Identifying causes of high pH in lamb meat

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Garrianne Fatima Hontiveros

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Abstract

Lamb meat export is a multi-billion-dollar New Zealand industry. Therefore, strategies to maintain and improve quality have immediate commercial benefit. Consumers judge quality by colour, tenderness, and flavour, all of which are affected by ultimate pH (pHu). Ideal meat pHu is 5.5 and is influenced by muscle glycogen concentration. Insufficient muscle glycogen at slaughter results in high pHu (>5.8) which results in tough meat, dark colour, and shorter storage life. The decrease in meat pH post-slaughter is caused by the build-up of H+ ions as a consequence of anaerobic glycolysis of glycogen for ATP formation. Physical stress depletes muscle glycogen, prompting consumers to conclude animal mistreatment. However, stress, does not necessarily equate to abuse as all animals experience some level of pre-slaughter stress and any negative effects depend on duration, type, intensity, and susceptibility. Studies have focused on post-farm-gate stressors (e.g. transport and lairage) but less clear is the role of on-farm stressors on the incidence of high pHu. The aim of this study was to investigate the variation of muscle glycogen of lambs on-farm, examine the use of heat shock protein 20 (HSP20) as a marker for physiological stress, and determine any relationship between residual glycogen level and meat quality attributes such as water binding capacity (WBC), drip loss, microbial abundance, fat, protein, and water content.

Two observations were conducted. Firstly, 60 animals from 2 farms with different production systems: wethers (castrated males) were raised with ewes in Farm 1, whereas, intact rams, wethers, cryptorchids, and ewes were all together and introduced at different times to the group in Farm 2. These animals were biopsied two months before slaughter, a month before, and post-rigor. On-farm, large inter-individual variation in muscle glycogen was observed in both farms but no significant difference in true glycogen between farms. Muscle glycogen levels seemed to fluctuate from one biopsy to another, contrary to previous belief that it remains constant throughout. There was no significant difference in HSP20 between farms and no correlation between muscle glycogen and HSP20 was observed. Post-rigor, 66% of carcasses from

Farm 2 had high pHu (≥5.8), compared to none from Farm 1. This may be due to stress brought about by exaggerated behaviours of rams (e.g. aggression and chasing) during close confinement at lairage. However, more observations on-farm and at lairage are needed. Lastly, 69 loin samples of varying levels of residual glycogen were tested for drip loss, (WBC), fat, protein, and microbial content a day post-rigor (Day 1) and again after eight weeks (Week 8). Results showed no correlation between residual glycogen content and any of the parameters tested at either time points. A decrease in total glycosyl units at Week 8 was observed but no decrease in true glycogen levels. This was likely due to microbes use of free glucose as a food source.

To conclude, the results of this study suggests that the co-habitation of intact rams and ewes on-farm causes stress, which is compounded by the close confinement at lairage, leading to high pHu of their carcasses. It is, therefore, recommended that, although intact rams have higher saleable weight, these rams be castrated before mixing with other rams and ewes to avoid high pHu and, hence, provide good quality meat and less stressed animals (better animal welfare). The fluctuation in muscle glycogen levels over time does also need closer scrutiny.

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Chapter 1

Introduction

Lamb meat production is a significant portion of the New Zealand economy with export providing $2.77 billion in the year ending September 2016, as reported by the Meat Industry Association of New Zealand (MIANZ) (2016). Export markets include populous countries such as China, the UK, and the US. Due to the popularity of New Zealand lamb, it is crucial that quality is maintained. Consumers judge meat quality firstly by colour, followed by tenderness, and finally, flavour; all of which are influenced by pH and temperature (Joo et al., 2013; Smulders et al., 2014). Ideal meat ultimate pH (pHu) is around 5.5 and should be gradually chilled post-slaughter to prevent toughening.

Insufficient muscle glycogen at slaughter results in high ultimate pH (pHu) above 5.8 (Immonen et al., 2000b). High meat pH has four main commercial consequences: darker meat colour (Calkins and Hodgen, 2007), earlier onset of spoilage, reduced chilled storage life, and tougher meat (if pHu falls between 5.8 and 6.0) (Farouk et al., 2013). The decrease in meat pH post-slaughter is caused by the build-up of H+ ions as a consequence of anaerobic glycolysis of glycogen (muscle energy storage) for ATP formation (McGeehin et al., 2001). Therefore, a high concentration of glycogen pre-slaughter leads to ideal pHu.

High pH in meat prompts consumers to conclude animal rights violations due to mistreatment prior to slaughter. Although stress depletes muscle glycogen, this does not necessarily mean animals are mistreated. All animals experience some level of stress prior to slaughter but the magnitude of any negative effects depend on the duration, type, intensity and the susceptibility of the animal to stressors (Ferguson et al., 2001). Furthermore, it is not clear whether inter-individual variability in stress responsiveness can account for unexplained variance in sensory traits such tenderness (Ferguson and Warner, 2008). For example, it was found that the average change in muscle glycogen in the *semitendinosus* and *semimembranosus* muscles of sheep between farm and slaughter varied greatly between consignments and was attributed to

difference in stress responsiveness (Jacob et al., 2005). Additionally, deliberately producing high pHu in meat experiments is difficult and often, despite relatively high levels of physical exercise, glycogen levels can remain relatively unchanged (Apple et al., 1994). In fact, the only reliable experimental method of *significantly* decreasing muscle glycogen levels is via the administration of adrenalin (McVeigh and Tarrant, 1982). The one exception are young bulls who, if mixed with other bulls from other farms- either during transport or on arrival at a processing plant, will then result in high pHu carcasses post-slaughter (McVeigh et al., 1982).

Jacob (2003) observed that “on-farm” factors can be as or more important than “post farm gate factors” on muscle glycogen concentration in lambs. However, there has been little research on muscle glycogen concentration on farm. The aim of this study, therefore, is to gain an understanding of muscle glycogen levels in lambs on-farm at different time periods and with different production systems. This would contribute to better management of muscle glycogen and subsequent ideal pHu and, possibly, better quality meat yield.

