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Lamb pH and meat quality: studies on biochemical changes in high pH meat associated with pre-slaughter farmyard stress.

A Dissertation
submitted in partial fulfilment
of the requirements for the Degree of
Master of Science in Food Innovation

at
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by
Benjamin Gibbs

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Producing meat of the highest quality is of the utmost importance to the red meat industry. This study looked at how energy metabolic changes and protein profiles in sheep were affected by stress before post-mortem, this gave critical insights into lamb meat quality. A study of biochemical shifts related to high-pH lamb meat with pre-slaughter stress was conducted. This study was conducted using a sample of 20, six-month-old ram lambs of the Coopworth breed (two individuals were of mixed Coopworth/Hampshire breeds) that were grazed on pasture until culling. Two treatments of control and stress were applied. The control treatment was minimal stress applied before slaughter, sheep were brought straight through the yards to the slaughterhouse. In the stress treatment, stressful conditions were applied via the use of heading dogs moving the sheep around in the yards for 10 minutes, at 30-minute intervals for 3 hours before slaughter. This caused an elevated metabolic rate in these individuals pre-slaughter. Animals were harvested immediately and tissue samples were snap frozen at -80 °C. Energy metabolic changes of the two intermediate twitch muscles *Gracilis* (G) and *Semimembranosus* (SM) were studied through the use of a D-Glucose and L-Lactic acid assay. A non-significant difference was seen in the muscles G and SM for their total glycogen levels between the stress and control treatment. In the lactic acid assay, a non-significant difference was seen in the total lactic acid levels between the two muscle fibres between the stress and control treatment. For the protein profile analysis, it was decided to study four muscles, the previous two mentioned, as well as the fast-twitch muscle *Longissimus lumborum* (LL) and the slow-twitch muscle *Supraspinatus* (SS). The protein profiles of these muscles were studied through the use of a Bradford protein determination assay and gel electrophoresis imaging. The Bradford assay revealed that there was no relationship between the total soluble protein concentrations and the stress treatment in all four of the muscle fibres. The gel electrophoresis images when analysed showed a significant difference in the relative frequency of two protein bands at around 100 and 16 kDa in the two muscles SM (P-values 0.05 and 0.01) and LL (P-values 0.03 and 0.05). These results show the potential effect of stress on protein

profiles, however further protein sequencing is needed before further conclusions can be made. An analysis of post-mortem pH values was also conducted from measurements after 90 minutes and 24 hours post-mortem (ultimate pH). A non-significant difference was seen after 90 minutes but, a significant difference was seen after 24 hours between all four muscle fibres and stress. Stressed carcasses had significantly higher pH values. Notably the slow twitch muscle SS exhibited higher ultimate pH values while the fast twitch LL muscle exhibited lower ultimate pH values. This indicated a fibre-type-specific response to the stress treatment in the muscle samples studied. Overall this study provided valuable insights into biochemical shifts occurring in sheep muscles under stress conditions. Further understanding of these processes is vital for the meat industry especially for the correct grading of meat before export/ sale. Future studies should look at sequencing the specific proteins that affect the stress treatment to see if they are related to meat quality parameters such as colouring, tenderness and the water-holding capacity (WHC) of meat.

Keywords: pH, Glycogen, lactate, *Semimembranosus*, *Gracilis*, *Longissimus lumborum*, *Supraspinatus*, *Post-mortem*, Glycolytic, Oxidative, Stress

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Chapter 1 Introduction and Literature Review

1.1 Introduction

The ability to produce meat of the highest quality should be of the utmost importance in the red meat industry. Higher quality meat can be sold for higher prices and in better markets, making the understanding of meat science vital for economies such as New Zealand's where lamb meat export is a multi-billion-dollar industry. Meat quality can be affected by several intrinsic and extrinsic factors including handling conditions, timing, cooling of the carcass after slaughter, different breeds, feeding practices, muscle fibre type, glycogen content and proteolytic activities (Raza et al., 2020). Extrinsic factors that directly affect the quality of meat can be exacerbated when animals are exposed to excessive stress pre-slaughter (Chulayo & Muchenje, 2013). Pre-slaughter stress can come from a variety of activities such as transportation, preparation beforehand on the farm, loading and off-loading of stock and external environmental factors at the abattoir (Adzitey., 2011). Glycogen is a storage molecule of glucose that can provide energy for metabolic processes. In normal post-mortem biochemical metabolism in slaughtered animals biochemical reactions continue but due to blood no longer circulating in the body oxygen and glucose can no longer be effectively delivered to muscles (Terlouw et al., 2021). locally stored glycogen reserves in the muscle are used as an energy source and catabolised in anaerobic glycolysis as a result. Due to the lack of the circulation of blood, the by-products of this reaction (in particular H⁺) accumulate locally in the muscle which results in a decline in the ultimate pH value (Terlouw et al., 2021). Excessive stress can cause fatigue in sheep which can lead to internal muscle damage and results in the depletion of glycogen reserves in muscles. (Nielsen et al., 2021, Chulayo & Muchenje, 2013). This depletion of glycogen levels means less of the by-products of anaerobic glycolysis are produced causing carcasses to fail to drop in pH values and meat having high pH values (Ferguson & Gerrard, 2014). High pH values have been commonly associated with the negative quality of meat and have thus been used as an indication of meat quality (Jacob & Pethick, 2014, Reis et al., 2014). Measuring the pH of carcasses has been a common method of grading red meat after culling at 24 hours (pH₂₄) post-mortem, this value is known as the ultimate pH value (Nache et al., 2016). There is a need in the industry to maintain high-quality meat and be able to grade lamb meat carcasses before the ultimate pH value is achieved after 24 hours. There is a need for other biochemical indicators of meat quality besides pH. This study hopes to further the research into potential biochemical markers that lead to high pH values in lamb meat products.

1.2 Literature Review

1.2.1 pH and Meat Quality.

The ideal ultimate pH (24 hours after post-mortem) for favourable sensory parameters such as tenderness and colouring in lamb meat is around 5.5, meat with a pH higher than 5.9 is deemed undesirable (Hontiveros., 2018, Belhaj et al., 2021). The effect of pre-slaughter stresses affecting the pH and subsequent quality of lamb meat has been proven by many studies. During the natural conversion of muscle to meat, pH will naturally fall from pH 7 to pH 5.4 - 5.7, 24 hours after post-mortem. The effect of stress can disrupt this natural pH drop, ultimately causing higher ultimate pH values of ~6 (Hemsworth et al., 2019). This higher ultimate pH value affects the final quality and grading of meat by leading to negative quality parameters such as darker-coloured meat (Hemsworth et al., 2019). One study by Sutherland et al. (2016) showed how stress factors could be caused by both physiological and psychological factors including stress from transport, feed and water deprivation and handling. This study looked at how the excessive use of heading dogs before slaughter would affect the pH and overall quality of the meat. This study showed that high-intensity handling caused higher pH in lamb and that all stress factors appeared to have an accumulative effect on lamb pH and overall meat quality. It was also found that high-intensity handling appeared to deplete muscle glycogen stores before slaughter resulting in a subsequently higher pH. Higher glycogen reserves in post-mortem lamb meat have been associated with the natural breaking of bonds between muscle proteins which can improve tendinous and other favourable sensory parameters (Martinez-Cerezo et al., 2005). This occurs when there are sufficient glycogen levels in muscles to allow the final pH value to drop into the ideal range of around pH 5.4 - 5.7, which allows muscles to mature into high-quality products. One study by Chulayo & Muchenje (2013) showed that a higher ultimate pH in lamb carcasses post-mortem was associated with tougher less tender meat. Tougher meat leads to negative sensory qualities of meat like chewiness. One study by Khatri & Huff-Lonergan (2023) used a panel of 10 trained sensory panellists to conduct a sensory analysis on 15 steers that were associated with stress-related syndromes including higher pH values. Samples that had higher pH levels (6.4-6.9) were scored significantly different ($p < 0.05$) from intermediate (6.0-6.1) and normal pH ranges (5.5). The trained panellist picked up negative sensory characteristics for not just tenderness but also connective tissue, flavour intensity, colour and juiciness related to their higher pH levels. An increase in pH doesn't just negatively affect the sensory parameters of red meat, it has also been shown to negatively affect its shelf-life which is vital for a successful red meat product (Nache et al., 2016, Hontiveros., 2018).

1.2.2 Muscle Fibres Types

Sheep muscle fibres are divided into three types due to their structure, metabolism pathway, and functionality. These include type I fibres which are oxidative, slow twitch, fatigue-resistant myofibers and type II fibres, which are glycolytic, fast twitch and fatigue-sensitive myofibers (Valentine., 2017). Type II muscle fibres can further be divided into two subclasses; type IIA or intermediate twitch muscles which use a mixed oxidative and glycolytic pathway of metabolism and type IIB, fast twitch muscles, which utilise the anaerobic glycolytic pathway. As stated each muscle fibre type has differing metabolic pathways associated with them which can affect the final quality of meat via altering the degradation of glycogen which subsequently affects their ultimate pH value. Type I fibres are characterised by having less glycogen content and a larger amount of myoglobin and triglycerides. These are what make up the slow twitch oxidative fibres made for low-intensity contractions (Radák, 2018). Type II muscles can withstand higher-intensity workloads due to them having higher levels of stored glycogen content and utilising the anaerobic glycolytic pathway. This allows them to withstand higher workloads and a lower ultimate pH value (Radák, 2018). It has also been shown in the literature that different muscle fibre types can have effects on the sensory parameters of red meat. This was shown by Bao et al. (2021) where different muscle fibre types in Tibetan sheep of differing ages were shown to influence final meat quality and fatty acid composition. These results also matched that of another study by Ithurralde et al. (2018) where it was found that there was a significant difference between muscle fibre type sizes and their Warner–Bratzler shear force which affects the tenderness of red meat. This study also found that differences in fibre size were also correlated with colour changes. It was found that fast-glycolytic muscles, larger fast-glycolytic fibres and intermediate muscles, were associated with higher L* values (whiter meat) while slow-oxidative muscles were associated with lower a* values (which leads to redder meat). This study also found that the redder meats had increased tenderness values making them of higher quality. Another study by Valin et al. (1982) also found that when tested by a trained sensory panel redder lamb meat tasted juicer with stronger more complex flavours than that of whiter meat. Similar results were found in a study by Maltin et al. (1998) where flavour and juiciness were significantly affected by intra-muscular fat that had higher proportions of slow twitch type I muscles. This study also stated that type I muscles are associated with higher levels of mitochondria which play a vital role in flavour formation during the cooking of meat. Due to the limitations of this dissertation including time and limited resources, only two intermediate twitch muscles *Gracilis* (G) and *Semimembranosus* (SM) were studied for their energy metabolic changes. For studying the changes in protein profiles four muscle samples (including the two previously mentioned) and the glycolytic fast twitch muscle *Longissimus lumborum* (LL) and the oxidative slow twitch muscle *Supraspinatus* (SS) will be utilised. These decisions were made due to samples already being collected and previous data being available from both 2021 and 2022 to compare. There was an immediate trade-off seen in the experimental design of this study. For the

study of the metabolic changes, two types of muscles were studied with ten samples of each used. For the change in protein profiles, four different muscles were studied however fewer were used (five). These trade-offs were a balance of having less statistical power (fewer samples) and having fewer muscle fibre types to compare to.

1.2.3 Muscle Physiology and Stress

Type I and II muscles are split into two types according to their function and physiology, each being used differently under certain conditions. Type I or slow twitch oxidative muscles have a low excitation threshold for the large amount of energy that is required for a large number of small contractions (Radák et al., 2018). Type I muscles also tend to have high amounts of slow oxidative mitochondrial cells that are required to power the numerous small contractions, with low levels of stored glycogen. They also tend to be redder because of the high amounts of mitochondria and myoglobin which improves the oxygen delivery to slow-twitch muscles (Cassens & Cooper, 1971). These type I muscles are designed for endurance and sustained work over longer periods of periods, with a more continuous supply of energy from the mitochondrial cells. Type I muscles are classified as oxidative as it is in the mitochondria where oxidative phosphorylation takes place which supplies energy through ATP. Type IIB or fast twitch muscles on the other hand have higher levels of glycogen content and thus higher levels of available glucose (Valentine., 2017). These muscles are designed for fast sporadic movements that are high intensity over a short period. Type IIB muscles are glycolytic meaning they rely on anaerobic glycolysis for the majority of energy metabolism which requires higher glycogen levels. Under stress conditions type IIB muscles tend to have higher lactic acid and lower glucose levels as during energy metabolism glucose is depleted for energy and lactic acid is produced as a by-product (Ferguson & Gerrard, 2014). This often occurs due to a lack of oxygen thus, after death muscles convert to anaerobic metabolism to revert to homeostasis (Ferguson & Gerrard, 2014).

1.2.4 Glycolysis

Another factor that can affect the quality of lamb meat post-mortem is glycolysis and the glycolytic pathway. Glycolysis is one of the most important biochemical pathways in our body and involves the enzymatic breakdown of glucose into pyruvate creating energy sources for the body in the forms of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH). Further anaerobic glycolysis can occur post-mortem which converts glycogen to lactic acid and H^+ anaerobically, this lowers the pH and can have a large effect on final meat quality (Bao et al., 2021). The effect of both insufficient and excessive glycolysis production has been associated with large economic losses in the red meat industry (Ren et al., 2022). One method used to measure anaerobic glycolytic glycolysis is by

measuring the production of glucose (TeSlaa & Teitell, 2014). For this study, the production of glucose sugars will be used to measure the rate of anaerobic glycolysis in post-mortem meat samples.

1.2.5 Protein Profiling

Protein expression profiling is the process of identifying what proteins are expressed in certain kinds of tissues, under certain conditions and at different times. This study will investigate if the protein profiles of different muscle tissues are affected by stress before slaughter. pH can have a large effect on meat quality and the final protein profiles of meat by affecting their interactions between molecules allowing or disrupting bonds. It makes sense that higher pH values in post-mortem muscle tissues can affect protein compositions due to the higher levels of glycolysis in the stress sheep model (Khatri & Huff-Lonergan, 2023). One study by Ren et al, (2022) looked at how different rates of glycolysis and pH affected the cooperative regulation of phosphorylation and acetylation of glycolytic enzymes in lamb meat. This study divided tissues into two groups according to their pH values and glycolysis rates. This study found that there was a significant difference between the protein profiles of muscle tissues with slow and fast glycolysis rates. In Lamb meat samples it was found that slower glycolysis rates and lower pH values were associated with the activity of the enzymes hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK). This study also found that phosphorylation and acetylation had a positive effect on the regulation of HK but a negative effect on the regulation of PFK activity. Another study by Gao et al, (2020) found that there was a strong correlation between the different protein profiles and the colour of muscle tissues in Small-tailed Han Sheep. This is particularly interesting because previous studies concluded that colour is strongly affected by stress and high pH values (Khatri & Huff-Lonergan., 2023). A study by Khatri & Huff-Lonergan (2023) found that the proteins Pyruvate kinase, Adenylate kinase isoenzyme 1, Creatine kinase M-type, and Carbonic anhydrase 3 were all closely related to final meat colour parameters due to their participating in glycolysis and other energy metabolism pathways differently post mortem. This dissertation looked at whether there were significant differences between the protein profiles of the two intermediate twitch muscle fibres SM and G. This study tried to identify possible changes in the protein profiles of proteins that are related to final meat quality. Previous literature has shown that excessive stress has a significant effect on internal muscle damage (Nielsen et al., 2021, Chulayo & Muchenje et al., 2013). This study wants to see if excessive stress had a significant effect on the protein profiles of muscles through mechanical stress causing microtears of muscles. This was done via a Bradford assay which is a quick and sensitive method of measuring the quantity of proteins from a solution. This method is based on the absorbance of the Coomassie Brilliant Blue G-250 dye from 465 to 595 nm after denatured proteins have been bound in the solution (Kielkopf et al., 2020).

