (n=21) and GH (n=21). Pigs were fed twice a day with diet shown in table 1. Water was provided *ad libitum.*

Table 1. Diet

Composition of pig diet		*Calculated chemical composition*	
Ingredient	**g/kg**	DE (digestible energy) MJ/kg	14
Wheat	600	Crude protein %	16.7
Barley	185.5	Crude fibre %	3.4
Soyabean meal	125	Ash %	5.2
Rapeseed meal	35		
Lysine	4		
Methionine	.9		
Threonine	.9		
Soya Oil	20.2		
Dicalcium phosphate	12.5		
Limestone	10		
Salt	3.5		
Premix	2.5		

Animals assigned to β-agonist group received 20ppm Ractopamine into the feed. Animals receiving GH (Reporcin) were injected intramuscularly (10mg/ml) every 2 days.
Blood and muscle samples were taken from animals at slaughter and stored for analysis.

Determination of Genotype

Genotype was determined by a blood-based test which involved extracting genomic DNA from the pig blood using a commercially available kit. DNA was amplified using the polymerase chain reaction (PCR) which uses an enzyme (in this case a commercial recombinant enzyme called *Pyrococcus furiosus* (PFu)) which copies the DNA strands at a high temperature creating more DNA to work with.

Glycogen Assay

Glycogen was extracted from *Longissimus dorsi* (LD) muscle after breaking down the tissue mechanically. Samples were then prepared with an enzyme called Amyloglucosidase and heated. The enzyme hydrolyses glucose to glucose bonds within glycogen specifically from the non-reducing end of the molecule. The amount of glucose can then be quantified by a colorimetric assay. Another enzyme, glucose oxidase is added which turns the solutions red. The amount of colour produced is

proportionate to the amount of glucose and this then then be quantified and can determine the amount of glycogen per gram of muscle compared to the original muscle weight.

AMPK determination

A protein assay was performed to provide a scale of molecular weights for known proteins. Protein was extracted from each LD muscle sample and separated by migrating through a gel apparatus known as SDS-PAGE at 200 volts (figure 5).

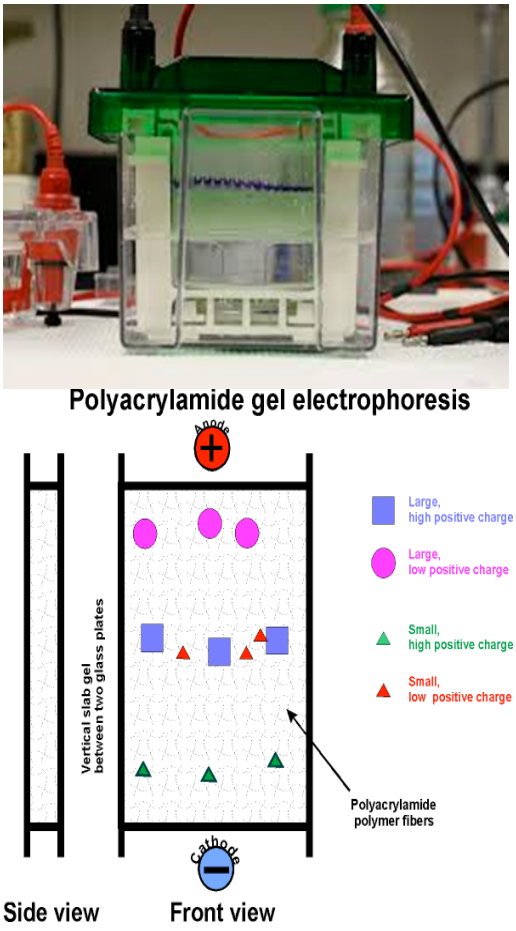

Figure 5. SDS-PAGE apparatus. Proteins can be seen migrating down through the gel.

Once the proteins have migrated, the gels are removed and the proteins are transferred to blotting paper (blots) by stacking gel between many layers soaked in a buffer (figure 6) and the unit was run at 20V for 60 minutes. This is called Western Blotting.

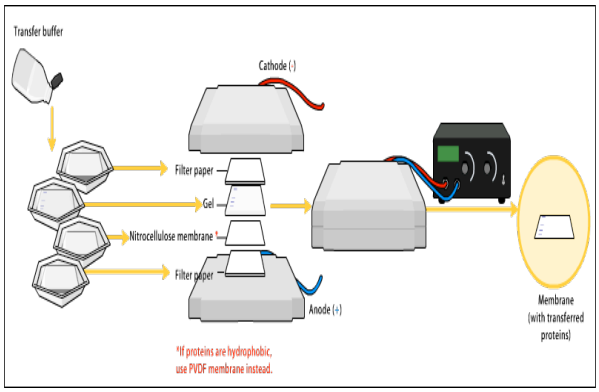

Figure 6. Transferring the gel to a blot.