1.1 Muscle Composition and Structure

Lamb skeletal muscle is composed of approximately 60% water, 22% fat and lipids (variable), 17% protein, 3.5% soluble organic compounds, some carbohydrates and a small amount of vitamins and minerals according to the United States Department of Agriculture (1963). There is considerable variation in biochemical and physical characteristics and visual appearance of skeletal muscle and reflects the proportion of fibre types in the cells (Smulders et al., 2014). Classically, muscle fibres are categorized by their contractile speed (fast or slow) and dominant metabolism into either: type I (slow oxidative), type IIa (fast oxidative), or type IIb (fast glycolytic) (de Rezende Pinto et al., 2015). The biochemical traits associated with these fibre types e.g. glycogen, myoglobin, ATP concentration, lipids, and enzymes reflect the diversity of muscle types and are related to their physiological function in the animal. As such, meat quality characteristics such as colour (influenced by myoglobin content, a red

pigmented protein that delivers oxygen to muscle cells), flavour (influenced by oxidation, lipid content, diet, myoglobin, and pH), juiciness (influenced by water content), and tenderness are fibre-type dependent (Calkins and Hodgen, 2007; Miranda-de la Lama et al., 2009; Listrat et al., 2016).

1.1.1 Composition

Water in the muscle is generally classified as: “free” water molecules which are immobilized by proteins (due to their configurations) but are not bound to the proteins themselves, and “loose” water which is extruded into the extracellular space and lost as drip (Ritchey and Hostetler, 1964; Stadnik et al., 2008; Pearce et al., 2011). A small percentage (5%) is directly bound to the hydrophilic groups in the protein. The ability of muscle to retain water despite external pressures is termed water binding capacity (WBC) or water holding capacity (WHC) (Huff-Lonergan and Lonergan, 2005).

Fat and lipid content is considerably varied in terms of quantity and composition and between muscles and species with males generally having less than females (Rowe et al., 1999; Bas and Morand-Fehr, 2000). Many intracellular lipids are associated with membrane structures with a considerable amount in the perimysium (Greaser, 1986) which consumers perceive as marbling. Lipid content, among others, is critical to consumer perception of flavour (Farmer, 1994; Calkins and Hodgen, 2007).

Muscle proteins are classified into: sarcoplasmic, myofibrillar, and stroma proteins (Brandebourg, 2013). Sarcoplasmic proteins which represents a significant proportion of muscle protein, are soluble in water or salt solutions of low ionic strength with many involved in the breakdown of glycogen (Huff-Lonergan and Lonergan, 2005). Myofibrillar proteins, subdivided into contractile and cytoskeletal proteins, are major components of the muscle’s contractile strength (de Rezende Pinto et al., 2015). Stroma proteins which include collagen, elastin, and reticulin (present in connective tissue) and proteins found in the membranes of muscle organelles such as mitochondria and sarcoplasmic reticulum (Francis, 2000). Collagen is an important factor in

meat tenderness due to its high mechanical strength and, hence, high shear force (Zapata et al., 2009).

1.1.2 Structure

The muscle is a highly structured tissue organised into progressively smaller fibrous components held together by connective tissue, of which there are three types: the epimysium (a fibrous sheath surrounding the entire muscle), the perimysium (a collagen network surround muscle fibre bundles), and the endomysium (fine connective tissue fibres encircling individual muscle fibres) (Fishman, 1994; Listrat et al., 2016). Figure 1 shows the organization of the muscle structure. Longitudinal dissection of myofibers show alternating patterns of dark areas (A-bands) and light areas (I-bands). Each I-band is sectioned into two parts by a Z-line and each repeating unit of two Z-lines is called a sarcomere, the smallest contractile functional unit of a myofibril (Brandebourg, 2013). A-bands are primarily composed of thick myofilaments while I-bands mainly consist thin myofilaments.

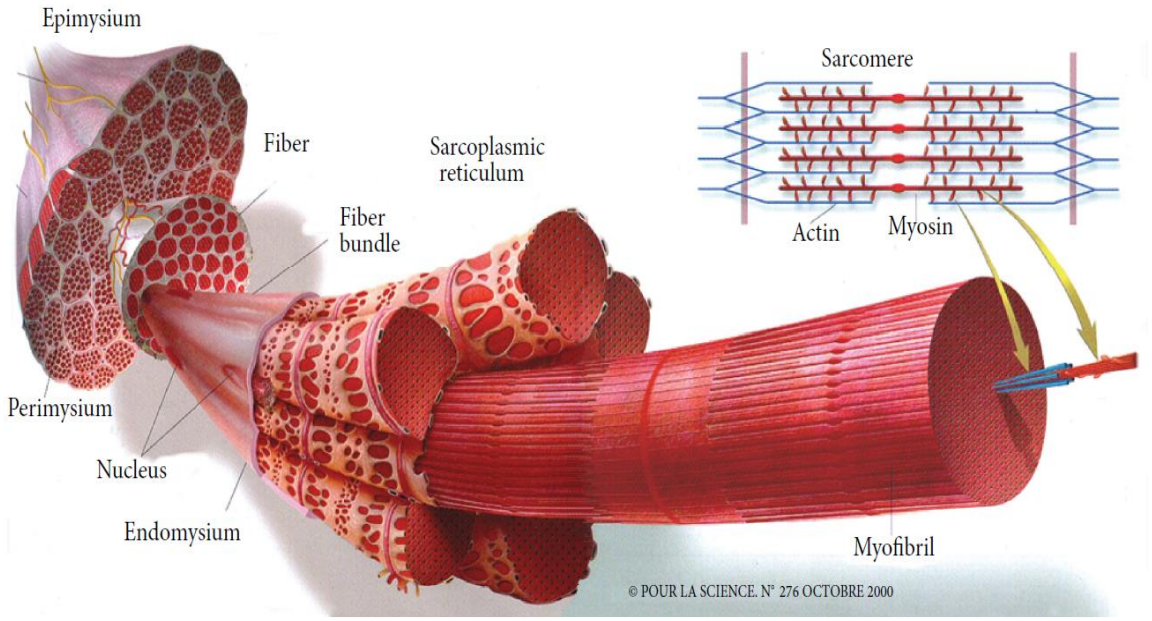

Figure 1.1. General organization of a muscle from the smallest contractile unit (sarcomere) to the full extent of the muscle (Listrat et al., 2016)