1.2.6 Lactic acid

Lactic acid or L-Lactic is one of the key end products and metabolites of anaerobic glycolysis and the production of excessive amounts of it has been correlated to a degradation of meat quality. Lactic acid is considered the most important free variable to determine the pH value of red meat at any given time including post-mortem (Puolanne et al., 2002). Previous studies such as that by Smutok et al. (2013) have used L-Lactic assays to measure the rate of glycolysis in food products. The detection of L-Lactic is important, especially in meat for pH-stabilizing additives and preservatives which can be critical in controlling the growth of pathogenic bacteria found in meat products such as *Listeria monocytogenes* (Mohan et al., 2012). For this dissertation, an L-Lactic assay was conducted to see if there is a significant difference in the total lactic acid levels between the stress and control treatment. This research may lead to the development of biochemical processes that allow lactic acid to be used as a successful biomarker for predicting an increase in pH values in lamb meat.

1.3 Hypothesis/Research Question:

1.3.1 Objective

The main objective of this study was to identify potential biochemical features which could potentially be used as biomarkers of an increase in pH values associated with pre-slaughter stress in lamb meat. These biomarkers could be used as alternatives to the ultimate pH value for determining final meat quality in the industry. The dissertation also aimed to provide evidence that minimising pre-slaughter stress on animals will improve final meat quality.

1.3.2 Hypotheses

This dissertation researched the effect of two biochemical shifts and how they were affected by stress which led to an increased ultimate pH value in stressed animals. This study had three different hypotheses, one for each of the two biochemical features studied and one for the analysis of the pre-existing pH data.

R1 Energy Metabolic Changes

Do high ultimate pH values caused by stress affect energy metabolic changes in the muscle tissues of sheep? Energy metabolic changes are one form of potential biochemical feature that may be affected by the stress model of sheep with higher pH values. This was analysed through the use of the commercially available L-Lactic and D-Glucose assays by Megazyme. This study hypothesised that the

stress treatment will have higher total levels of lactic acid and lower levels of glucose in the ultimate post-mortem carcasses due to the higher levels of anaerobic glycolysis caused during stress.

R2 Protein Profiles

Do high ultimate pH values caused by stress affect the protein profiles in the muscle tissues of sheep? Proteins are expected to be expressed differently under increased pH values due to changes in energy biochemical shifts. This study hypothesised that the protein profiles of proteins related to the final quality of lamb meat will be affected by the shift in pH and stress. This was expected to be seen in the statistical analysis of the gel electrophoresis images, the relative frequency of proteins associated with meat quality will be significantly different between the two treatments. It was hypothesised that a statistically significant difference would be seen in the relative quantity of the molecular weight of protein bands associated with meat quality. It was also hypothesised that mechanical stress caused during excessive exercise and an increased metabolic rate during the stress treatment will cause micro-tears in muscles. These microtears will cause significant differences between the treatments when a Bradford protein determination assay is conducted.

R3 Changes in Ultimate pH

Does the treatment of stress cause significant differences in the ultimate pH values in the muscle tissues of sheep? An analysis of the pH data was conducted at both 90 minutes and 24 hours after sheep were culled. It was hypothesised that there would be a statistically significant difference between the final post-mortem pH values of the stress and control treatments. It was also hypothesized that the ultimate pH value of the control treatment would be lower than that of the stress treatment.

Chapter 2 Materials and Methods

2.1 Raw Sample Preparation

2.1.1 Sample Preparation:

This study utilized data from 20 six-month-old lamb rams that were culled and processed in 2021 (Lincoln University Trial 1). Most sheep were of the breed Coopworth besides but two mixed Coopworth/Hampshire sheep (1032 and 247) with one in each treatment respectively. Two treatments were used to study the effect of stress on meat quality. A control treatment of 'no stress' was applied where animals were put under as minimal stress as possible and were put straight through the yards to slaughter. Under the stress treatment, 'stressful' conditions are caused by heading dogs moving the sheep around in the yards for 10 minutes, at 30-minute intervals for 3 hours before slaughter. Animals were killed directly after exercise. This caused both physiological and psychological stress on these sheep. Sheep were culled and processed straightaway. Muscle tissues were harvested immediately and subsampled (divided into their muscle types, ladled and cut/ diced into individual samples wrapped in tin foil) and snap-frozen after killing at 0 hours at -80°C.

2.2 Experiment One – Energy Metabolic Changes

It is expected that stressed meat will undergo maturation and certain metabolic changes will occur associated with it. It is expected because of these changes in metabolic rate that there will be different levels of both lactic acid and glycogen in stressed meat tissue samples. In Experiment One these metabolic changes will be analysed to see if there is a difference between the stressed and non-stressed models of sheep.

2.2.1 Sample Extraction

Sample extraction methods were based on those done by Choe et al. (2008), Arshard et al. (2021) and the Masters proposal by Bentley, (2021). One shared base extraction solution was used for both the glycogen and the lactic acid assays. Samples were first cut into about 2cm cubes. 1.5 – 2.0 g of muscle was weighed out according to similar methods by Choe et al. (2008) and Arshard et al. (2021). Samples were then diced and put into 15ml falcon tubes. In a fume hood, the addition of a 10-fold 0.5M perchloric acid (PCA) to the sample according to modified methods from Choe et al. (2008). The samples were next homogenised using the ultra-turrax at, 10,000 RPM for around 15 seconds multiple times until satisfactorily. Care was taken to not overheat the samples which could degrade them. Samples produced here were used as the base solution for further metabolic analyses.

For the glucose assay, it is recommended that two solutions be prepared with non-hydrolysed (for the indications of the free non-bound glucose) and hydrolysed for indications of the total glucose content (glycogen broken down and the free glucose in the system). To prepare for the non-hydrolysed sample 500µl base sample and 1150µl reverse osmosis water (RO water) was prepared. The hydrolysed samples need 500µl of the base sample with 50µl 30% KOH and 1000µl of amyloglucosidase (AMG) (3,260 U/mL) (Megazyme, Ireland). This was incubated in a water bath at 37°C for 3 hours. After the incubation was complete the additional 100µl 3M PCA was added to stop hydrolysis. Next both samples were centrifuged at 10,000 g for 15 minutes at 4°C. The supernatants were then extracted from both sample sets after centrifuge, care was taken not to disturb the precipitate.

The pH meter was calibrated via the pH standard solutions of; 4.0, 7.0, and 10.0. The lactic acid assay extraction was prepared using 10ml of base sample and 20ml of RO water and the pH was adjusted to 10.0 with 2M KOH. After the sample pH was adjusted, RO water was added up to the 50ml mark on the beaker. Samples were covered and placed in the fridge for 20 minutes, this precipitates any fat in the sample to form on the top. The samples were then filtered using filter paper and the filter paper and solids were discarded as recommended by the commercial Megazyme L-Lactic Acid (L-Lactate) procedure booklet. The filtered samples were then centrifuged at 3828 g for 15 minutes at 4°C, and supernatants were pipetted and distributed appropriately into named tubes.

2.2.2 Glucose Assay

The glucose assay sample prep was based on methods derived from Choe et al, (2008), and Hammelman et al, (2003). Both studies utilised an assay to measure the total glycogen and lactic acid content of meat tissues from pigs. These methods were further refined in the Master's thesis by Bentley, (2021) on which the methods of this study were based. The assay methodology was based on that given in the commercial Megazyme D-Glucose (HK/G6P-DH) kit instructions. As directed by the microplate assay procedure, the glucose standard curve was made from the D-Glucose kit instructions at standard concentrations of 50, 100, 150, 200, 300 and 400 µg/mL using the 4 D-Glucose standard solution. Samples were recorded in triplicates. The assay was conducted via the microplate assay procedure in the Megazyme D-Glucose (HK/G6P-DH) kit instructions. The original methods for this analysis used RO water however it was deemed that a more accurate standard curve for our sample was achieved with the 0.5 M PCA, so it was used instead. After the final solution was added readings were taken every 5 minutes until 20 minutes had passed at 320nm. The standard curve was plotted using the readings after 20 minutes. Standard curves were formed in the Microsoft Excel software. Using the linear equation of the standard curve the amount of glucose was calculated. Total glycogen

was calculated by subtracting the values of the hydrolysed and the non-hydrolysed samples which gave the total glycogen content.

After completing the original glucose assay it was deemed that further testing of the methods was needed using a glycogen spike assay. Methods for the D-Glucose spike assay were again based on that given in the Megazyme D-Glucose (HK/G6P-DH) kit instructions. Only the samples SM1 and SM2 were used for this assay due to both time and resource constraints. Spike processing was done by adding 100 μ l of standard with D-Glucose spike (0.4 mg/ml) to both the hydrolysed and non-hydrolysed samples. In the non-hydrolysed samples, 500 μ l of 0.5 M PCA, 1150 μ l of RO water and 100 μ l of the D-Glucose spike standard were combined. For the hydrolysed samples 500 μ l of 0.5 M PCA, 1000 μ l of AMG, 50 μ l of 30% KOH and 100 μ l of the D-Glucose spike standard were combined. Both solutions had a total volume of 1750ml and had the same volume of the D-Glucose spike (0.4 mg/ml) thus their concentration was the same (Equation 1). Because the concentration of the D-Glucose spike is known in the solution this allows us to compare the amount of glucose to our original glucose assay to see if there was any loss of glucose during the procedure. Samples were remixed with the homogeniser at 10,000rpm in the same method as before in 15-second intervals until satisfactory. The following glycogen assay procedure was the same, samples were loaded into 96 well plates and read 320nm with a standard and a 0.5 M PCA blank, absorbance was recorded every 5 minutes with the values after 20 minutes used to create the standard curve.

2.2.3 Lactate Assay

The lactate assay sample prep was based on the methods derived from Choe et al, (2008), and Hammelman et al, (2003). These methods were further refined in the Master's thesis by Bentley, (2021) on which the methods of this study were based. The methodology for the assay itself was based on that given in the instructions of the commercial Megazyme L-Lactic Acid (L-Lactate) kit instructions. The lactic acid standard curve was made from the L-Lactic acid solution on 0.02% (w/v) sodium azide, at the standard concentrations of 15, 31.875, 45.75, 82.5, 116.25, and 150 μ g/mL. Samples were recorded in triplicates. The assay was conducted via the microplate assay procedure in the commercial Megazyme L-Lactic Acid (L-Lactate) kit instructions. After the final solution was added readings were taken every 5 minutes until 20 minutes had passed at 340nm. A standard curve was formed via the final 20-minute readings. A Standard curve was formed in the Microsoft Excel software. The concentration of lactic acid was calculated from the linear equation given from the standard curve for each equation.

2.3 Experiment Two - Protein Profiles

It is expected that the protein profile concentrations will be different between the stressed and non-stressed models due to the expected increase in pH value affecting the physiology of the different muscle fibres. Measuring protein profile concentrations can give vital insights into the biochemical processes that are occurring between the two treatments in post-mortem muscles. Changes in total protein profile concentrations can give powerful insights into the biochemical processes that are occurring in meat post-mortem. This study could have used either the BCA or the Bradford assay for determining soluble proteins. Both assays have slightly different methods and are beneficial in different situations. For this study, the Bradford assay (also known as the Coomassie blue method) was used to determine the soluble proteins. This was done as available data to compare to was available from the SCIE 601 course which also conducted a Bradford assay.

2.3.1 Sample Extraction

Sample preparation methods for the base sample extraction were adapted from those used by Ren et al. (2022) and Li et al. (2018). From the muscle tissues stored 1g of tissue sample was finely chopped up using a sharp scalpel and placed into a 5-fold label-free lysine buffer (100 mM Tris Base, 150 mM Glycine, 1% w/v SDS, 0.07%v/v β -mercaptoethanol) this contains a protease inhibitor cocktail (complete™, Mini Protease Inhibitor Cocktail, Sigma Aldrich, ST Louis, MO). Care was taken to only prepare pure samples while trying to avoid fat and other connective tissue. Using a Polytron 2500 (Kinematica, Switzerland) the samples were then homogenised at intervals at 10000 rpm for about 15 seconds until satisfactory. Care was taken to not overheat the samples which would denature and destroy them. After the hominization process, the samples were then vortexed and centrifuged at 11000 rpm for 10 minutes at 4°C. Base sample extracts were stored in the fridge after extraction.

2.3.2 Bradford Assay

Bradford assay methods were based on those explained in the SCIE 601 Laboratory: Protein Determination Assay manual (Lee, 2023). The commercial Bio-Rad Protein Assay (Bio-Rad Laboratories, Life Science Group, USA) was used following its recommended procedures. The only variation from the procedure was that microplates were used, instead of the recommended test tubes. Bovine Serum Albumin (BSA) (0.5 $\mu\text{g}/\mu\text{l}$) from Thermofisher Scientific/Pierce, New Zealand protein standard solution was used for this assay. The linear range of the assay for the BSA is from 0.05 mg/mL to approximately 0.5 mg/ml. 2.5ml of the diluted BioRad Dye reagent was needed for 50 μl sample/standard. Final BSA concentrations made were 0.1, 0.2, 0.3, 0.4, 0 and 0.5 (mg/ml) made using RO water and 0.5 mg/mL BSA. A singular blank value was made using 0.5 M PCA with no protein sample loaded. Measurements

were made in triplicates. Samples were then properly mixed and vortexed. 50 μ l of each of the standards and sample solutions were pipetted into the 96-well microplate. 50 μ l of diluted Bio-Rad Dye Reagent (x1) was added to each of the microplates. The plate was then incubated at room temperature for 5 minutes (absorbance increases over time so care was taken not to incubate for longer). Absorbance was measured at 595 nm using the spectrophotometer which had been properly blanked. Absorbance was then graphed against protein concentration in Excel to get a linear equation. A linear equation was used to determine the amount of soluble proteins present.