The blots were then removed and incubated with antibodies for AMPK, which bind to the desired protein. The bands were visualised by placing the blots in a cassette with a sheet of photographic film after incubating blot with a fluorescent solution which binds to the proteins and the image was developed in the same way as a photograph. The position of the bands were then calculated in relation to the proteins of known molecular weights. This determined the molecular weights of the unknown samples.

Results

Verification of Genotype

All pigs were confirmed as halothane negative or *NN* (table 2)

Table 2. Frequency of genotype* (n=63)

	Time (days)				
Genotype	**1**	**3**	**7**	**13**	**27**
nn	0/12	0/12	0/12	0/12	0/15
NN	12/12	12/12	12/12	12/12	15/15
Nn	0/12	0/12	0/12	0/12	0/15

*Genotype verified as being *nn* (282bp), *NN* (150BP) and *Nn* (280,150,132bp)

Glycogen Assay

The assay (figure 6) showed no significant difference (less than 0.05 is deemed significant) in glycogen per unit muscle weight between treatments and control (P=0.389). There was also no effect between glycogen content and length of treatment (P=0.939) and no effect was seen between treatment and time (P=0.867).

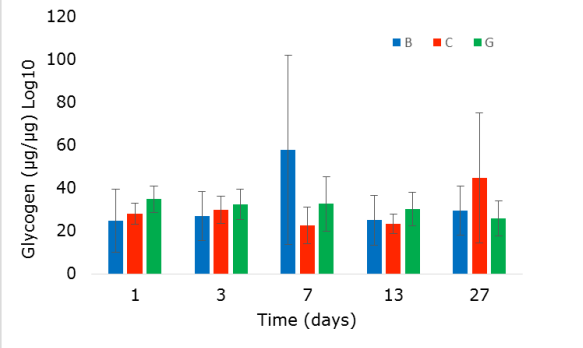

Figure 6. Glycogen content (µg/µl) LD muscle from pigs administered either β-agonists (B), growth hormone (GH) or control (C) for set time-points (days). Days 1-13 (n=12), day 27 (n=15). Data are presented as ±s.e.m. P>0.05.

AMPK

Western blot shows immunodetection with anti-AMPK and anti-phospho AMPK antibodies (figure 7). The phosphorylation of Thr172 in AMPKα at 62 kDa is associated with activation of both the α1 and α2 subunits of AMPK

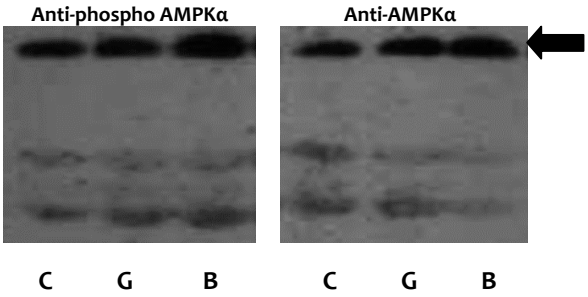

Figure 7. Western blots showing phospho-AMPKα and AMPKα at 62kDa (arrow).

There was no significant difference between AMPK activity and treatment (figure 8) P=0.707, none between AMPK activity and days of treatment (figure 9) P=0.345 and none between AMPK activity, time and treatment P=0.713.

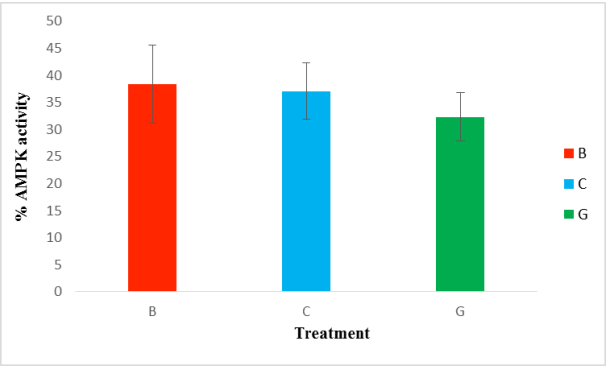

Figure 8. AMPK activity (%) of LD muscle administerd different treatments. Data are presented as ± s.e.m. (n=10) P>0.05.