Thin myofilaments are composed of filamentous F-acting (linear polymer of globular G-actin). Two strands of F-actin form a helix and is associated with tropomyosin and the troponin complex, composed of three peptide sub-units: troponin C (binds calcium ions), troponin I (inhibits interaction of actin and tropomyosin), and troponin T (the tropomyosin-binding subunit) (Pearce et al., 2011). Thick myofilaments mainly consist of packed myosin molecules, each of which consist of a helical tail and a head with two globular units. Each head subunit contains a site with ATPase activity and a site that forms cross-bridges with actin to form an actomyosin bond during muscle contraction (Smulders et al., 2014). In normal muscle contraction, calcium ions are released from the sarcoplasmic reticulum and bind to the troponin complex covering actin leading to a conformation change that allows myosin heads to bind to actin filaments, forming an actomyosin complex. ATP hydrolysis provides the power for contraction and is used to release this bonding either because the muscle is relaxed or if more force is needed (in which case myosin will bind to the next actin) (Brandebourg, 2013). Figure 2 shows the structure of actin and myosin.

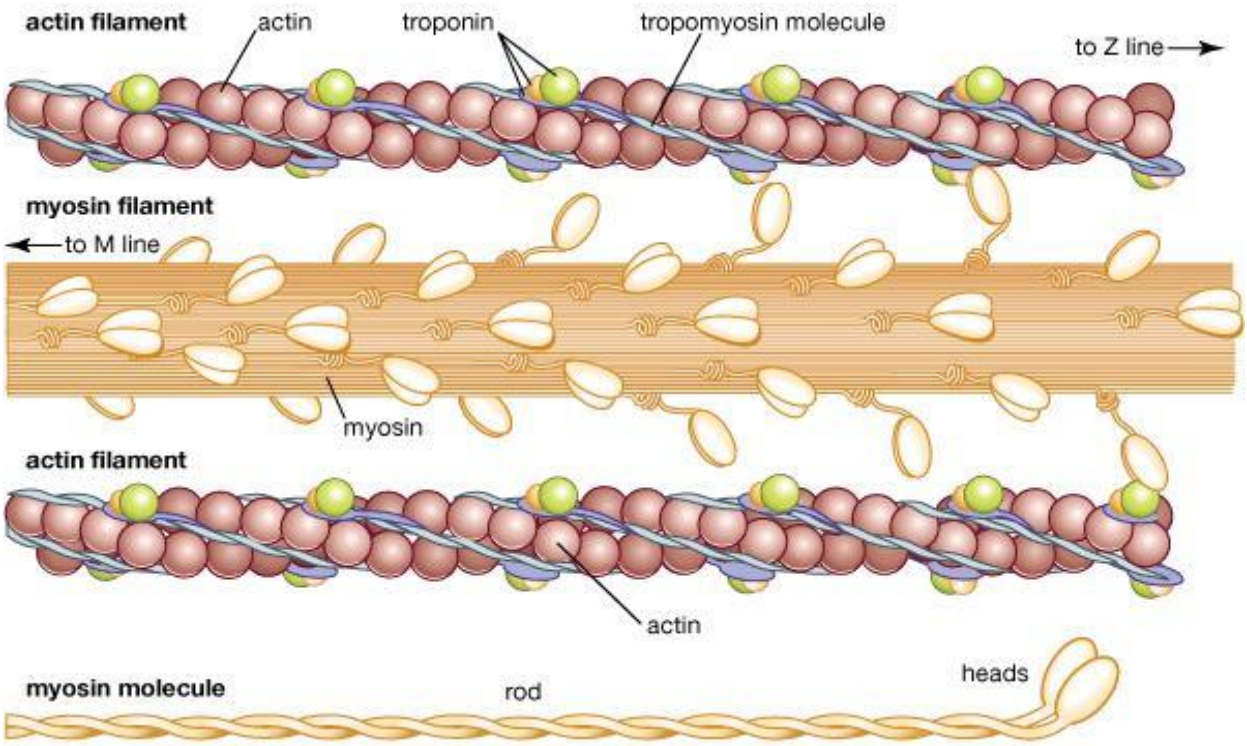

Figure 1.2. Structure of actin and myosin filaments (Wood et al., 2017)

1.2 Conversion of Muscle to Meat

At slaughter, blood circulation ceases leading to oxygen and carbohydrate deprivation (essential components for oxidative phosphorylation) of muscles. Muscle cells then become dependent on anaerobic glycolysis for ATP synthesis from the moment muscle-specific creatine phosphate reserves are depleted (Smulders et al., 2014). However, compared to oxidative phosphorylation, anaerobic glycolysis forms much less ATP and its products (lactate, hydrogen ions, and adenosine monophosphate) leads to a gradual decrease in pH. Rigor mortis sets in when too little ATP is available to release the actomyosin complex (Braden, 2013). There are three stages: delay phase, when metabolic processes are still functioning and muscle extension can occur by applying enough force; onset, when muscles are inextensible; and resolution, which is not completely understood although thought to be related to protein degradation.

1.2.1 Post-mortem Cellular Changes

Upon exsanguination, an animal loses approximately 50% of circulating blood (Honikel, 2004). Acute blood loss causes a decrease in central venous pressure and cardiac filling leading to reduced cardiac output and arterial pressure. Eventually, the heart fails and the supply of glucose and oxygen to cells cease (Klabunde, 2011). In normal muscle contraction, the binding of myosin heads and actin filaments is released via ATP. This is formed through oxidative phosphorylation (OXPHOS) which requires both oxygen and glucose, producing 36 molecules of ATP per molecule of glucose. When oxygen and glucose supply ceases, oxygen becomes limited to the small amount stored in muscles, but is quickly depleted, forcing cells to use anaerobic glycoglysis of glycogen (muscle energy storage, polymeric form of glucose) for ATP generation. However, anaerobic glycolysis is less efficient than OXPHOS, producing only two molecules of ATP per glucose molecule or three ATP molecules for every glucosyl unit derived from glycogen (Hedrick, 1994). ATP is, therefore, quickly depleted resulting in the permanent actomyosin complex causing inextensible muscles characteristic of rigor mortis (Francis, 2000).