2.3.3 Gel Electrophoresis

Due to the differing concentrations of the supernatants used sample volumes were adjusted by adding differing amounts of RO water according to their true sample concentration. 50 μ l of 1x LDS solution was added to each sample. This gave the wanted protein concentration of 1.0 μ g / μ l for each of the samples used. Gels were next loaded with samples according to their. It was decided to use commercially available gels purchased over stain-free, in-house-made gels due to preliminary testing of the stain-free gels being unsuccessful. Four NuPAGE 4-12% Bis-Tri 12 well gels (Biorad, New Zealand) were prepared in an electrophoresis tank with 1x MES SDS Running Buffer (Biorad, New Zealand). 8 μ l of the Precision Plus Protein™ Dual Xtra Prestained Protein Standards by Bio-Rad was added to act as a molecular marker and pipetted into the first well. BSA solution was made according to the SCIE 601 Laboratory: Protein Determination Assay manual (Lee, 2023). LDS was first incubated at 70 °C for 15 minutes. 24 μ l of prepared BSA, 30 μ l of LDS loading buffer and 60 μ l of RO water were combined. 5 μ l of BSA were pipetted into the 12th well as a potential reference point to compare to in the Image lab software (BioRad). BSA was loaded into the last far right lane.

Gels were run for 50 minutes at 200 V in the tanks. Progress was tracked by carefully tracking the protein lanes forming as they travelled down the tank against the molecular marker. Gels were then next carefully removed and placed in a plastic container ready for the rocker in 30ml of the mixing solution methanol-fixing solution (50% methanol, 7% acetic acid solution) for around 15 minutes. The gel was then rinsed with RO water several times to properly remove excess fixing solution and stained in 15 ml of the GelCode blue stain reagent (ThermoFisher, New Zealand) for 1 hour on the rocker. After this, the gel was then rinsed again and placed on the rocker in RO water for one hour. Bands were originally accessed to be too faint so they were rinsed in RO water and put on the rocker overnight. After all of the primary antibodies were identified, the membrane was first airdried and then the membrane was imaged using Molecular Imager Gel doc™ XR+ with the Image Lab Software (BioRad).

Gel images were analysed in the Image Lab software. In this software, each of the four images was first flipped to be orientated the right way, with the protein standard on the left-hand side, then trimmed according to the size protein band length. Individual lane profiles and bands were automatically applied to the gels. Band sensitivity was set to 'low'. Bands once applied were then edited manually, this was done to remove bands caused by external factors that might affect the results such as bubbles etc. The conditions of the statistical analysis were that lanes that had significantly larger relative quantity values to the reference band were removed from the statistical analysis. This is because these bands were considered to be outliers due to them being overloaded which skewed the results. Data from the Image Lab software was then downloaded and copied into Excel. Band data was then analysed by comparing the relative frequency of each lane band to the molecular marker (Precision Plus Protein™ Dual Xtra Prestained Protein Standards by BioRad).

2.4 Experiment Three – Changes in Ultimate pH

Statistical analysis of all the assay data produced was completed in the commercially available software Minitab. Statistical analyses performed were either a 2 sample T-test of mean values (when only 2 variables were being analysed for significance) or an analysis of variance (ANOVA) (when comparing more than two variables for significance), followed by a Tukey post hoc test when applicable. Significance was determined if the p-value ≤ 0.05 . Samples were considered statically significant in the Tukey post hoc test if they were not assigned the same subscript letter.

2.4.1 pH data.

pH data was collected on the muscle tissues of all of the sheep at both 90 minutes and 24 hours after post-mortem. This data was readily available for this study. This data was used to complete a two-sample T-test to compare the differences between each of the treatments (control and stress) of each of the four muscle types at both 90 minutes and after 24 hours. This was done to compare the change in pH until it reaches its ultimate pH value after 24 hours, as is commonly done in the industry (Nache et al., 2016). A one-way ANOVA was used to analyse the samples at both 90 and 24 hours to see if there was a significant difference between the samples.

Chapter 3 Experiments & Analysis of Data

3.1 Experiment One - Energy Metabolic Changes

3.1.1 Introduction

The purpose of the energy metabolic work was to study the biochemical shifts associated with changes in pH triggered by the stress treatment. This was done using the determination assays of the D-Glucose and L-Lactic acid assays by Megazyme. The results of this section will further the knowledge of these processes which could potentially allow them to be used as successful biomarkers of the quality of meat. This section will be used as supporting data for the Master's work of Kelly-Anne Bentley and will be used to direct future work at Lincoln University around sheep stress trails. In particular in refining the methods of the glycogen assay for future studies.

3.1.2 Results

3.1.2.1 Glycogen Assay

Two glucose assays were conducted on the two intermediate muscles of G and SM. Both the total hydrolysed and non-hydrolysed glucose results ($\mu\text{mole/g}$) can be seen in Tables 1 and 2 respectively. Total glycogen ($\mu\text{mole/g}$) levels before and after stress were calculated by subtracting the hydrolysed results from the non-dry hydrolysed results, this can be seen in Table 3. It was found that there was a non-significant difference in the levels of glucose in the hydrolysed samples of both the SM (P-value $0.074 > 0.05$) and G muscles (P-value $0.624 > 0.05$). It was also found that there was a non-significant difference seen between the levels of non-hydrolysed glucose levels of both the SM (P-value $0.136 > 0.05$) and G muscles (P-value $0.73 > 0.05$). Most importantly for this study, there was no significant difference seen in the total glycogen content in both the muscle samples of SM (P-value $0.255 > 0.05$) and G (P-value $0.243 > 0.05$). A trend was seen that the muscle SM decreased in its total glycogen content in the stress treatment dropping from 5.16 to 3.19 mole/g. The opposite trend was seen for muscle G which saw an increase in the total glycogen content in the stressed treatment rising from 2.76 to 3.76 mole/g. This is interesting as both of these muscles are intermediate-type IIA muscles and should have comparable levels of oxidative glycolytic pathways during energy metabolism (Radák., 2018). The increase in total glycogen levels in the G muscle is most likely due to the results of the hydrolysed assay, where a higher glucose content was recorded in the stress treatment than in the control treatment (16.69 vs 15.7 $\mu\text{mole/g}$). This could have affected the final results of the total glycogen. This study found SM muscle showed higher levels of variation in the stress treatment.

Table 1 Total hydrolysed glucose ($\mu\text{mole/g}$) levels of *Semimembranosus* (SM) and *Gracilis* (G) between control and stress treatments

	SM	G	Biological Control
Control (n=10)	19.2 \pm 2.03	15.7 \pm 4.66	
Stress (n=10)	13.5 \pm 7.84	16.69 \pm 4.2	
P-Value	0.074	0.624	0.13

^mean \pm standard deviation, P-values given indicate a significant difference in the total hydrolysed glucose between different muscle tissues (calculated using T-test ($P < 0.05$) in the Minitab software). The biological control column was an analysis conducted on the two muscle control treatments to compare if there was a significant difference in the natural levels of hydrolysed glucose between the two muscles.

Table 2 Total non-hydrolysed glucose ($\mu\text{mole/g}$) levels of *Semimembranosus* (SM) and *Gracilis* (G) between control and stress treatments

	SM	G	Biological Control
Control (n=10)	14.02 \pm 2.03	13.49 \pm 4.91	
Stress (n=10)	10.31 \pm 7.07	12.928 \pm 0.975	
P-Value	0.136	0.73	0.748

^mean \pm standard deviation, P-values given indicate a significant difference in the total non-hydrolysed glucose between different muscle tissues (calculated using T-test ($P < 0.05$) in the Minitab software). The biological control column was an analysis conducted on the two muscle control treatments to compare if there was a significant difference in the natural levels of non-hydrolysed glucose between the two muscles.

Table 3 Total glycogen content ($\mu\text{mole/g}$) levels of *Semimembranosus* (SM) and *Gracilis* (G) between control and stress treatments

	SM	G	Biological Control
Control (n=10)	5.18 \pm 4.34	2.212 \pm 1	
Stress (n=10)	3.19 \pm 3.1	3.76 \pm 03.83	
P-Value	0.255	0.243	0.064

^mean \pm standard deviation, P-values given indicate a significant difference in the total glycogen content between different muscle tissues (calculated using T-test ($P < 0.05$) in the Minitab software). The biological control column was an analysis conducted on the two muscle control treatments to compare if there was a significant difference in the natural levels of total glycogen between the two muscles.

3.1.2.2 Glycogen Spike Assay

After completing the two glucose assays and calculating the total glycogen content (Table 3) it was noticed that the results were lower than that seen in the papers the methods were based on, and other literature (Choe et al., 2008, Hammelman et al., 2003, Li et al., 2018). To investigate if there was a loss of total glycogen during the sample extraction and purification process It was decided to complete D-Glucose testing spike assay. This was conducted from the base homogenised meat sampled. This was done to see if there was a flaw in the original assay methods conducted in this trial that would lead to such low levels of glucose in the final results.

Equation 1 The concentration of the D-Glucose spike added in the assay:

$$\text{D – Glucose spike concentration} = \frac{0.04\text{mg}}{1.750\mu\text{l}} = 22.8\mu\text{g/}$$

Table 4 Comparison of the total glucose levels (ug/mL) between *Semimembranosus* (SM) control samples of the original D-Glucose and spike D-glucose assays

	Muscle Samples	Original D-Glucose Assay	Spike D-Glucose Assay	Blank spike
NH	SM1	2514.08	1383.63	1097.25
	SM2	2906.57	562.97	
H	SM1	3732.76	2245.83	2684.84
	SM2	2729.63	4232.38	

Results from the D-Glucose spike assay can be seen in Table 4. Which compared data from the original glycogen assay of SM1 and SM2 to that of the glucose spike assay results. SM3 was also used in the D-Glucose spike but was removed from the results due to an error in the data most likely sourced due to pipetting of the samples into the 96 well plate.

As shown in Equation 1 above, the total concentration of the glucose spike added to the sample was 22.8 µg/ml. It is expected that an extra 22.8 µg/ml will be seen in the glucose spike assay results when compared to the original glucose assay. However, further loss of total glucose from the glucose spike assay was seen. This loss in the total glucose content could have occurred due to several reasons. One reason for the loss of glucose in the spike glucose is that the base sample solution was too old when the spike assay was completed. As stated after extraction and purification the base samples were

simply stored in the fridge. The base samples for the glucose assay were first made on 27/11/2023 while the spike work was first started on 15/1/24. There is a possibility that the samples could have degraded over this period. One study by Kjellstrand et al, (2004) stated that the most important factor for glucose degradation during storage is time. Another paper by Leitzen et al, (2021) stated that heat (in this paper heat sterilisation) can have a significant effect on the degradation of glucose. The heat was shown to trigger many glucose-degrading products (GDPs) due to oxidation which significantly degraded glucose. GDP's in glucose solution include glyoxal, methylglyoxal, glucosone, 3-deoxyglucosone/3-deoxygalactosone, 3,4-dideoxyglucosone-3-ene, and 5-hydroxymethylfurfural (Leitzen et al., 2021). There is a possibility that the glucose base solution had been left in the fridge for too long and had sufficiently degraded during the time between the original assay and the spike work. More compressive testing of the method is needed to validate the data.

3.1.2.3 Lactic Assay

After the original lactate assay was completed and the absorbance readings were viewed it was decided to redo the assay for some of the samples. It was noticed some of the samples had an unusually high absorbance reading of ~300 AU. This was most likely due to human error during the pipetting stage into the 96 well plates. These included the samples G11, SM6, SM11, SM12, SM13, SM14, SM15, SM16, SM17, SM18, SM19 and SM20. The assay was redone and the data was added to the original assay data for the statistical analysis.

Total Lactic acid levels ($\mu\text{mole/g}$) of the two intermediate samples SM and G were measured in the L-lactic assay (Table 5). A statistical analysis was conducted on the control samples of both muscle types to compare if there is any significant biological variation between the two muscle types. It was found that there was a non-significant difference in the levels of lactic acid (mole/g) between the control and stress treatments of the muscle type SM (P-value $0.271 > 0.05$) and G (P-value $0.107 > 0.05$). Interestingly total lactic acid levels in SM increased while it decreased in the G muscles with stress, however, these are still both considered to be non-significant. There was also a non-significant difference (P-value $0.107 > 0.05$) seen between the control samples of the muscles SM and G lactic acid levels. A non-significant difference between the control samples' lactic acid levels means that natural biological differences or variations in the animals do not explain the change in lactic acid levels. This can be seen as a 'baseline' and that biological variation does not explain the change in lactic acid levels. Notably, the standard variation in both muscle tissues was higher in the stressed treatment than in the control treatment. This is particularly notable in the SM samples where the standard deviation of the stress treatment is 3.09 while the control sample is only 1.14.

Table 5 Total lactic acid ($\mu\text{mole/g}$) between *Semimembranosus* (SM) and *Gracilis* (G) between control and stress treatments

	SM	G	Biological Control
Control (n=10)	4.03 \pm 1.14	4.77 \pm 1.12	
Stress (n=10)	5.24 \pm 3.09	3.8 \pm 1.43	
P-Value	0.271	0.107	0.159

^mean \pm standard deviation, P-values given indicate a significant difference in the levels of lactic acid between different muscle tissues (calculated using T-test ($P < 0.05$) in the Minitab software). The biological control column was an analysis conducted on the two muscle control treatments to compare if there was a significant difference in the natural levels of lactic acid between the two muscles.

3.1.3 Discussion

3.1.3.1 Glycogen Assay

The low levels of total glycogen content seen compared to the literature could be due to several reasons including preparation error. The methods used for sample preparation were based on that of Choe et al. (2008) and Hammelman et al. (2003). The methods used in this study were adapted to fit the conditions and capabilities of the lab used. There were some notable differences in the methods used by Choe et al. (2008). This paper used 10 mL of 9% PCA while this study used 25 ml of 5.03% PCA. After this, their samples were then centrifuged at 15,000g (a higher g level than the 10,000g used in this experiment) at 4 °C. These differences in the method particularly the difference in the PCA concentration could have caused the discrepancies seen between the results. There were also some key differences seen in the Hammelman et al. (2003) methods. This study used substantially less meat for their samples, only using around 0.1–0.3 g compared to around 2g in this study. Frozen samples were also crushed with a mortar and pestle instead of being homogenised in a solution. These discrepancies in methodology could explain the difference in the results seen. Another possible cause of the error could have been during pipetting of the homogenised meat solutions. It was noticed that after the homogenisation process, there were still visible meat solids in the solution. These sizable meat solids could sometimes get stuck in the end of the pipette tips which would obstruct the flow of liquid. This could have led to less of the solution being pipetted into the samples and thus a lower actual glycogen concentration in the solutions than was calculated. If this were true, however, it would be expected in Tables 1 and 2 that the standard deviation values would be much higher if the pipetting error between samples was causing these issues.

It is also possible that other factors in this trial could have also affected the results. These include different diets/ grazing habitats, variations seen in individual physiology, different levels of individual excitement and other underlying stressful conditions (for example pre-existing sickness) that could have acted as pseudo-stress treatment in sheep. It is also possible that hormones in particular testosterone could have played a significant role. Previous literature has shown that rams compared to wethers (rams that have been castrated) have higher feed utilization rates, and have a higher ratio of lean meat on their carcass due to their higher levels of testosterone (Liu et al., 2022). These higher levels of testosterone have negative effects on meat quality parameters such as tenderness.