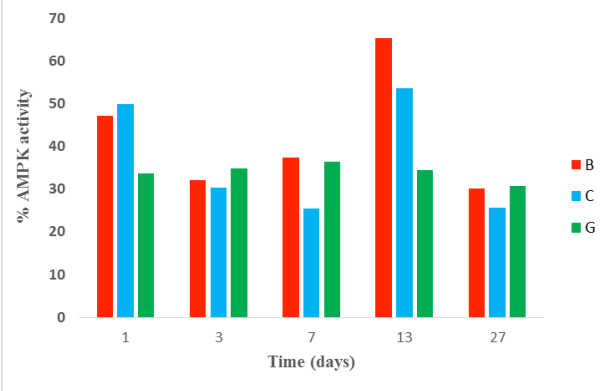

Figure 9. AMPK activity (%) for set time-points (days). Days 1-13 (n=12), day 27 (n=15): P>0.05.

pH

Following slaughter, pH values were recorded at 45 minutes post mortem (pH45) and after 24 hours (pH24). Values were only taken from carcasses treated for 13 and 27 days so data was pooled to give n=4 per treatment. Correlations were performed between AMPK activity and pH45 (figure 10) and pH24 (figure 10).

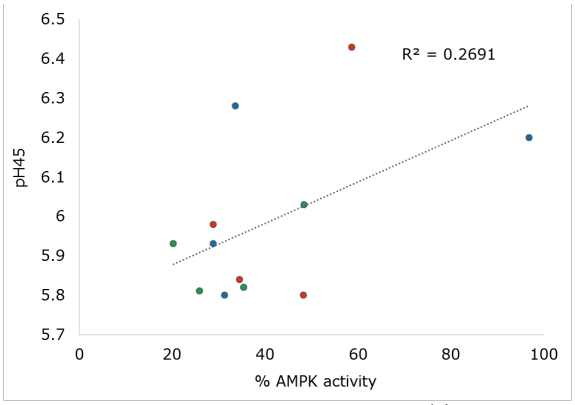

Figure 10. Correlation between AMPK activity (%) and pH at 45 minutes post slaughter (n=12) P>0.05.

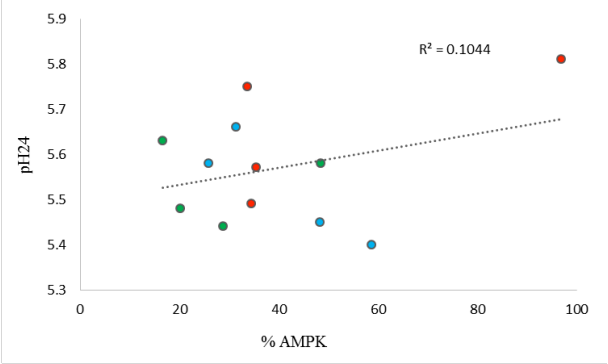

Figure 11. Correlation between AMPK activity (%) and pH at 24 hours post slaughter, n=12 (●B-agonist, ●control, ●GH) P>0.05.

Similarly, pH was measured against muscle glycogen content at 45 minutes (figure 12) and 24

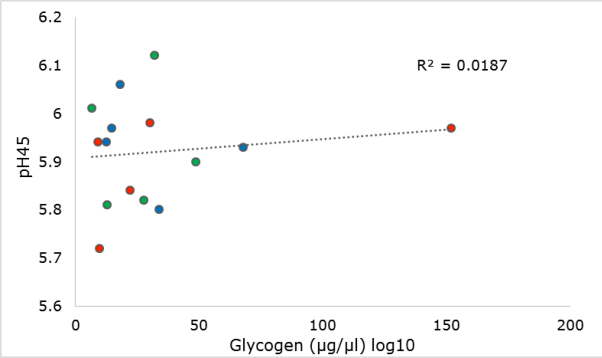

Figure 12. Correlation between glycogen content (µg/mg) and pH at 45 minutes post slaughter, n=12 (●B-agonist, ●control, ●GH) P>0.05.

hours (figure 13) post slaughter and no significant correlation was found between these factors.

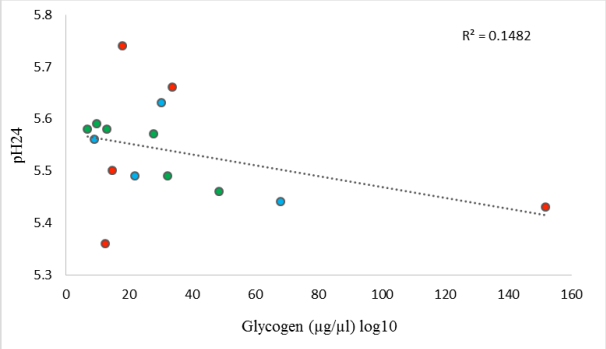

Figure 13. Correlation between glycogen content (µg/mg) and pH at 24 hours post slaughter, n=12 (●B-agonist, ●control, ●GH) P>0.05.