With the cessation of circulation comes the loss of waste elimination mechanisms such that hydrogen ions and lactic acid produced from anaerobic glycolysis accumulate, gradually decreasing muscle pH (McGeehin et al., 2001). Immediately after blood loss, cells increase production of factors that stabilise cells and proteins, called heat shock proteins (HSPs) to preserve cellular function (Ouali et al., 2013). However, pH decline begin to render still-functioning glycolytic enzymes non-responsive and all manner of cellular metabolism gradually stops due to ATP depletion. Muscle pH in live animals is about 7.4 and drops to between 5.4 and 6.3, averaging 5.5 to 5.6 in meat (Honikel, 2004). pH decline is dependent on the type of fibres within the muscle. For example, aerobic fibres enter rigor more quickly than anaerobic fibres because the former have much lower glycogen reserves than the latter. In life, the high capillary density of aerobic fibres provide continuous supply of oxygen and glucose for OXPHOS (Vollestad and Blom, 1985; Rehfeldt, 2004).

The principal physical change post-mortem is cell shrinkage. In the early stages of rigor, myofilament spacing increases due to sarcomere shortening, a consequence of continued binding of myosin heads and actin filament (Huff-Lonergan and Lonergan, 2005). The extent of longitudinal shrinkage depends on residual glycogen levels and post-mortem muscle temperature. Lateral shrinkage is also observed as biochemical changes in the environment alter myofibrillar protein structure. This shrinkage leads to the extrusion of water into the extracellular space which is easily lost as drip (Pearce et al., 2011). The ability of muscle to retain water despite external pressure is called water binding capacity (WBC) and is a critical component of meat quality (Apple and Yancey, 2013).

1.2.2 Reversal of Rigor

The reversal of rigor occurs due to major changes in the myofibrillar structure, predominantly via proteolysis of key myofibrillar proteins which include inter-myofibril linkages (e.g. desmins and vinculin), intra-myofibril linkages (e.g. titin and nebulin), those that link myofibrils to the sarcolemma by costameres (e.g. dystrophin), and the attachment of muscle cells to the basal lamina (Ouali et al., 2006). In particular, structural proteins such as titin and vinculin are targets of proteolytic enzymes (Di Luca et al., 2013). Titin is critical for intra-myofibril linkage while vinculin is integral for inter myofibril linkages and the linking of myofibrils to the sarcolemma via costameres. Further, tubulin (a major component of microtubules) and cofilin 2 (which binds actin and stabilizes actin filaments) are also degraded shortly post-mortem (Jia et al., 2007). The calpain proteolytic system is thought to play a central role in post-mortem proteolysis and tenderization (Herrera-Mendez et al., 2006). The calpain system consists of three proteases: μ-calpain, m-calpain, and calpain 3. Calpains, when activated by calcium, degrade their substrates and autolyze, causing a loss in activity. It has been reported that μ-calpain is largely responsible for post-mortem muscle tenderization (Koohmaraie and Geesink, 2006). Geesink and colleagues (2006) observed that μ-calpain knockout mice showed significantly

decreased post-mortem proteolysis compared to controls, further giving evidence to the importance of the calpain proteolytic system in tenderization and rigor reversal. Other enzymatic systems proposed to reverse rigor, and thus aid tenderness, include the cathepsins and proteasomes (Di Luca et al., 2013). More recently, apoptosis has been proposed to also play a role in rigor reversal and tenderness (Herrera-Mendez et al., 2006).

1.3 Major Sensory Characteristics of Meat

Eating quality and palatability are related to the interactions of many chemical and physical properties of meat both ante- and post-mortem (Andrés et al., 2007). For example, juiciness and tenderness depend not only on the fat content but also on the water binding capacity as well as on the structural characteristics of the meat such as contractile state of muscle fibres. Furthermore, consumers do not perceive attributes in isolation but in relation to other aspects. Colour, tenderness, juiciness, and flavour are the most important eating qualities of meat and are influenced mainly by pH (Warriss, 2000).

1.3.1 Colour

Myoglobin is the red pigmented protein primarily responsible for the colour of meat (Smulders et al., 2014). Myoglobin has a higher affinity than haemoglobin for oxygen and receives oxygen molecules from the haemoglobin in the capillaries (Listrat et al., 2016). Upon binding to oxygen, myoglobin turns a cherry-red colour forming oxymyoglobin. However, when the haem-iron oxidises post-mortem (Fe^{2+} to Fe^{3+}), metmyoglobin is formed, resulting in unattractive greyish-brown meat. The stability of the colour of fresh meat is dependent on preventing metmyoglobin formation (Faustman and Cassens, 1991). The longest shelf-life, in terms of colour, is achieved with 100% CO_2 at a storage temperature of -1.5 °C (Gill and Gill, 2005)

The longest shelf-life, is achieved with 100% CO_2 with storage at -1.5 °C (Gill and Gill, 2005) but the volume must be two to three times the meat mass to prevent collapse from CO_2 solubility (Sun and Holley, 2012).

1.3.2 Tenderness

The main components determining tenderness are myofibrillar protein degradation, connective tissue content, and fat (Guillemin et al., 2011). Firstly, post-mortem improvement of meat tenderness is due to softening of myofibrillar structure by endogenous peptidases (Ouali et al., 2006). The calpain proteolytic system is thought to play a central role in post-mortem proteolysis and tenderization (Herrera-Mendez et al., 2006). In skeletal muscle, the calpain system consists of three proteases: μ-calpain, m-calpain, and calpain 3. Calpains, when activated by calcium, degrade their substrates and autolyze, causing a loss in activity. It has been reported that μ-calpain is largely responsible for post-mortem muscle tenderization (Koohmaraie and Geesink, 2006). Geesink and colleagues (2006) observed that μ-calpain knockout mice showed significantly decreased post-mortem proteolysis compared to controls, further giving evidence to the importance of the calpain proteolytic system in tenderization. There have also been reports that cathepsins (calcium-dependent proteases) influence meat tenderness during ageing (Calkins and Seideman, 1988). Secondly, connective tissue is also a central component in tenderness. The main component of connective tissue is collagen which has a high mechanical strength brought about by the formation of intramolecular cross-links within the collagen fibre which becomes more stable (stronger, more rigid, and less susceptible to enzymatic degradation) as the animal ages (Francis, 2000). Younger animals have less collagen and a higher proportion of collagen with weaker bonds, and therefore, have more tender meat (Zapata et al., 2009). Lastly, fat contributes to tenderness by acting as a “diluent” of the muscle matrix with a high degree of marbling generally being associated with better tenderness (Cross et al., 1980; Smulders et al., 2014).