Another possible cause of the discrepancies seen between the results and the literature is possibly different feeding strategies used. Sheep used in this study were fed on pasture which is the standard practice for grazing sheep in New Zealand. However many other countries use different feed strategies for their livestock including the use of grains like corn and maize. The two papers on the methods of the glycogen assays were based off did not explicitly say what feeding programme was utilised for their livestock (pigs). Hammelman et al. (2003) was conducted in the U.S.A. while Choe et al. (2008) was conducted in South Korea. Thus, it could be possible that these studies used a different feed strategy such as grains over pasture. The use of grain as feed especially in feedlots is very common in places like the U.S.A. Previous studies have shown that different feeding strategies can have large effects on the final glycogen content of meat (Brisky et al., 1959, England et al., 2003). One study conducted in Australia stated that cattle fed on grain tended to have higher levels of glycogen in comparison to that of pasture-fed cattle (Steel et al., 2022). This is due to grain being a more energy-dense source of food. Grains are high in carbohydrates when compared to pasture which has a higher fibre content (Priolo et al., 2001). Priolo et al, (2001) stated that it was this higher concentration of fibre in pasture-fed cattle that led them to have a higher pH due to the fibre affecting the ratio of acetate/propionate. Daly et al, (1998) reported that cattle fed on pasture showed significantly ($P < 0.05$) lower residual glycogen levels ($\mu\text{mole lactate equivalent g}^{-1}$ wet wt) (2.4 ± 2.4) than that feed on grain-based cattle (11.9 ± 8.4). This same study also found that pasture feed cattle also had significantly lower ($P < 0.05$) glycolytic potential ($\mu\text{mole lactate equivalent g}^{-1}$ wet wt) (81 ± 13) than that of based cattle (106 ± 22). One study by Immonen et al, (2000) reported that grain-fed strategies that are characterised by higher energy feed sources were purposely done in the beef industry to stop significant levels of glycogen loss pre-slaughter. A review paper by Priolo et al, (2001) argued that pasture-fed cattle typically have higher ultimate pH values due to them having lower levels of stored glycogen which can become depleted faster pre-slaughter due to stressors such as handling etc. One paper by Apaoblaza et al, (2020) also found that there were significantly higher pH levels of grass-fed cattle compared to grain-fed cattle. However, this paper found no difference in the levels of glycogen or lactic acid between the two fed methods. Most of the previous research done on pasture vs grain feed diets and their effect on the

total glycogen content was conducted with cattle so consideration must be taken when comparing these results to sheep. One study by Belhaj et al, (2021) compared the meat quality of two breeds of sheep (Beni-Guil (BG) and Ouled-Djellal (ODj)) in eastern Morocco. This trial compared the ultimate pH values of three age groups of lambs A1 six months to one year, A2 between one and two years and A3 three and four years at which they were slaughtered. It was found that the ultimate pH values were significantly affected ($p < 0.05$) by the age at which lambs were slaughtered (A1 = 5.82 ± 0.05^a , A2 = 5.77 ± 0.08^{ab} , and A3 = 5.70 ± 0.07^b) for the breed BG but not ODj (A1 = 5.81 ± 0.07^{ab} , A2 = 5.75 ± 0.02^{ab} , and A3 5.72 ± 0.07^{ab}). In this study notably, adult lambs were fed on both pasture and additional barley and grains as they were weaned and matured. A negative correlation was seen between ultimate pH and age supposedly due to the differing levels of stored glycogen between young and adult lambs. The adult lambs were found to absorb more carbohydrates through cereals in comparison to the younger lambs. This high-energy diet was said to promote ruminal fermentation and thus the production of propionic acid which is the precursor of muscle glycogen and thus a higher pH. These results differ from those done on the pH of beef and the effect of grain-fed vs pastured diets. This may be due to other factors between young and adult lambs affecting the results besides feed. There is a current gap in the literature for a study that looks at the effect of pasture and grain-fed diets of sheep of the same age, similar to what has been done in the literature for beef.

It was hypothesized that the stress treatment would have lower levels of total glycogen than the control treatment. This is because it was expected that the stress treatment would have used higher levels of stored glycogen in the glycolytic pathway. This trend was seen in the muscle SM but not the muscle G. This may be due to the anatomy of the muscles themselves. Both G and SM are intermediate twitch fibres (type IIB muscles) meaning they are both slightly oxidative and glycolytic. They have moderate levels of glycogen in comparison to fast-twitch muscle fibres which have higher levels of glycogen.

If this experiment were to be completed again or extended one change that would be made is to the methodology of the glycogen assay. The paper by Schaubroeck et al, (2022) described different methods for the quantification of glucose levels that may be more reliable than that used in this study. This study described an updated glucose protocol coupled with an optimized phenol-sulfuric acid quantification protocol. This method was able to extract significant glycogen for an assay from only 20 mg of sample and only used one centrifuge stage with higher levels of glycogen sensitivity (proportionality constant for glycogen = $0.07279 \text{ A.U./}\mu\text{g}$) assay. There has been a debate in the literature for the optimal method for glycogen extraction for many years (Passonneau & Lauderdale, 1974). As new methods are made and others fall out of favour it is important to stay updated with these changes.

3.1.3.2 Lactic Acid Assay

This study hypothesised that the final lactic acid levels would be higher in the stress treatment than in the control treatment for both the SM and G intermediate muscles. This was not seen in the results. The muscle SM increased in total lactic acid content ($\mu\text{mole/g}$) as hypothesised while G decreased in the stress treatment. According to their assigned P-value (Table 5), all of these results were non-significant so no significant statements can be made about these muscles and their total lactic acid content (mole/g) with stress. Interestingly these results mirror that seen in the glycogen assay in part 3.1.2.1 of this study. Non-significant results were seen in both muscles, SM went with what was predicted in the literature (decreased in total glycogen and increased in total lactic acid levels) while G went against the literature (increased in total glycogen and decreased in total lactic acid levels) (Choe et al., 2008, Hammelman et al., 2003). This indicates that there may be some key differences in how these two muscles respond to stress.

The discrepancies in the results seen may be due to the differences between the two intermediate muscles G and SM physiology and structure. SM is located in the thigh of sheep and is a major leg muscle. SM is located on the posterior of the thigh and is quite broad, giving it a more cylindrical shape compared to G (Alvarenga et al., 2021, Ithurralde et al., 2015). On the other hand, G due to its placement on the inside of the thigh has more of a flat shape and is narrower and more compact than SM (Ithurralde et al., 2018). It is possible that these muscles were worked unequally before slaughter. This could be due to the different locations of the two muscles and their differing physiology exposing them to unequal levels of work. Thus one muscle was potentially subjected to higher levels of glycogen depletion and degradation of the muscle fibres. These differences in physiology and anatomy between the muscles could have led to the difference in lactic acid levels seen. These results could show that G may be of better eating quality because of its lower levels of lactic acid than SM in the stressed treatment due to it potentially being worked less. This could be vitally important for the correct grading of meat samples in the industry. However, the lactic acid results for G were not significant so more testing will need to be done before any conclusions can be made. This study could have benefited highly from more samples and subsamples of each muscle to have a larger population. This larger population would have given this study more explaining power during the statistical analysis.

3.2 Experiment Two - Protein Profiles

3.2.1 Introduction

The purpose of the protein profiles work was to study if the expression of the muscle sample proteins would differ under the stress treatment conditions and higher pH values. Particular interest was taken

into the hypotheses of whether mechanical stress during excessive exercise caused microtears in proteins that could negatively affect meat quality. The results of this section will be used as supporting data for the Master's work of Kelly-Anne Bentley and will be used to direct future work at Lincoln University around sheep stress trails.

3.2.2 Results

3.2.2.1 Bradford Assay

A Bradford assay was completed to compare the total protein concentrations ($\mu\text{g}/\mu\text{L}$) of the four muscle samples. Data was then analysed and the results can be seen in Table 6. The results of the Bradford assay were convincingly non-significant. When a one-way ANOVA was completed comparing all 4 samples to each other a P-value of 0.976 was given which was a highly non-significant result being close to 1. When T-tests were completed comparing the protein concentrations between the stress and control treatment all of the P-values were substantially larger than 0.5. The subscript lettering from the Tukey pairwise comparison method was also all the same meaning a non-significant difference was seen for all of the data.

Table 6 Soluble protein concentration ($\mu\text{g}/\mu\text{L}$) between *Semimembranosus* (SM), *Gracilis* (G), *Longissimus lumborum* (LL) and *Supraspinatus* (SS) between stress and control treatments

	SM	G	LL	SS	Biological Control
Control (N=5)	35.79 \pm 3.17 ^a	32.12 \pm 2.24 ^a	32.38 \pm 7.03 ^a	33.73 \pm 4.96 ^a	33.5 \pm 4.58
Stress (N=5)	29.8 \pm 13.83 ^a	32.25 \pm 4.92 ^a	32.3 \pm 13.83 ^a	32.18 \pm 2.49 ^a	31.64 \pm 9.38
P-value	0.398	0.959	0.996	0.56	0.432

^amean \pm standard deviation, P-values are given and the assigned subscript letters indicate a significant difference in the soluble protein concentration between the muscle tissues (calculated using ANOVA ($p < 0.05$) in the Minitab software).

3.2.2.2 Gel Electrophoresis

Gel electrophoresis images were edited in the Image Lab software. It was originally planned to use a band from the BSA sample as a molecular marker. This is because BSA bands are commonly used as molecular markers and have clear bands with known proteins. However, the BSA samples in the gels created were stretching over their limits when compared to other bands and looked overloaded and over-saturated. It was decided to use a band from the 8 μl of the Precision Plus Protein™ Dual Xtra

Prestained Protein Standard by Bio-Rad for this study instead. For this study, it was decided to choose the band at 37 kDa band. This band was decided because it was mid-range, and was deemed not too saturated but still visible.

During the editing of the gel electrophoresis images, It was decided to remove data from the lanes SM13 and SS15 from further statistical analysis. This was done due to quality discrepancies seen in these lanes most likely caused by human error. This was done as it was deemed that their relative frequency to the reference band values generated from the Image Lab software was too high due to errors caused during the analysis and it would subsequently skew results. This can also be seen visually in Figures 2 and 1 respectively where these two lanes seem to be overloaded. It was found that there were some quality discrepancies seen in some of the gels. It is notable that in the SM gel, two of the stressed samples appeared to have an extra band compared to other lanes in both treatments, which appeared at 75 kDa. This was seen in the samples SM14 and SM15. No statistical analysis was done on these bands as there was no band at the same molecular weight in the control samples.

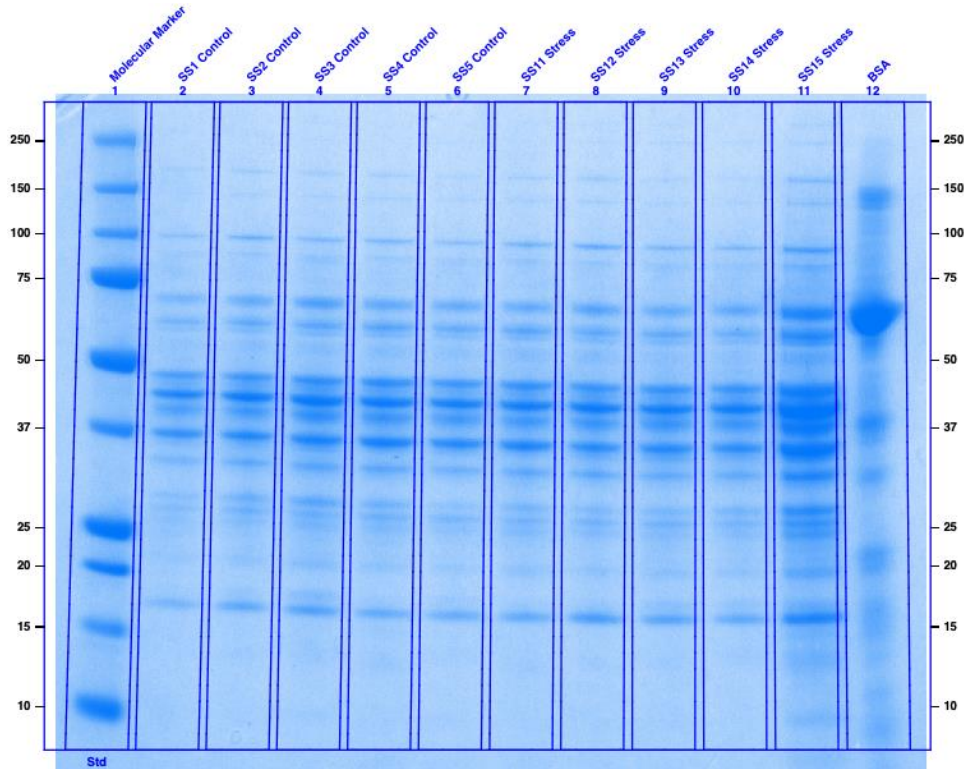
For the statistical analysis of these gels, it was decided to only complete further analyses if the band was deemed significantly visible in each of the gel images (Figures 1, 2, 3 and 4). Bands that were too saturated or too faded were removed from the analysis. This was done as the bands that were not visible in the gel images were deemed irrelevant for this study. This meant that each muscle had a statistical analysis done on different bands. For both the muscles G and SS the bands deemed worth doing a statistical analysis on had molecular weights of around 100, 70, 60, 45, 42, 40, 35 and 16 kDa. The muscle LL had statistics conducted on the bands that had a rough molecular weight of 100, 45, 42, 40, 36, 33, 28 and 16 kDa. While the muscle SM had statistics done on the bands that had a rough molecular weight of 100, 60, 45, 42, 36, 33 and 16 kDa. All four muscle types had statistics done on four bands to compare the differences between the control and stress treatments. The four bands compared had the average molecular weight of 100, 45, 42 four and 16 kDa. These bands were chosen for statistical analysis as they were the only bands that were shared between all of the four muscles and were still significantly visible in the gel images. These four bands could be compared between the control samples of each muscle to compare for biological variation between the muscle types (Table 7).

Biological variation was tested via a one-way ANOVA of the four muscle sample control samples to compare to each other (Table 7). There was found to be a significant result for three of the four bands. It was found that there was a significant difference in the relative frequency between the band that had a rough molecular weight of around 100 kDa to the reference band. This was shown by the assigned subscript letters through the Tukey analysis (P-Value $0.00 < 0.05$). The muscle samples SM

(intermediate) and LL (fast twitch muscle) were deemed significantly different from the muscle samples G (intermediate) and SS (slow twitch muscle) for the control only. For the band that had a rough molecular weight of around 45 kDa, it was found that there was a significant difference in the relative frequency to the reference band in the muscle samples G, LL and SS. It was also found that the muscle sample SM was significantly different to G but not LL and SS muscle samples. This was shown by the assigned subscript letters through the Tukey analysis ($P\text{-Value } 0.00 < 0.05$). A significant difference was also seen in the band that had a rough molecular weight of around 42 kDa between the relative frequency to the reference band in the muscle samples slow twitch muscle SS and fast twitch muscle LL. The intermediate muscle SM was significantly different to LL (fast twitch muscle) but not G (intermediate muscle) and SS (slow twitch muscle). The intermediate muscle G was significantly different to SS (slow twitch muscle) but not SM (intermediate muscle) and LL (fast twitch muscle). This was shown by the assigned subscript letters through the Tukey analysis ($P\text{-Value } 0.001 < 0.05$). These results were interesting as it was able to split the different muscles into their distinct fibre types just by this one protein band. A non-significant difference was seen in the four muscle samples with the relative frequency of the protein that had the rough molecular weight of 16 kDa to the reference band.