Carcass and muscle weights

As expected, BA significantly increased carcass weight at later time-points (figure 14) compared to control and liver weight increased with GH treatment (figure 15) at all time-points.

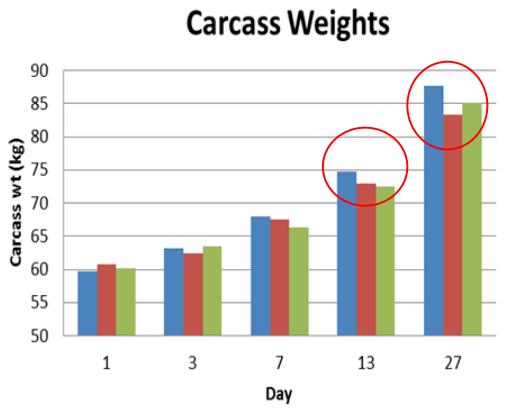

Figure 14. BA increased carcass weight at days 13 and 27, P=0.009 (●B-agonist, ●control, ●GH)

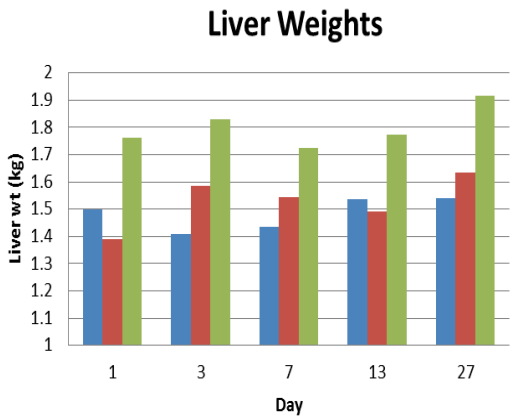

Figure 15. GH increased liver weight at all time-points (P<0.001) (●B-agonist, ●control, ●GH)

In the *Vastus Lateralis* (VLat) muscle, BA increased weight significantly (figure 16) compared to GH and control at all time-points.

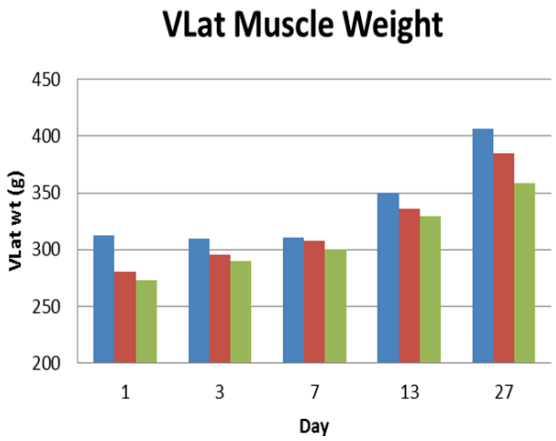

Figure 16. BA increased VLat weight at all time-points, P<0.001 compared to control (●B-agonist, ●control, ●GH).

Discussion

A clearer understanding of the mechanisms involved in growth and metabolism will be of benefit to both the meat industry and for the treatment and prevention of metabolic disorders and aging effects seen in humans.

In this study however, results showed no significant effect of either treatment or time upon glycolysis or AMPK activity compared to the negative control.

Due to time constraints the glycogen assay was only performed once and ideally this could have been repeated to ensure consistent results. Some studies have indicated that the use of growth promoters may induce stress and therefore

increase glycolysis. In this case it appeared unlikely as the glycogen content of the muscle was not significantly different to the control.

Glycolysis

However, other studies have noted an increase in glycolysis in relation to PSE meat and have studied genotypes likely to induce this. In this case all pigs were proved to be Halothane negative and so it is difficult to compare findings. PSE occurs more often in pigs as a species, as post mortem glycolysis is normally faster in the pig and this is further exacerbated if the animal is stressed shortly before or after slaughter (Offer, 1991). Despite extensive research into the mechanisms associated with abnormal glycolysis and PSE mechanisms have not been fully elucidated.