1.3.3 Water Binding Capacity

The water binding capacity of meat is of great economic relevance as it directly affects saleable weight and the perception of “juiciness” (Smulders et al., 2014). Water in the extracellular space (extruded by sarcomere shrinkage) is easily lost as drip and abnormally low water binding (high drip loss) equals lower saleable weight. It has been reported that as much as 50% of pork produced has unacceptably high drip. Additionally, drip loss involves the loss of a significant amount of protein (mostly water-soluble, sarcoplasmic proteins), as much as 112 mg of protein per mL of fluid (Huff-Lonergan and Lonergan, 2005).

1.3.4 Flavour

Complex interactions between hundreds of compounds contribute to the flavour and aroma of meat (Calkins and Hodgen, 2007). Flavour comprises two main senses: taste and smell. Although the human nose can recognise thousands of different odours, receptors in the mouth can recognize four main taste sensations: sweet, sour, salt, bitter (Farmer, 1994) and, more recently described, umami (Kurihara, 2009). While odour is generally caused by low molecular weight volatile compounds, taste substances are usually larger and water soluble. Odour compounds may reach receptors either through the nose or through posterior nares at the back of the nose and throat while food is chewed. Therefore, odour plays a major role in defining the flavour characteristics of food. Meat flavour can be influenced by diet, lipid content, oxidation, myoglobin, and pH. The diet of the animal appears to play a critical role in flavour composition: high polyunsaturated fatty acids in the diet (such as canola oil) may be a contributing factor to off-flavours in pork (Myer et al., 1992; Shackelford et al., 1995)

1.4 Factors Affecting Meat Quality

Central to post-mortem development of colour and tenderness are pH and temperature, which, themselves, are affected by ante- and post-mortem factors (Braden, 2013; Marino et al., 2013; Kim et al., 2014). pH itself is affected by temperature: the higher the temperature during rigor, the higher the rate of pH decline (White et al., 2006). Ideal ultimate pH (pHu) is between 5.5 and 5.8 at which point meat is bright red in colour, firm but not hard, non-exudative (low drip loss), lean meat surface. Residual glycogen concentration in muscle at slaughter is affected by ante-mortem factors such as diet and stress (both on- and off-farm).

1.4.1 pH

pH is perhaps the most important factor affecting meat quality being involved in tenderness, colour, and flavour (Hedrick, 1994). At the ideal pHu, proteolysis of myofribillar proteins occurs aiding rigor reversal and tenderness. Watanabe and colleagues (1996) reported significantly higher shear force values at intermediate pHu (5.8 to 6.2) compared to ideal (5.5 to 5.8). Similarly, they reported that the myofibrillar fragmentation index (MFI), a marker of proteolysis and tenderness, was lowest at intermediate pHu and highest at low pHu. pH, itself, is affected by temperature such that the higher the temperature, the faster the drop in pH (White et al., 2006). However, this rapid pH decline can lead to low pHu (<5.4) and low pHu leads to pale, soft, exudative (PSE) meat. On the other hand, low temperature can lead to slow pH decline which can lead to dark, firm, dry meat (DFD). These two are major quality defects facing the meat industry and both are affected by pHu (Adzitey and Nurul, 2011).

Buffering capacity, the ability of muscle to resist change in pH, is critical in pH decrease in rigor. The first study of meat buffering capacity was produced by Smith (1938) who determined buffering capacities of several muscle types from different species and discussed the roles of different proteins, carnosine (a dipeptide molecule of histidine and alanine), and orthophospates (Smith,

1938). Since then, it has been shown that white muscles (predominantly composed of fast glycolytic fibres e.g. M. *longissimus dorsi)* have higher buffering capacities than red muscles (mainly composed of slow oxidative fibres e.g. *Mm. supraspinatus)*. Post-rigor, this means that white muscles have lower pHu than red muscles (Rao and Gault, 1989). This is attributed to their higher contents of carnosine and inorganic phosphorus. White muscle fibres require high buffering capacities because they rely on glycolytic metabolism, the by-product of which is lactic acid and H+ ions which lower pH (Puolanne and Kivikari, 2000). Buffering capacity in these muscles, therefore, must be high to prolong the time of effective fibre activity. The same compounds that regulate pH in living muscle fibre also regulate pH in post-rigor meat. The compounds that most affect the buffering capacity at pH range 5.5 to 7.0 are: phosphate compounds having pK_a between 6.1 and 7.1, histidylimidazole residues of myofibillar proteins, and dipeptides carnosine and anserine (Sewell et al., 1992).

1.4.2 Anomalies in Ultimate pH (pHu)

1.4.2.1 Dark, firm, dry (DFD)

This is observed when pHu is abnormally high (>6.0). High pHu results in little protein denaturation, leading to little or no shrinkage of the myofilament lattice causing water to be tightly bound within the muscle (high water holding capacity) leading to high shear force (tough) (Francis, 2000; Adzitey and Nurul, 2011). The refractive index of myofibrils and sarcoplasm is reduced so that muscles absorb light, making meat appear darker. Furthermore, oxygen penetration is reduced resulting in only a thin layer of oxymyoglobin forming, allowing the purple colour of the underlying metmyoglobin be visible underneath. Smulders and colleagues (2014) explains that there are three main mechanisms that explain abnormally high pHu: a) poor pre-slaughter handling resulting in substantial depletion of muscle glycogen reserves while animals are not allowed sufficient recovery time; b) adrenergic activation of

glycogenolysis by increased adrenaline concentration in the blood; and c) long-term depletion of glycogen as a result of malnutrition or starvation.