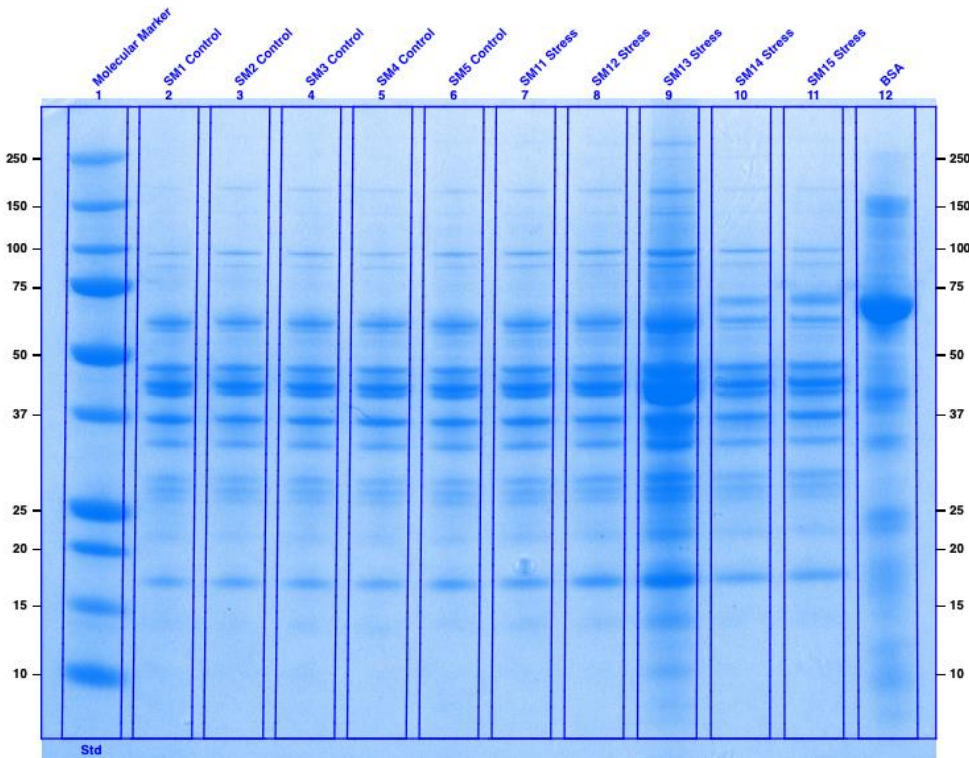
As stated statistical analysis was also conducted to compare the relative frequency of the stress and control of the four muscles' bands to the reference band. This was conducted by a series of T-test analyses for each band. For the muscle sample G, a non-significant difference was seen between the stress and control treatments for all of the eight bands tested (Table 8). A non-significant difference was also seen in the SS muscle treatments for all of the bands tested (however, the 100 kDa band was close to being significant ($P\text{-value } 0.06 > 0.05$) (Table 9)). Interestingly for the muscle samples SM and LL, there was a significant difference seen in both the 100 kDa ($P\text{-value } 0.05 \leq 0.05$) ($P\text{-value } 0.03 \leq 0.05$) and the 16 kDa bands ($P\text{-value } 0.01 \leq 0.05$) ($P\text{-value } 0.03 \leq 0.05$) in both muscle samples respectively (Table 10 and Table 11 respectively). These results are interesting as they show us that there is no biological variation seen in the relative quantity of the 16 kDa band between any of the muscle types but, there was a significant difference seen in the relative frequency of the 16 kDa band when comparing the stress and control treatments of the muscles SM and LL.

Figure 1 Gel electrophoresis image of *Supraspinatus* (SS)



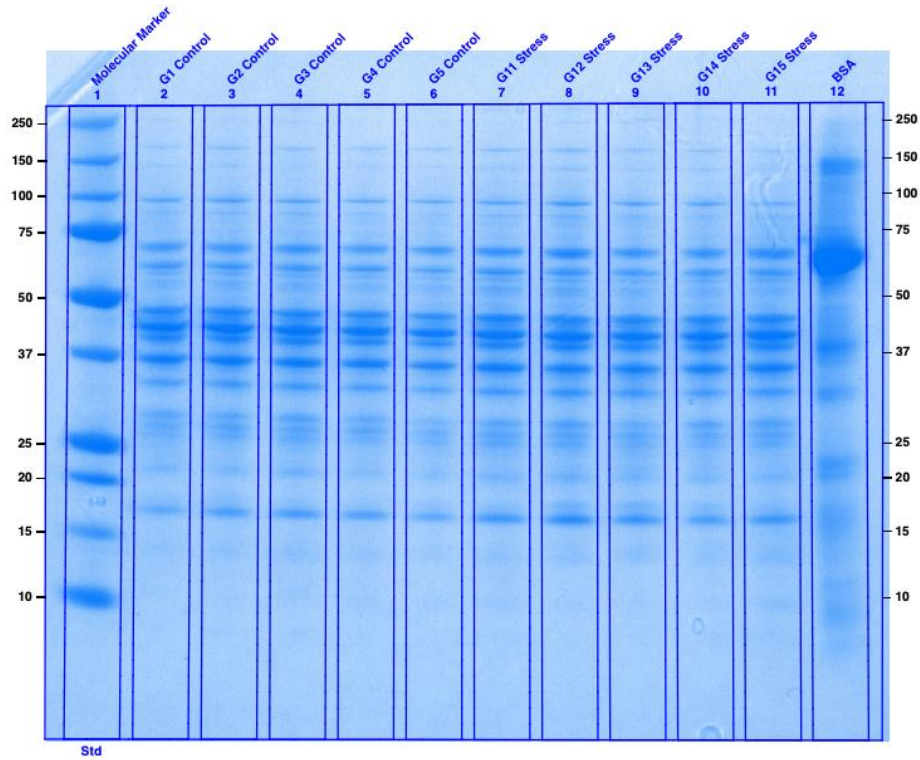
Molecular Marker is the Bio-Rad Precision Plus Protein™ Reference Band and the BSA is the Bovine Serum Albumin standard recommendations from Thermofisher Scientific/Pierce. Note the cover-saturated BSA lane and the overloaded SS15 lane. Analysed in the Image lab software.

Figure 2 Gel electrophoresis image of *Semimembranosus* (SM).



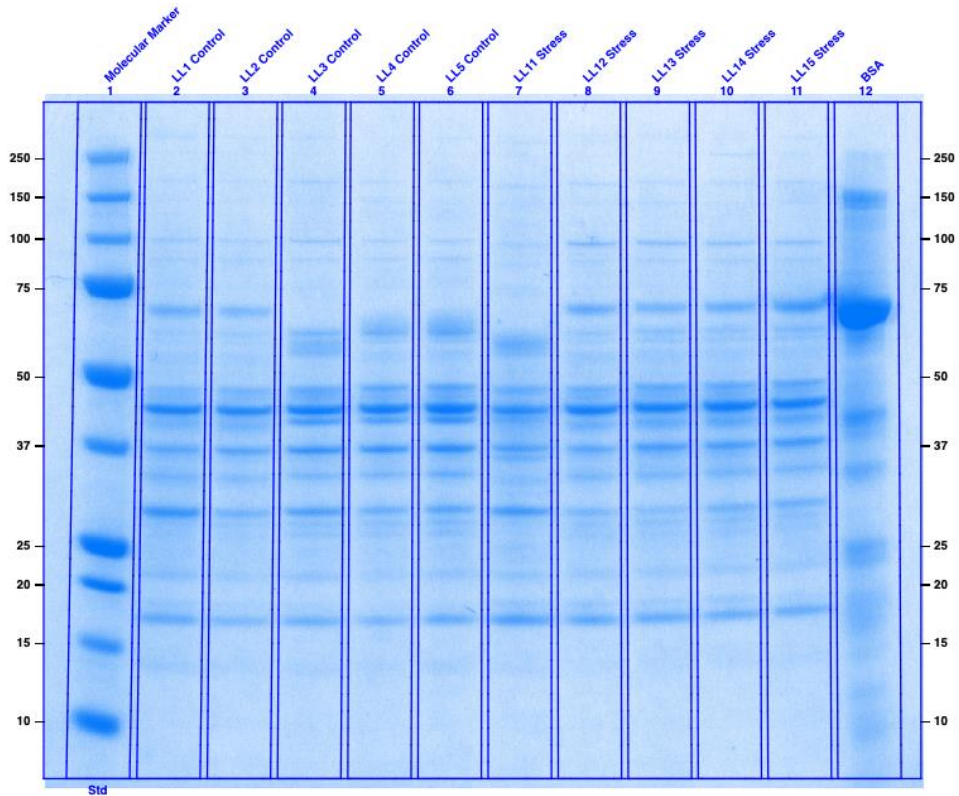
Molecular Marker is the Bio-Rad Precision Plus Protein™ Reference Band and the BSA is the Bovine Serum Albumin standard recommendations from Thermofisher Scientific/Pierce. Note the over-saturated BSA lane and the overloaded SM13 lane. Analysed in the Image lab software.

Figure 3 Gel electrophoresis image of *Gracilis* (G).



Molecular Marker is the Bio-Rad Precision Plus Protein™ Reference Band and the BSA is the Bovine Serum Albumin standard recommendations from ThermoFisher Scientific/Pierce. Note the over-saturated BSA lane. Analysed in the Image lab software.

Figure 4 Gel electrophoresis image of *Longissimus lumborum* (LL).



Molecular Marker is the Bio-Rad Precision Plus Protein™ Reference Band and the BSA is the Bovine Serum Albumin standard recommendations from ThermoFisher Scientific/Pierce. Note the over-saturated BSA lane. Analysed in the Image lab software.

Table 7 Ratio of the protein bands to the reference band in the control muscle samples of *Semimembranosus* (SM), *Gracilis* (G), *Longissimus lumborum* (LL) and *Supraspinatus* (SS)

45 kDa (n=5)	0.45±0.03^{bc}	0.62±0.05^a	0.37±0.1^c	0.69±0.12^b
42 kDa (n=5)	1.08±0.01 ^{ab}	1±0.08 ^{bc}	0.093±0.05 ^c	1.16±0.11 ^a
16 kDa (n=5)	0.4±0.06 ^a	0.48±0.06 ^a	0.43±0.09 ^a	0.4±0.11 ^a

^mean ± standard deviation, the assigned subscript letters indicate a significant difference in the biological variation seen in the control muscle tissues (calculated using ANOVA ($p < 0.05$) in the Minitab software). A Tukey pair-wise comparison analysis was completed on each of the band lengths.

Table 8 Ratio of the protein bands to the reference band in the muscle *Gracilis* (G)

Band length (kDa)	100	70	60	45	42	40	35	16
Control (n=5)	0.25±0.03	0.47±0.05	0.32±0.05	0.62±0.05	1.0±0.08	0.59±0.06	0.96±0.08	0.48±0.06
Stress (n=5)	0.24±0.05	0.43±0.23	0.35±0.13	0.59±0.07	0.92±0.1	0.49±0.12	0.87±0.18	0.59±0.1
P-value	0.8	0.77	0.67	0.45	0.19	0.17	0.316	0.1

^mean ± standard deviation, P-value indicates a significant difference in the Relative Frequency of the *Gracilis* (G) muscle samples Protein Bands to the 37 kDa Bio-Rad Precision Plus Protein™ Reference Band (calculated using T-test (P -value < 0.05) in the Minitab software).

Table 9 Ratio of the protein bands to the reference band in the muscle *Supraspinatus* (SS)

Band length (kDa)	100	70	60	45	42	40	35	16
Control (n=5)	0.16±0.04	0.42±0.09	0.09±0.04	0.69±0.12	1.16±0.11	0.80±0.09	1.14±0.15	0.45±0.11
Stress (n=5)	0.21±0.03	0.55±0.07	0.42±0.18	0.8±0.12	1.27±0.06	0.89±0.09	1.19±0.14	0.53±0.1
P-value	0.06	0.1	0.5	0.2	0.12	0.34	0.63	0.31

^mean ± standard deviation, P-value indicates a significant difference in the Relative Frequency of the *Supraspinatus* (SS) muscle samples Protein Bands to the 37 kDa Bio-Rad Precision Plus Protein™ Reference Band (calculated using T-test (P -value < 0.05) in the Minitab software).

Table 10 Ratio of the protein bands to the reference band in the muscle *Semimembranosus* (SM)

Band length (kDa)	100	60	45	42	36	32	16
Control (n=5)	1.13±0.04	0.70±0.08	0.45±0.03	1.08±0.01	0.67±0.02	0.30±0.01	0.4±0.06
Stress (n=5)	0.22±0.06	0.45±0.42	0.55±0.08	1.09±0.10	0.67±0.07	0.67±0.34	0.60±0.08
P-value	0.05	0.3	0.1	0.8	1	0.12	0.01

^mean ± standard deviation, P-value indicates a significant difference in the Relative Frequency of the *Semimembranosus* (SM) muscle samples Protein Bands to the 37 kDa Bio-Rad Precision Plus Protein™ Reference Band (calculated using T-test (P-value < 0.05) in the Minitab software).

Table 11 Ratio of the protein bands to the reference band in the muscle *Longissimus lumborum* (LL)

Band length (kDa)	100	70	60	45	42	40	35	16
Control (n=5)	0.07±0.02	0.37±0.10	0.93±0.05	0.31±0.09	0.55±0.10	0.24±0.02	0.46±0.19	0.43±0.09
Stress (n=5)	0.15±0.05	0.32±0.03	0.97±0.09	0.32±0.10	0.48±0.12	0.23±0.07	0.21	0.56±0.08
P-value	0.03	0.32	0.51	0.85	0.39	0.72	0.37	0.05

^mean ± standard deviation, P-value indicates a significant difference in the Relative Frequency of the *Longissimus lumborum* (LL) muscle samples Protein Bands to the 37 kDa Bio-Rad Precision Plus Protein™ Reference Band (calculated using T-test (P-value < 0.05) in the Minitab software).

3.2.3 Discussion

3.2.3.1 Bradford Assay

This study hypothesized that there would be a statistically significant difference between the control and stress treatments' total protein concentrations ($\mu\text{g}/\mu\text{L}$) due to mechanical stress and microtears caused during the stress treatment. This hypothesis was not supported in the results. There were no statistically significant differences seen in any of the four muscles studied in this dissertation and thus it would be fair to conclude that mechanical stress caused by stress did not significantly affect the soluble protein concentrations. One observation made in this study however is that the LL stress and SM stress treatments had particularly high standard deviations when compared to the other samples (13.82 and 13.83 respectively n=5 (Table 6)). This meant in these two stress treatments there was a lot of variation seen in the results of the four muscle samples tested. It was also noted that when the

mean values of the four muscle protein concentrations from the stress treatment were compared it also had a high standard deviation of 9.38 (Table 6). This means the protein profiles may vary widely from individual to individual in the stress treatment in the LL and SM muscles. This means that there was a large amount of variation in the protein concentrations caused by the stress treatment. This could be due to several reasons including having a small sample size of just five replicates for each muscle, which could have been influenced greater by outlier numbers.