AMPK

As for glycolysis, no significant differences were seen between treatment and control groups and also no effect with duration of treatment. This may however be consistent with studies that have suggested AMPK does not reach maximal activation until 1 hour post mortem. On the other hand, other studies have noted no significant differences in glycogen levels during the post mortem period (Shen & Du, 2006). As some of the AMP is converted to Inositol Mono Phosphate (IMP), it may be that measuring the AMP alone is not viable and that measuring the total AMP+IMP/ATP would be a more reliable indicator of AMPK activation. Unfortunately, it was not possible to test this theory within this investigation.

pH

In regards to meat quality, pH is a vital component. Whilst in this investigation, no significant association was seen between pH and glycogen content or pH and AMPK activity, pH remains as the most confounding factor in the post mortem conditioning process despite extensive research being carried out in this area.

Carcass weight

As growth promoters clearly serve to promote growth, the fact that in this case catabolic mechanisms were not significantly activated between groups can only be seen as a positive aspect. It has been well demonstrated that the responsiveness to ractopamine is attenuated with prolonged administration (Kim et al, 2005). No significant change in carcass weight at the earlier time-points at 1, 3, and 7 days suggests that treatment for longer periods is necessary. Carcass weights increased with b-agonist treatment at days 13 and 27. Evidence suggests that pigs show the greatest response to ractopamine during the first 14 days and the results here support this theory.

Liver weight increased significantly at all time-points with GH administration. This is supported by other trials showing carcass weight may not significantly increase with GH, but liver weight does (Brameld et al, 1996).

Muscle weight was seen to increase significantly with time in the *Vastus lateralis* (Vlat). This is a ham muscle, therefore an increase in weight is beneficial. Evidence suggests that ractopamine use leads to a higher ultimate pH (pHu) in these muscles (Boler et al, 2010) which would alter processing characteristics leading to better protein interaction therefore quality. Unfortunately in this experiment it was not possible to observe this as pH measurements were not recorded from these muscles.

Future work

The effect of growth promoters on nutrient repartitioning are well documented. Whist in terms of meat quality, a reduction in lipid synthesis may be seen as a healthier choice for the consumer, the loss of intramuscular fat will affect the succulent quality of the meat.

In terms of metabolic conditions though, understanding how AMPK interacts with these mechanisms may lead to development of treatments.

References

Boler D.D., Holmer S.F., Duncan D.A., Carr S.N., Ritter M.J., Stites C.R., Petry D.B., Hinson R.B., Alle G.L., McKeith F.K., Killefer J. (2010) Fresh meat and further processing characteristics of ham muscles from finishing pigs fed ractopamine hydrochloride. *Journal of Animal Science*, **89:** 210-220

Brameld J.M., Atkinson J.L., Saunders J.C., Pell J.M., Buttery P.J., Gilmour R.S. (1996) Effects of growth hormone administration and dietary protein intake on insulin-like growth factor I and growth hormone receptor mRNA expression in porcine liver, skeletal muscle and adipose tissue. Journal of Animal Science, **74:** 1832-1841

Essén-Gustavsson B., Karlström K., Lundström K Muscle fibre characteristics and metabolic response at slaughter in pigs of different

halothane genotypes and their relation to meat quality. Meat Science, **31**: 1-11

Etherton T.D. (2001) Porcine growth hormone: A central metabolic hormone involved in the regulation of adipose tissue growth. *Nutrition,* **17**: 789-792

Etherton T.D and Smith S.B. (1999) Somatotrophin and β-Adrenergic agonists: their efficacy and mechanisms of action. *Journal of Animal Science,* **62**: 2-26

Hardie D.G., Scott J.W., Pan D.A., Hudson E.R. (2003) Management of cellular energy by the AMP-activated protein kinase system. *FEBS letters,* **546**: 113-120

Hardie D.G. (2004) The AMP-activated protein kinase pathway- new players upstream and downstream. Journal of Cell Science, **117**: 5479-5487

Mersmann H.J. (1998) Overview of the effects of beta-adrenergic receptor agonists on animal growth including mechanisms of action. *Journal of Animal Science,* **76**: 160-172.

Offer G. (1991) Modelling of the formation of Pale, Soft and Exudative meat: Effects of the chilling regime and rate and extent of glycolysis. *Meat Science,* **30**: 157-184

Shen Q.W and Du M. (2005) Role of AMP-activated protein kinase in the glycolysis of post-mortem muscle. *Journal of the Science of Food and Agriculture,* **85**: 24012406

Author Profile

"Sarah was a mature student who studied at the School of Biosciences graduating in 2013 with a 2:1 degree, BSc. Nutritional Biochemistry. Sarah was particularly interested in muscle biochemistry and had an interest in production animals from a nutritional aspect. Sarah is now been appointed as research co-ordinator in the Animal Feed industry with a company specialising in enzymes and micro ingredients for pigs and poultry"