1.4.2.2 Pale, soft, exudative (PSE)

Lower than ideal pHU (<5.4) leads to pale, soft, exudative (PSE) meat (Francis, 2000). Acute stress before slaughter, such as the use of electric goads, fighting, and overcrowding in lairage causes early acidification in muscles post-mortem due to glycogen breakdown (Adzitey and Nurul, 2011). This acidification prior to slaughter leads to lower pH values in carcass when temperatures are still high. The combination of low pH and high temperature causes denaturation of muscle proteins (high levels of proteolysis) leading to lower than normal water holding capacity. Abnormally low water holding capacity (high drip loss) leads to lower saleable weight (Huff-Lonergan and Lonergan, 2005). It has been reported that as much as 50% of pork produced in the US has unacceptably high drip. This also involves the loss of a significant amount of protein, as much as 122 mg of protein per mL of fluid (mostly water-soluble, sarcoplasmic proteins).

Two major factors contributing to suboptimal glycogen levels at slaughter are excessive glycogen depletion from stress during pre-slaughter handling (loading animals at the farm, transport form farm to abattoir, unloading animals at the abattoir, and slaughter), lairage (housing), and poor quality diet (Speer et al., 2001). Daly and colleagues (1996) have reported that sheep lairaged overnight and swim washed had significantly less muscle glycogen than a tailgate group (transported directly to slaughter) and consequently, their pHu values were significantly higher. These findings were supported by Miranda-da Lama and colleagues (2009) who reported that lairage time had a significant effect on meat quality attributes, independent of season. However, they also found there was no significant difference in pHu animals who were and were not subjected to a long, double transport, agreeing with Adnoy and colleagues (2005). Dehydration in holding yards during transport before exsanguination may also contribute to high pHu by increasing glycolytic response to stress (Daly and Wright, 1996). Furthermore, there is evidence than changes in nutritional value of the feeding regime, whether beneficial or not, decreases

muscle glycogen (Devine et al., 1983). The highest pHu were found in animals who have received a high level of nutrition following an earlier low level (Daly et al., 1996). However, deliberately producing high pHu in experiments can be difficult and often, despite relatively high levels of exercise, glycogen levels can remain relatively unchanged (Apple et al., 1994). The only reliable method experimentally is by adrenalin administration. The one exception are young bulls, who, if mixed with bulls from other farms, either during transport or on arrival at the processing plant, will suffer high pHu at slaughter (McVeigh et al., 1982).

Seasonality may also influence pH and other meat quality attributes. It has been reported that changes in environmental temperatures stress animals and increases the rate of DFD (Miranda-de la Lama et al., 2009) by as much as 30.3% as was observed during winter in the Spanish plateau (Sotelo et al., 2008)

1.4.3 Temperature

Carcass temperature decreases at the same time as it enters rigor mortis. Different muscles lose heat at different rates, mainly due to size and inherent metabolic differences (Jacob and Hopkins, 2014; Kim et al., 2014). Temperature directly affects pH as it influences glycolysis rate. For example, as temperature increases, so does glycolysis, and, in turn, leads to more rapid pH decline. The degree of sarcomere shortening is also dependent on temperature: shortening was minimal when pre-rigor muscles were held at 15°C to 20°C (Kim et al., 2014). A shortening of 30% less than normal length was observed at temperatures of 42°C (heat-induced toughening), whereas, severe shortening (50% less than normal length) was observed at 0°C (cold shortening).

1.4.3.1 Heat-Induced Toughening

Numerous studies have reported on the negative effects of accelerated pH decline combined with high temperature on post-mortem tenderness, called

heat-induced toughening, in beef (Devine et al., 1999; Thompson, 2002; Rosenvold et al., 2008) and lamb (Geesink et al., 2000; Devine et al., 2002; Rosenvold and Wiklund, 2011). Generally, meat entering rigor at 15°C produces more tender loin meat than meat that entered rigor at 35°C to 38°C (Kim et al., 2014). Rosenvold and Wiklund (2011) reported that lamb loins from non-electrically stimulated carcasses held at 42°C pre-rigor had significantly higher shear force values that those held at 15°C pre-rigor, even after 7 weeks of ageing at -1.5°C. This indicates that extended ageing cannot overcome the negative effects of high temperature and rapid pH decline on meat tenderness development.

1.4.3.2 Cold Shortening

Cold shortening is the result of the rapid chilling of carcasses immediately after exsanguination, before glycogen is converted to lactic acid and before rigor has taken hold, causing irreversible muscle contraction (Daly and Wright, 1996; Braden, 2013). It can be prevented if temperatures below 10 ^{0}C are avoided until pH falls below 6. Daly and Wright (1996) observed that the lowest pHu is attained when the muscle is at 15^{0}C but increases significantly, from 5.5 to 6.0, if the temperature is lowered to 0^{0}C. Cold shortening tends to be more severe than heat-induced toughening. Generally, shortening is one of the major contributors to tough meat because, the shorter the sarcomere, the tougher the meat (Kim et al., 2014).

1.5 Physiological Stress Markers

Heat shock factors are transcriptional factors that activate under physiological stress resulting in the rapid synthesis of heat shock proteins (HSPs) which are found in virtually all organisms (Feder and Hofman, 1999; Kregel, 2002; Shamovsky and Nudler, 2008). HSPs are molecular chaperones that bind and stabilise unstable proteins to facilitate correct assembly, but are not, themselves, components of the final structure (Hendrick and Hartl, 1993). Small HSPs (sHSPs) function in an ATP-dependent manner and are integral for proper functioning of mammalian cells under stress conditions (Welch,

1992). HSPs have been widely used to measure physiological stress levels of organisms from fish (George et al., 1999) to rats (Goering et al., 1993) to humans (Rossi et al., 2002) and are ubiquitous in research that involves cellular stress. Currently, HSP20, HSP27, and αβ-crystallin have been implicated in meat quality attributes (Lomiwes et al., 2014). Oxidative stress and eccentric contractions have been reported to increase HSP27 and αβ-crystallin expression in skeletal muscle (Paulsen et al., 2007) where they translocate from the sarcoplasm to the I-band and Z-disc of the myofibril (Koh and Escobedo, 2004). In this thesis, HSP20, a sHSP family with an average molecular weight of 20 kiloDaltons (kDa), was used as a marker for lamb physiological stress (Groenen et al., 1994).