It is also possible that the sheep showed varying levels of response to the stress treatment, simply put some were more affected by stress than others. This could have been caused by several things such as biological and genetic variation seen between individual sheep. It has been shown that sheep can display high levels of variation to stress. One study by Collier et al., (2008) found that sheep show high levels of variations when responding to the stress treatment of heat. One study by Kadim et al. (2008) showed how heat stress in sheep and goats affected their meat quality. Sheep killed in ambient temperatures (over 35 °C) had higher pH values and lower colour than of sheep killed in cooler temperatures (20 °C). Interestingly though this study found that the heat stress affected meat samples' lipid and protein oxidation. Protein oxidation can affect proteins' secondary and tertiary structures which can thus affect protein concentrations ($\mu\text{g}/\mu\text{L}$). Previous studies have also shown how temperature can have a significant effect on protein concentrations (Beliciu & Moraru, 2011).

3.2.3.2 Gel Electrophoresis

It was noticed in the gel electrophoresis images that there were some obvious quality discrepancies. The two lanes SS15 and SM13 were removed from the statistical analysis for having high relative frequency to the reference band values in comparison to the other lanes. This can be seen visually in Figures 1 and 2 respectively which resulted in bands that appear to be overloaded. These samples would have been useless in the analysis because they were overloaded and did not accurately represent the true band frequencies. It is assumed that this occurred due to human error during pipetting, potentially too much sample solution was pipetted into the wells. It was also notable that in the gel on the LL samples (Figure 4) there was a large quality discrepancy with a large amount of variation seen between some of the sample bands' molecular weight between 50-75 kDa. Samples LL1, 2, 12, 13, 14 and 15 can be seen to have a consistent bright band at around 70 kDa. While the samples LL3, 4, 5 and 11 all have bright bands that seem to match in brightness as those previously mentioned at 70 kDa, however, their molecular weight varies and all appeared to have shifted down the gel in a random fashion. This discrepancy could be due to several reasons. Gels are based on soluble solutions, because of this not all soluble proteins can be transferred to the soluble solution and may be lost (Kurien & Scofield, 2012). Proteins can be lost during the shift from the physical sample to the soluble solution. Protein solubility is an important factor for successful gel electrophoresis results, proteins

need to be solubilized before they go through the separation procedure. There are methods around this including methods where you would choose to solubilise only a select subset of proteins. This can be done to reduce the sampling complexity and background noise of other proteins. These methods are based on a study by Molloy et al. (1998) for the selective solubilization of different protein pools. The quality discrepancies seen may also be because proteins can aggregate together which can affect their solubility (Vihinen, 2020). Protein aggregations can cause irreversibly altered conformations of proteins that can affect their molecular weight. It has been shown that protein aggregation can affect the results of gel electrophoresis images especially due to acid or heat treatment (Sakuma et al., 2011). BSA, in particular, is sensitive to aggregation because it contains 17 disulfide bonds which facilitate aggregation after limited reduction (Yang et al., 2015). This may be why the 75 kDa bands can be seen in the SM14 and SM15 and none of the other samples. If there was more time in this study further gels of the LL samples would have been done to fully troubleshoot the quality discrepancies seen.

One of the bands that had a statistically significant difference between the stress and control treatment was the bar at around 16 kDa. This was seen in both the SM (P-value $0.01 \leq 0.05$) and LL samples (P-value $0.05 \leq 0.05$). These bands are thought to be either a subunit of the protein β globin chain (theoretical molecular weight = 16 kDa) (Yamaguchi et al., 1996, Kranen et al., 1999) or the protein myoglobin (theoretical molecular weight = 17 kDa) (Zaia et al., 1992) or even both at the same time. β globin chain is a subunit of a larger protein complex called haemoglobin which is located in the red blood cells in sheep (Bank, 2005). Haemoglobin is made up of a pair of two α globin chain and β globin chain dimers combined. Interestingly, the speculated 16 kDa proteins are β globin subunits that are significantly damaged by stress because studies have found that haemoglobin is an important factor that determines meat quality including colour and haemorrhage (Kranen et al., 1999). The role of haemoglobins is to transport oxygen around the body, if the β globin chain is damaged or degraded during stress potentially the body cannot transport oxygenated blood around the body efficiently. It has been found that the extent of haemorrhage in muscle tissues is directly related to the amount of haemoglobin in muscle samples (Kranen et al., 1999). Measuring the amount of haemoglobin is an effective method for determining haemorrhage in meat samples. As stated this protein band could also represent the protein myoglobin. Even though myoglobin's kDa is higher in the literature than it is in this study (16 vs 17 kDa respectively), these values are only an estimation and variation can be seen naturally between the molecular weight of some proteins. If this band was myoglobin it would be particularly interesting as myoglobin is a sarcoplasmic-heme-protein that is the protein primarily responsible for the final colour of red meats (Suman & Joseph, 2013). Type I slow low twitch muscles have higher amounts of myoglobin naturally which gives them their more red colouration compared to type IIB fast twitch muscles which are more white (Radák, 2018). If this protein band is the theoretical protein myoglobin this could mean that there could be significant differences in the meat

colour between the two treatments of LL and SM. A study by Liu et al, (2022) found that the oxidation of both haemoglobin and myoglobin greatly affected the quality of beef meat by reducing its WHC. This study's results are interesting when you consider a significant difference between the two treatments in the fast twitch muscle LL but not the slow twitch muscle SS at the 16 kDa band. This could potentially be because SS is a slow-twitch muscle and thus would have larger amounts of the oxygen-binding protein myoglobin. Further analyses of this 16 kDa band and final meat colour may be interesting for future studies. More testing would be beneficial with more samples for example duplicates of each muscle to make any final conclusions.

The natural biological variation seen between the relative frequency of the four different bands to the reference band was tested and the results can be seen in Table 7. For the 100 kDa bands, there was a significant difference found between the relative frequency of two muscles SM (intermediate twitch muscle) and LL (fast twitch muscle) and the muscle samples G (intermediate twitch muscle) and LL (slow twitch muscle) to the reference band. These results are interesting as they split LL the fast twitch and SS the slow twitch muscle into two significantly different groups (each has an intermediate muscle grouped in as well). This means that naturally in sheep, there is biological variation between these two groups of muscles at the protein around 100 kDa. Without sequencing the proteins it's impossible to definitively say what this protein band represents, but we can speculate. This band at around 100 kDa could be a lot of things including enzymes and could even represent multiple proteins at the same molecular weight. One paper claimed that a band around 100 kDa in the two muscles *semitendinosus* (SD) and *longissimus dorsi* (LD) in Wuzhumuqin Sheep were two single peptide chains of $\alpha 1$ and $\alpha 2$ that help to form type 1 collagen (He et al., 2023). These two chains with the addition of type 2 collagen $\alpha 1$ chains help to form the triple helix structure of the collagen-heavy chain (Shoulders & Raines, 2009). Because the gel Electrophoresis results split the fast twitch and slow twitch muscles into two significantly different groups this 100 kDa band may represent a structural protein like myosin heavy chain fragments (Bao et al., 2021). This theoretical protein may be expressed significantly differently between the fast and slow twitch muscle groups due to their differing physiology but again, further sequencing would need to be done to be sure.

A statically significant result was also found for the biological variation and the band that had the rough molecular weight of 45 kDa to the frequency of the reference band in the muscle samples G, LL and SS. It was also found that the muscle sample SM was significantly different to G but not LL and SS. This is interesting as again there was biological variation seen between all three muscle fibre types fast (LL), slow (SS) and intermediate (G) twitch muscles. Again it is hard to identify this protein without proper sequencing of the protein so the identity of what exactly this protein is uncertain.

Another band that was tested for biological variation had a rough molecular weight of 42 kDa. The results for this band were particularly interesting as it was able to significantly group the fast twitch muscle (LL) and the slow twitch muscle (SS) in two significantly different groups. 42/43 kDa is commonly associated with actin monomer (G-actin) subunit proteins in the literature (Glyakina & Galzitskaya, 2020, Cooper, 2000). This water-soluble globular protein helps form the actin protein filament structure which is a protein complex consisting of the G-actin subunits, troponin and tropomyosin that form microfilaments that form the thin firmaments that make up muscle fibres. Each G-actin subunit has tight binding sites that mediate head-to-tail binding with two other actin subunits. This allows them to polymerize to form long actin filaments of F actin. This study predicts that this 42 kDa band seen in the gels is the G-actin subunit. One study was able to sequence G-actin subunits at 43 kDa in the *longissimus thoracis* muscle of Duolang and Hu sheep (Yan et al., 2018). This is important as it highlights the major physiological differences between the fast twitch and slow twitch muscles. Previous research has shown that slow-twitch muscle fibres have longer actin filaments than fast-twitch muscles (Robaszekiewicz et al., 2020). This means that the significant difference seen in the relative frequency of what is suspected to be the actin subunits is unsurprising.

The last band that was analysed to see if there was any biological variation between the samples was the 16 kDa band. A non-significant difference was seen between the relative frequency of this protein between the four muscle samples and the reference band. A non-significant difference in biological variation was seen for this band. This theoretical protein is thought to be either a β globin chain subunit of haemoglobin or the myoglobin protein. Protein sequencing of this band will need to be done to determine indefinitely if this band is haemoglobin, myoglobin or something else entirely different. This study hypothesised that the protein profiles of proteins of lamb meat will be significantly affected by the treatment of stress. This hypothesis was proven correct for both the SM and LL gels with both of them having a statically significant difference between the relative quantity of their 100 and 16 kDa protein bands to the reference band. No statistically significant difference was seen in the G and SS muscles. Due to the limited time in this dissertation, protein bands were not sequenced. protein sequencing would be needed to confirm if the hypotheses of stress-affected proteins are related to meat quality or not, however, these results suggest it is.

One study by Bai et al. (2020) looked at the phosphorylation rate of phosphorylase enzymes and their effect on the rate of glycolysis in 60 samples of the fast twitch muscle *longissimus thoracis* of sheep. This study found that the phosphorylation level of phosphorylase enzyme significantly affected the glycolytic rate and thus would also affect the final glycogen levels post-mortem. Another study by Chen et al. (2016) showed that the phosphorylation rate in lamb meat can have significant effects on the

final meat quality parameters such as tenderness. This study showed that meat that had low tenderness values had significantly higher rates of phosphorylase phosphorylation. These studies and others show that the phosphorylation of the phosphorylase enzyme plays an important role in glycolysis and the ultimate glycogen levels post-mortem (Ren et al., 2022). If there was more time and resources allocated to this dissertation it would have been interesting to see if the phosphorylation rate of phosphorylase changed between the stressed and non-stressed treatments and how this affected final glycogen content.

3.3 Experiment Three - Analysis of Supporting pH Data

3.3.1 Introduction

The purpose of the pH data analysis was to create a source of comparative data to support the results seen in the previous two experiments. The ultimate pH (after 24 hours) is an extremely important value in the industry as it is used to grade the quality of meat carcasses. Data used in this experiment were measures of the pH values of carcasses 90 minutes and 24 hours after post-mortem. Particular interest was taken into the comparison of the different muscle fibre types to see if there were any trends between ultimate pH values and the type of muscle fibre.

3.3.2 Results

Data for the pH values were analysed using both T-tests and a one-way ANOVA in the Minitab software. Statistics were done on all four muscle samples and a mean of all four samples to try to explain the results of the previous two experiments. It was found that there were no significant differences between any of the muscles after 90 minutes (Table 12). However, the slow twitch muscle SS was close to being significant (P-value $0.06 > 0.05$). When the mean of all four muscle samples combined were compared at 90 minutes there was a strong non-significant difference seen (P-value $0.96 > 0.05$). This means after 90 minutes post-mortem negligible differences in pH values can be expected in the muscle samples tested. The opposite was true for the results of the ultimate pH values after 24 hours (Table 13). It was found that there was a significant difference between the final pH values of all four muscle samples (SM (P-value $0.02 < 0.05$), G (P-value $0 < 0.05$), LL (P-value $0.05 \leq 0.05$) and SS (P-value $0 < 0.05$)). It was also found that there was a highly significant difference between the mean of all four muscle samples combined after 24 hours (P-value $0.00 < 0.05$). The stress treatments had higher ultimate pH values than the control treatment due to it failing to fall into the ideal pH range values (6.15 vs 5.84). It was also noted that the slow twitch muscle SS had both the highest mean pH value after 90 minutes (7.06 ± 0.14) and the ultimate value after 24 hours (6.37 ± 0.12) in the stress treatment. This is particularly notable when you consider the results of the different fibre types coming in the

same order in both pH statistical analyses; SS was the highest followed by the two intermediate twitch fibres (SM and G respectively) then lastly LL the fast twitch muscle fibre. Another notable result is that the two different muscle twitch fibres SS (slow twitch) and LL (fast twitch) were deemed to be significantly different from each other as shown by the subscript letters assigned in Table 13 by the Tukey pairwise analysis. The two intermediate twitch fibres (G and SM) on the other hand were not significantly different from each other. This shows that statically the ultimate pH values of the muscle samples in the stress treatment can be categorised by their different fibre types.

Table 12 PH values of the muscles *Semimembranosus* (SM), *Gracilis* (G), *Longissimus lumborum* (LL) and *Supraspinatus* (SS) at 90 minutes post-mortem.

	SM	G	LL	SS	All Muscles
Control (n=5)	6.62±0.33 ^b	6.77±0.22 ^{ab}	6.8±0.4 ^{ab}	6.9±0.2 ^{ab}	6.77±0.32
Stress (n=5)	6.84±0.3 ^{ab}	6.73±0.2 ^{ab}	6.7±0.3 ^{ab}	7.06±0.14 ^a	6.83±0.27
P-value	0.14	0.67	0.57	0.06	0.96

^mean ± standard deviation, the assigned subscript letters indicate a significant difference in the muscle samples pH values (calculated using ANOVA (p < 0.05) Tukey pairwise comparison method was completed on each treatment in the Minitab software).

Table 13 Ultimate pH values of the muscles *Semimembranosus* (SM), *Gracilis* (G) and *Longissimus lumborum* (LL) at 24 hours post-mortem

	SM	G	LL	SS	All Muscles
Control (n=5)	5.77±0.1 ^d	5.85±0.11 ^{cd}	5.79±0.08 ^d	5.96±0.11 ^{bcd}	5.84±0.12
Stress (n=5)	6.25±0.5 ^{ab}	6.09±0.18 ^{abc}	5.9±0.14 ^{cd}	6.37±0.12 ^a	6.15±0.33
P-value	0.02	0	0.05	0	0

^mean ± standard deviation, the assigned subscript letters indicate a significant difference in the muscle samples pH values (calculated using ANOVA (p < 0.05) Tukey pairwise comparison method was completed on each treatment in the Minitab software).