1.6 Summary

Lamb meat production is a significant part of the New Zealand industry, providing billions in profit, yet little is research has been done on muscle glycogen levels on-farm. Elucidating normal muscle glycogen in on-farm lambs at different time points and with different production systems will give a clearer picture of how glycogen levels can be managed and preserved. In order to ensure the competitiveness and quality of New Zealand lamb meat production system. Defining a relationship between heat shock proteins and glycogen, would have high commercial implications as they can be used to determine cellular stress, and therefore, muscle glycogen levels of lambs prior to slaughter. Keeping high residual glycogen will have immediate commercial value to farmers because consumers are willing to pay a premium for high quality meat, especially in overseas markets. Therefore, the aim of this study is to investigate the variation in glycogen levels on-farm, examine the use of HSP20 as a marker for physiological stress, and determine any relationships between residual glycogen levels and meat quality attributes such as water binding, drip loss, microbial populations, and to a lesser extent, fat, protein, and water content.

Chapter 2

Methods

2.1 Serial Biopsies

To identify muscle glycogen variation in lambs, 60 lambs from 2 farms with different production systems were followed through to slaughter. Lambs from Farm 1 were born on-farm and raised to slaughter. Males were castrated early on (wethers) and mixed with ewes (females). Farm 2 lambs were bought from other farms, fattened on-farm, and sent to slaughter. Rams (left intact), cryptorchids (males with undescended testicles), wethers (castrated males), and ewes were all together in a group on this farm. They were biopsied with a biopsy gun (16-gauge needle) removing approximately 20 mg from the loin. Samples were placed on ice for transport, and immediately placed in liquid nitrogen in the lab for later analysis. From this sample the following assays were conducted: HSP20 sandwich ELISA assay, hexokinase glycogen assay, and BCA assay. HSP20 here was used as an indication of physiological stress, glycogen was used to measure glycogen levels, and BCA assay was used to determine soluble protein as a check that samples taken were not full of fat. Muscle volume is usually calculated by dividing muscle mass by muscle density. Density was calculated at 1.0597 g/cm^3 (or 1.06 g/mL) (Mendez et al., 1960). It should be noted, however, that there may be a potential for species-specific difference in muscle density (Ward and Lieber, 2005) but as of yet, this is the value used in research.

These 60 lambs were grouped into 3 (n=20). The first group (red, n = 20) were biopsied a few weeks after birth (biopsy 1, B1), again a month later (biopsy 2, B2), and then 24 hours after slaughter i.e. post-rigor (post-rigor, PR). The second group (yellow, n = 20) were sampled in B1 and PR. The third group (green, n = 20) were sampled at B2 and PR. This was done to identify any extra stress that animals may have encountered during the initial biopsy. Post rigor

carcasses were first measured for pH then samples were taken with a core borer that removed approximately 200 mg of meat from the loin. The same assays were done as with samples from on farm biopsies with the addition of a glucose assay.

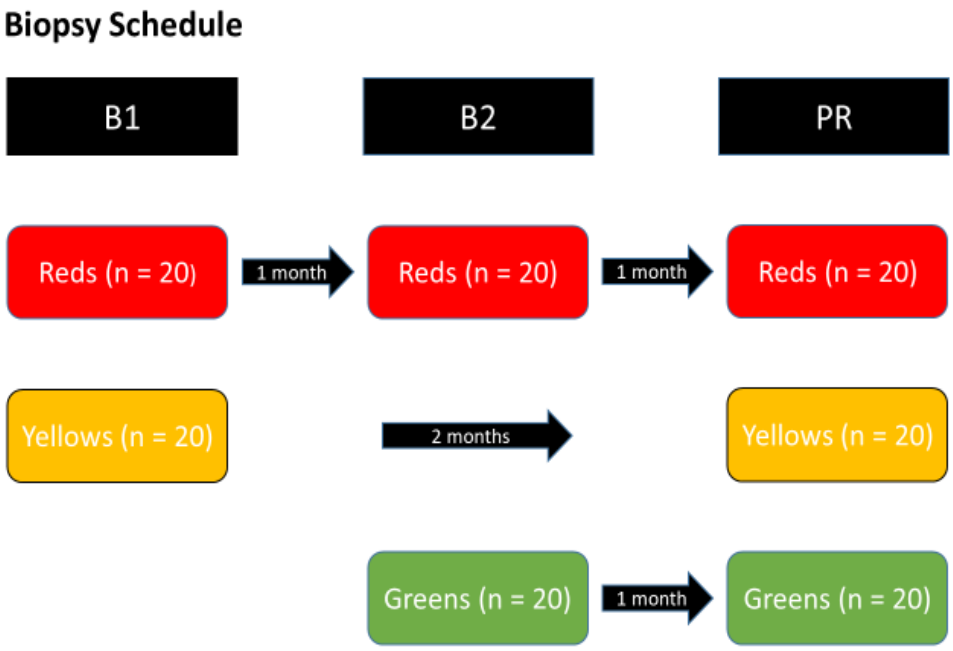

Figure 2.1. Flow diagram of when lamb groups were biopsied. 60 animals per farm and 20 animals per biopsy.

2.1.1 HSP20 Sandwich ELISA Assay

1. ***Coating:*** 100 uL of coating solution (see Appendix 1) were pipetted into each well in a 96-well microtiter plate, covered, and incubated overnight at 4^0C.
2. ***Blocking:*** Coating solution was removed and the plate was washed three times with 250 uL of PBS-T (see Appendix 1).
3. Remaining protein-binding sites were blocked with 250 uL blocking buffer per well. Plates were again covered and incubated at room temperature for one to two hours.