3.3.3 Discussion

The results from the pH data analysis proved the hypothesis correct for the pH data. It was hypothesised that there would be a statistically significant difference between the final post-mortem pH values of the stress and control treatment which was proven in the results (Table 13). These results match that seen in previous literature where a significantly higher (P-value = 0.01) ultimate (after 24

hours) pH (5.58) was seen in the treatment exposed to stress before slaughter than the control group (5.52) (Hambrecht et al., 2005). It was also hypothesized that the ultimate pH value of the control treatment will be lower than that of the stress treatment which was also seen in the results (Table 13). A trend was seen in the results that the fast twitch muscles had lower ultimate pH values in both the control and stress treatment than that of the intermediate and slow twitch muscles. These results also matched that in the literature where the three different muscle fibre types were studied and their relationship with the ultimate pH (Ithurralde et al., 2018, Lee et al., 2022). This makes sense as fast twitch muscles naturally have higher levels of stored glycogen due to their reliance on the anaerobic glycolytic pathway. This means that the slow twitch muscle SS naturally has a higher ultimate pH value due to its physiology and fibre type. This indicates that post-mortem fast twitch muscle fibres can mature more favourably and drop their pH lower due to them having higher levels of stored glycogen.

One notable observation of the data is that the fast twitch muscle LL was close to having a non-significant difference between the two treatments in its ultimate pH values ($P\text{-value } 0.05 \leq 0.05$). This makes sense as LL is a fast-twitch muscle and thus relies fully on the anaerobic glycolytic pathway for energy metabolism. This means that even in the control sample because these muscles can't undergo oxidative metabolism they can only use the stored glycogen as energy. These results were mirrored by previous literature that also found that under stress conditions type IIA fast twitch muscles tend to have higher high lactic acid levels and low glucose levels than type I slow twitch muscles as it is being depleted faster for energy (Ferguson & Gerrard, 2014). Similar results were also seen in the study where lower ultimate pH was linked to type IIA fibres with a higher glycolytic activity.

It was also noted that the pH values in this study were generally higher than that seen in previous literature. A study by Ithurralde et al., (2018) had a mean ultimate pH of 5.65 for LL, 5.67 for SM and 5.5 for SS in sheep while our study had a mean ultimate pH value of 5.79 for LL, 5.77 for SM and 5.96 for SS (all control samples). This study did not use the muscle sample G or add a stress treatment however these results are still comparable. This means there is something different about these trail sheep that could have led to the differing pH results including breed, genotypes, body weight and sex. The study by Ithurralde et al., (2018) used Poll Dorset cross-bred sheep and no mention of what sex the sheep was given. Other studies have linked different sex to significantly different pH values post-mortem (McGeehin et al., 2001). This study used Coopworth sheep and mixed Coopworth breeds that were all lamb rams.

This is notable as other studies have linked ram lambs to having higher ultimate pH values compared to ewes of the same age (between 5 and 8 months) (Johnson et al., 2005). This was seen in both the muscles LL (with ewes having a pH of 5.60 and rams 5.74) and SM (with ewes having a pH of 5.63 and

rams 5.66). The same study also linked this higher ram lamb pH to a lower overall eating quality than females ($P < 0.001$). This was determined by higher Warner-Bratzler shear values (peak value 109.8 vs 97.0 N in the muscle SM) and lower quality parameters of redness (a^*) and lightness (L^*) values ($P < 0.001$). This means there is a chance the difference in pH values seen in this trial may just be due to the trial's design of using all ram lambs over a mix of both sexes.

WHC is defined as the ability of meat to hold native moisture during processing, fabrication and storage (Zuo et al., 2022). There is a possibility that the WHC of sheep could have affected the results of this study too. Previous studies have shown that without sufficient levels of stored glycogen before slaughter, glycogen can have a larger effect on the final pH ultimate pH value after 24 hours (Chauhan & England, 2018). This change in the ultimate pH can affect the final water-holding capacity of meat samples (Zuo et al., 2022). Thus there is evidence in the literature that these values are linked but there is no evidence of the final water-holding capacity of meat samples affecting the final glucose levels in meat directly. There is evidence, however, of the structure of muscle tissues affecting both the final glycogen levels and the WHC of meat. One study by Listrat et al, (2016) explained how altered muscle structure through genetic, environmental factors and excessive stress could affect both the WHC and the final stored glycogen levels. The literature has shown that glycogen levels and the final WHC are indirectly linked but there is no evidence of the final WHC significantly affecting total glycogen levels in meat samples.

3.4 General Trial Discussion

This study used a sample of 20 Coopworth ram lambs. It is also notable that the only two mixed Coopworth/Hampshire sheep (1032 and 247) were noted to be particularly stressed and highly muscular compared to the other 18 pure Coopworth sheep. This is notable as in a trial with such small sample sizes as 20, having different sheep can add variation and greatly affect the results. This is even more relevant when you consider that this trial is around the biochemical shifts in muscles and having one breed of breed of sheep visually more muscular and stressed has the opportunity to skew the results. One study by Monaco et al, (2015) looked at the eating quality of lambs from six different breeds including Ile de France, Santa Inês, Dorper with Santa Inês, Suffolk, Hampshire Down (which is the same as the Hampshire breed in our study) and lambs without definite breed or mixed lambs (WDB). This study also looked at the muscle *longissimus lumborum* (LL) and found that the Hampshire breed had the lowest shear force 3.6 ± 0.1 (kg) and after sensory analysis testing was significantly the most tender. This study also found that this breed had the second highest final pH value (after 24 hours) at 5.87 ± 0.01 , after WDB (6.11 ± 0.08). Notably, also this study's final pH values are highly comparable to the final pH values found in this study for LL (LL control 5.79 ± 0.08 , LL stress 5.90 ± 0.14). These pH results are interesting as pH is closely linked to stress and subsequent biochemical shifts in lamb meat (Jacob & Pethick., 2014, Khatri & Huff-Lonergan, 2023)). Thus there is a possibility that the two mixed Coopworth/Hampshire sheep could have affected the results of this study. My recommendation next time a trial such as this is conducted is to use a population of just one breed of sheep. These two sheep samples were only used in the Bradford assay as the other analyses only used five samples not all 10 of each treatment.

The experimental design of this study was ultimately restricted due to limitations in both time and resources. If this study had not been conducted in a dissertation style some changes would have been made to the methodology. One change that this study would have benefited from would have been more samples taken of each muscle, Ideally having duplicates of each muscle from the base sample homogenate. A compromise was made with the protein profiles work to only use five samples of the stress/ control treatment. If there had been more time in this project all 10 samples would have been used. All these sampling restrictions were taken due to time and resource constraints, which would have ultimately affected the final sample size. This ultimately meant that this study had less power when it came to statistical analysis. A larger sample size would also mean that the effect of possible outliers in the population would have less skew on the results. This should theoretically make the results more significant. Another change to benefit this study could be to use an updated methodology for the glucose assay methods. As shown in the spike assay work, this study's glucose assay methods possibly suffered from a loss of glucose somewhere in its procedure. It also probably did not help that the samples for the glucose spike work were taken from the original base homogenate which could

have possibly degraded over time. This study would have also benefited from adding protein sequencing of the different muscle samples into its methodology. Techniques such as mass spectrometry and Edman degradation can be used to identify what proteins are present in the solution. This would have been beneficial as the results would have been able to confirm the identity of the different protein bands that were given in the gel electrophoresis images. Knowing the identity of these bands would have allowed us to see if the bands that were deemed statistically different between the control and stress treatments (100 kDa and 16 kDa) were related to the quality of meat or not (as is speculated in the 16 kDa band).

Chapter 4 Overall Conclusions

In conclusion, this study looked at the effect of stress and a high pH on the metabolic changes and protein profiles of the four sheep muscles *Gracilis* (G), *Semimembranosus* (SM), *Longissimus lumborum* (LL) and muscle *Supraspinatus* (SS). The rationale of this study was to study the biochemical changes associated with pre-slaughter stress in lamb meat to try and further the development of potential biomarkers of lamb meat quality. This study aimed to do this through the use of three research questions to study the effect of stress and a high pH value on biochemical shifts in lamb meat.

R1: Do high ultimate pH values caused by stress affect energy metabolic changes in the muscle tissues of sheep? This study showed that stress had a mixed effect on the metabolic changes of the muscles. The results on the SM muscle matched that of previous literature, it saw a decrease in its total glycogen content and an increase in its total lactic acid levels post-mortem under stress. Muscle G went against previous literature and saw an increase in its total glycogen content and a decrease in its total lactic acid levels under stress. However, none of these results were deemed significant (P-value > 0.05).

R2: Do high ultimate pH values caused by stress affect the protein profiles in the muscle tissues of sheep? The result of the Bradford assay was non-significant and no obvious trends was seen. The results from the gel electrophoresis images showed that two protein bands at around 100 and 16 kDa in the two muscles SM and LL showed significant differences in their relative frequency to the reference band between the two treatments. Further protein sequencing research would be needed to draw any meaningful conclusions on what proteins these bands represent.

R3: Does the treatment of stress cause a significant difference in the ultimate pH values in the muscle tissues of sheep? The results showed a significant difference between the two treatments' pH data after 24 hours (ultimate pH), with the stress carcasses maintaining a higher pH value. It was also found that the slow twitch muscle SS exhibited the highest mean ultimate pH value, followed by the two intermediate muscles SM and G (respectively) then the fast twitch muscle LL. This indicated a fibre-type-specific response to the stress treatment between the four muscles tested.

This study found that the results of the glycogen assay were lower than those of previous literature. This could have been due to several reasons including methodology used, sample prep, age of sheep, testosterone levels and even different feeding strategies of livestock. Further work developing the methods of the glucose assay is needed before any meaningful conclusions can be made. One interesting finding of this study was that there were significant differences in the protein bands that had a rough molecular weight of around 16 and 100 kDa in the stress treatment of muscles SM and LL. It is speculated that the 16 kDa band represents either a β globin chain subunit (subunit of haemoglobin) or the protein myoglobin. Both of these proteins are commonly associated with meat

quality. These results could make up the basis of an interesting follow-up study that looked at the effect of stress on sequenced proteins that have been proven to affect meat quality such as haemoglobin and myoglobin. Further research on how specific meat proteins are affected by stress and how this affects final meat quality parameters such as colour and tenderness would be insightful.

Currently, ultimate pH after 24 hours is the standard for assessing final meat quality. This study looked at furthering the development of other potential biomarkers for the indication of final lamb meat quality such as total glycogen, lactic acid and protein profiles. Further research is needed to fully characterise and develop these biomarkers into reliable tools as well as establishing linked technology to reliably measure them in the industry.

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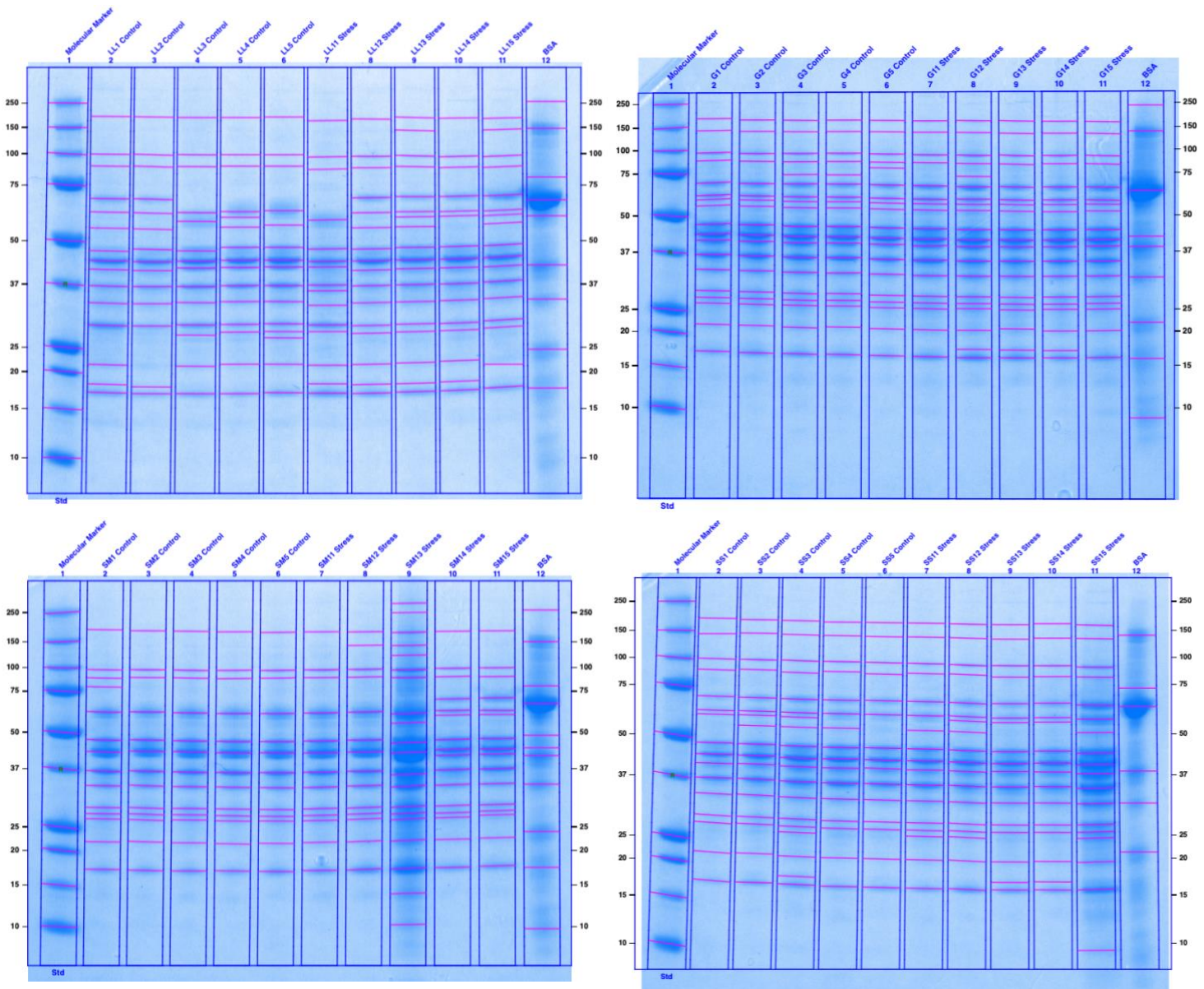
Appendix A Raw Sample Extraction Data

Table 1 Raw sample data from the sample extraction

	Animal ID Number	Kill Order	Tube #	LL (g)	5x Lysine Inhibitor	SS (g)	5x Lysine Inhibitor	G (g)	5x Lysine Inhibitor	SM (g)	5x Lysine Inhibitor
Control	56	1	1	0.554	2.77	0.544	2.77	0.573	2.865	0.567	2.835
	261	2	2	0.606	3.03	0.59	2.95	0.511	2.55	0.59	2.95
	50	3	3	0.624	3.12	0.565	2.82	0.536	2.58	0.59	2.95
	1132	4	4	0.542	2.71	0.61	3.2	0.59	2.68	0.511	2.55
	38	5	5	0.574	2.86	0.542	2.71	0.629	3.19	0.549	2.74
Stress	1036	11	11	0.645	3.22	0.534	2.67	0.519	2.59	0.585	2.92
	6	12	12	0.628	3.14	0.562	2.82	0.595	2.98	0.632	3.16
	1113	13	13	0.595	2.98	0.556	2.77	0.585	2.82	0.571	2.85
	78	14	14	0.517	2.55	0.6	3	0.6	3	0.564	0.83
	216	15	15	0.533	2.67	0.645	3.22	0.58	2.9	0.557	2.77

Appendix B Gel Electrophoresis images with Bands

Figure 1 Gel electrophoresis images with the automatically generated bands from the Image lab software



All four gel electrophoresis images with the bands that were used during statistical analysis of the four muscle types *Longissimus lumborum* (LL), *Gracilis* (G), *Semimembranosus* (SM) and *Supraspinatus* (SS) from left to right, top to bottom. Molecular Marker is the Bio-Rad Precision Plus Protein™ Reference Band and the BSA is the Bovine Serum Albumin standard recommendations from ThermoFisher Scientific/Pierce. Note the over saturated BSA lanes in all gels. Analysed in the Image lab software.

Appendix C Assay Standard Curves Produced.

A.1 Example standard curve for the D-Glucose assay

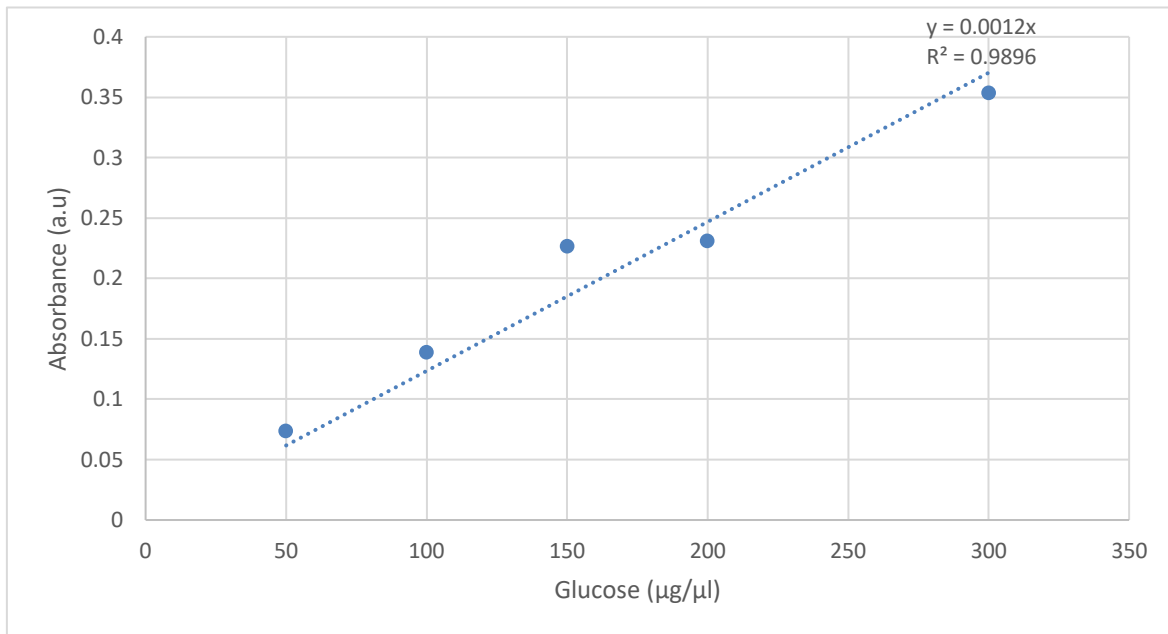


Figure 1: Total glucose standard curve made in the Excel software

A.2 Example standard curve for the D-Glucose spike assay

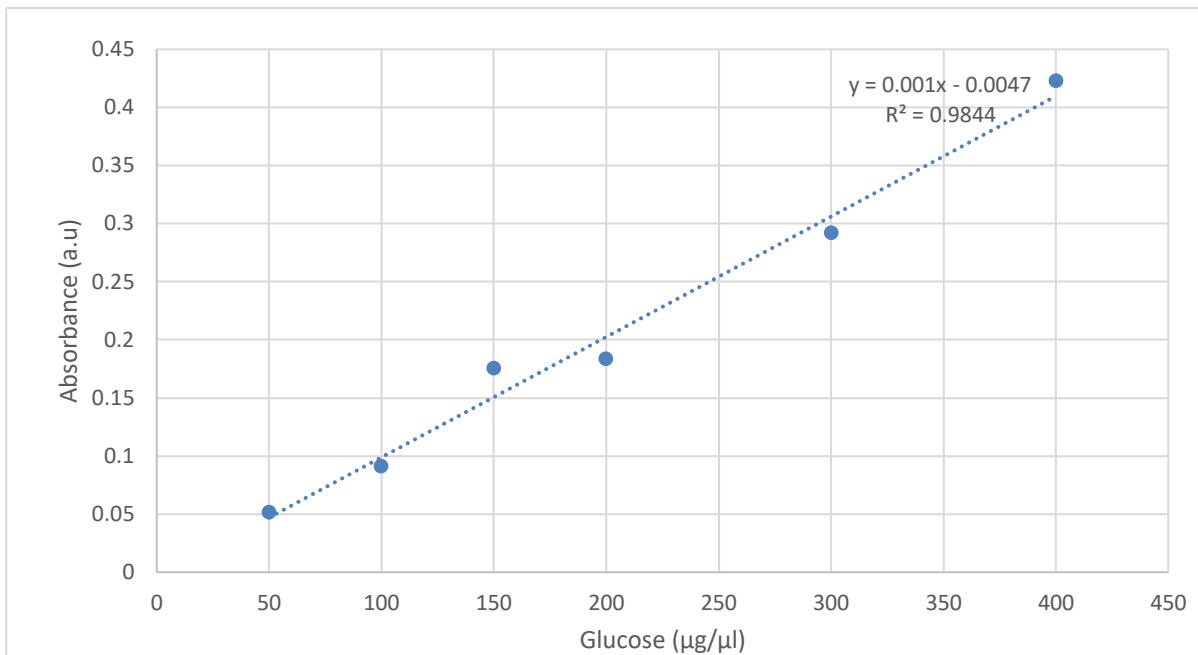


Figure 2 Total glucose spike standard curve made in the Excel software

A.3 Example standard curve for the L-Lactic assay

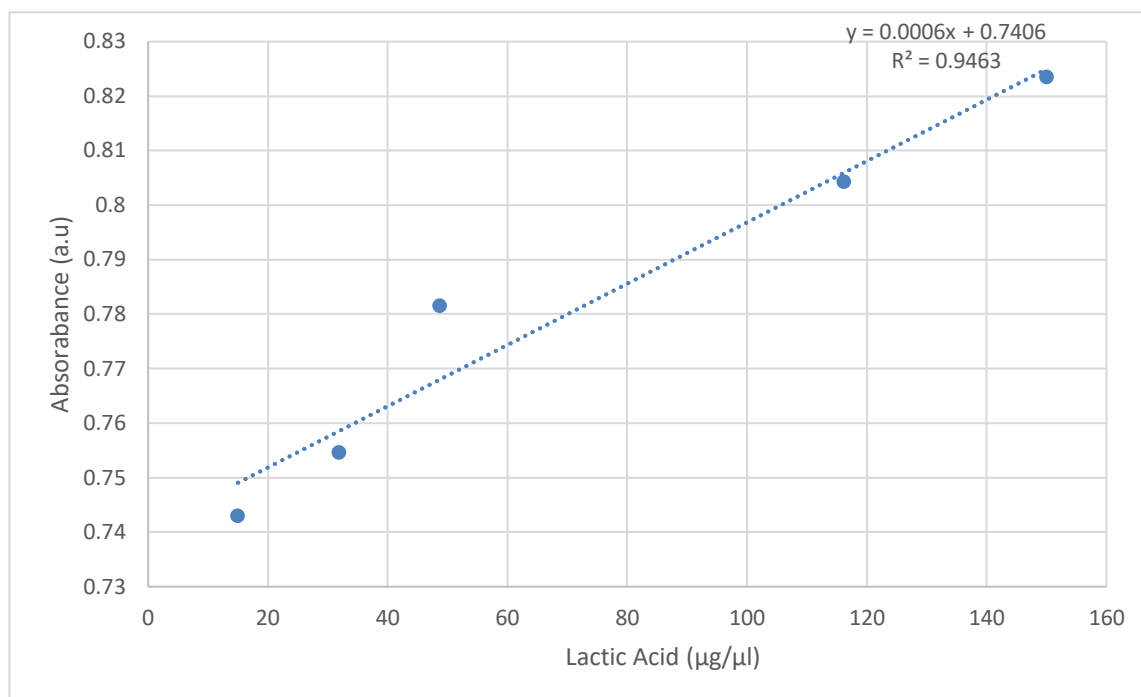


Figure 3 Total lactic acid standard curve made in the Excel software

A.4 Example standard curve for the L-lactic redo assay

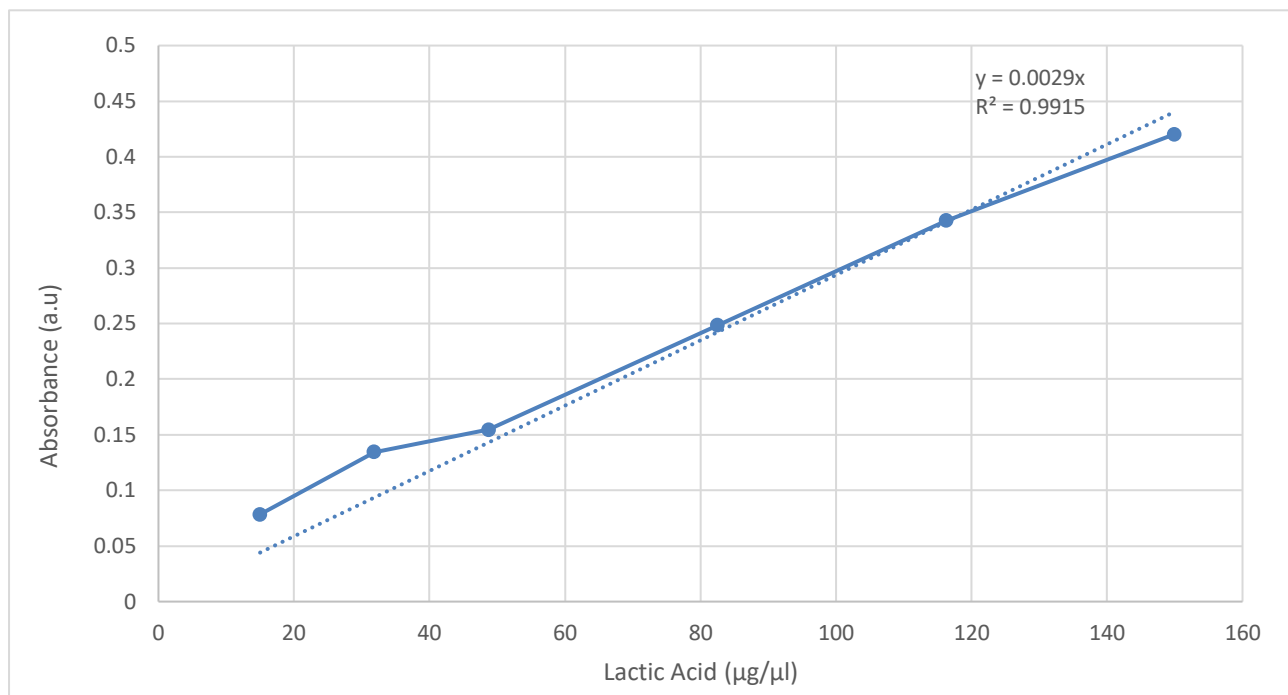


Figure 4 Total lactic acid standard curve made in the Excel software

A.5 Example standard curve for the Bradford assay

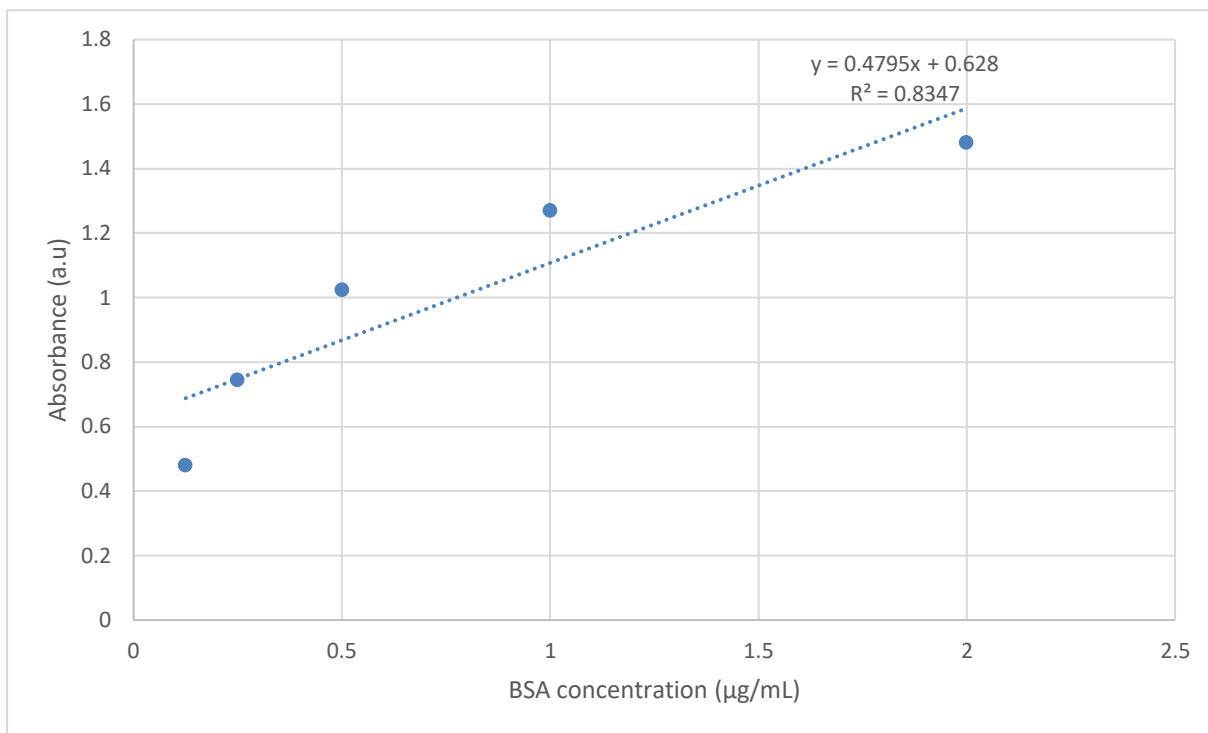


Figure 5 Bradford assay standard curve made in the Excel software