4. ***Sample Preparation:*** Biopsied samples were weighed and added to 1 mL of homogenisation buffer (see Appendix 1) and homogenised at 12 000 rpm until completely homogenised. 300 uL was removed for glycogen determination
5. The remainder was centrifuged at 2 400 rpm for 3 minutes at 4 ^{0}C.
6. Samples were serially diluted into 1:2, 1:4, 1:8, and 1:16 using PBS-milk as diluent.
7. 100 uL of standard and sample were aliquoted into corresponding wells in duplicate with the diluent as blank. Plate was covered and incubated at room temperature for 1 hour.
8. ***Detection:*** Samples were removed and the plate washed again with 250 uL of PBS-T 3 times.
9. 100 uL of detection antibody (see Appendix 1) was added to each well, covered, and incubated for 1 hour at room temperature.
10. Detection antibody was removed and the plate washed 3 times with 250 uL of PBS-T.
11. 100 uL of secondary antibody (see Appendix 1) was added to each well, covered, and incubated again for 1 hour at room temperature.
12. Secondary antibody was removed and washed four times with 250 uL PBS-T.
13. 100 uL of OPD solution was added to each well, covered with tinfoil, and incubated for 10 to 30 minutes at room temperature.
14. 50 uL of 2M sulfuric acid was then added to each well to stop the reaction.
15. The absorbance was then measured at 490 nm within 30 minutes of ending the reaction.

Standard Curve

In microtiter tubes, a serial dilution of the HSP20 standard was done as follows:

Table 2.1. HSP ELISA standard curve.

Standard Concentration (ng/mL)	HSP20 Standard (uL)	Diluent (uL)	Total Volume (uL)
50	25	475	500
25	240	240	480
12.5	240	240	480
6.25	240	240	480
3.125	240	240	480
1.56	240	240	480
0.78	240	240	480

Concentration Calculation:

i. Corrected Absorbance:
   Average absorbance (sample or standard) – Average absorbance (blank)
ii. Standard curve formed using corrected absorbance with the standard concentrations on the y-axis. Slope and R^2 value provided.
iii. Wells (ng/mL):
   Corrected Absorbance (sample) x Slope (standard curve)
iv. Pre-dilution (ng/mL):
   Wells (ng/mL) x 2 (1:2 dilution)
   Wells (ng/mL) x 4 (1:4 dilution)
   Wells (ng/mL) x 8 (1:8 dilution)
   Wells (ng/mL) x 16 (1:16 dilution)
v. Per sample (ng/g):
   Dilution (ng/mL) / Sample weight (g)
vi. Volume correction (ug/g):
   [(ng/g) x [1 +(sample weight (g) x 1.06)]] / 1000
vii. Final Concentration (ug/g):
   Average= [1:2 (ug/g) + 1:4 (ug/g) + 1:8 (ug/g) + 1:16 (ug/g)] / 4

2.1.2 Hexokinase Glycogen Assay

Glycogen content of muscles can be determined by an acid hydrolysis method (Passonneau and Lauderdale, 1974). Glycogen is hydrolysed into glucose sub-units (enzymes are inactivated by the acid) and converted into glucose-6-phosphate (G-6-P) by hexokinase in the presence of ATP. With a supply of NADP, G-6-P is further converted to 6-phosphogluconic acid by G-6-P dehydrogenase. During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration and has a sensitivity range of 0.05 to 5 mg/mL (Zhang, 2012). The following method was adapted from Zhang's protocol (2012) and follows the commercial protocol from Sigma-Aldrich .

1. 300 uL of homogenised sample from HSP20 assay (before centrifugation) was added to 74 uL of concentrated HCl (for a final concentration of 2N HCl) in a 2 mL microfuge tube with a hole punched on the lid to allow vapours out.
2. Tubes were boiled for two hours with occasional vortexing.
3. Tubes were then centrifuged at 4 000 rpm for 3 minutes at 4 ^{0}C.
4. 200 uL of supernatant was removed and added to 200 uL of 2 N NaOH (to neutralise the acid).
5. 40 uL was aliquoted from each sample, standard, and blank into wells in a microtiter plate in duplicate.
6. 200 uL of hexokinase reagent (*Sigma Aldrich GAHK20*) was added to each well and incubated at room temperature for 20 minutes.
7. Absorbance was read at 340 nm.

Table 2.2. Hexokinase glycogen assay standard curve

Concentration (µM)	7 mM Glucose solution (µl)	HCl/NaOH mix
1500	214	786
1000	143	857
700	100	900

350	50	950
200	29	971
100	14	986
50	7	993
Blank	0	1000

Concentration Calculation:

i. Corrected absorbance:
   Average absorbance (sample or standard) – average absorbance (blank)
ii. Standard curve was made with concentration on the y-axis. Concentrations were converted to ug/mL.
iii. Wells (ug/mL):
   Corrected absorbance x Slope (standard curve)
iv. NaOH Dilution (ug/mL):
   Wells (ug/mL) x [0.4 / 0.2]
v. HCl Dilution (ug/mL)
   NaOH dilution (ug/mL) x [0.374 / 0.3]
vi. Per sample (ug/g):
   HCl dilution (ug/mL) / Sample weight (g)
vii. Volume Correction (ug/g):
   (ug/g) x [1 +(sample weight (g) x 1.06)]
viii. Convert to uM/g:
   Volume correction / 180.1 (MW glucose)

2.1.3 BCA Assay

The bicinchoninic acid assay (BCA assay) was used to determine the total concentration of soluble protein in solution with a range of 0.5 ug/mL to 1.5 mg/mL. It was used as a check to ensure that the sample was not a clump of fat which is common for biopsies. This procedure was followed as per Pierce BCA Protein Assay Kit #23225.

1. Incubator was first set at 37 ^{0}C.
2. 300 uL of homogenised supernatant sample from HSP20 assay was taken and serially diluted to 1:2 and 1:5 as follows

Dilution	Sample Volume (uL)	Diluent (uL)
Neat	300	0
1:2	30	30
1:5	24	36

3. 10 uL of each sample dilution and standard was aliquoted into corresponding wells in duplicate. Distilled water was used as blank.
4. 200 uL of BCA reagent were added to each well, covered, and incubated for 1 hour at 37 ^{0}C.
5. Plate was read at 560 nm.

Table 2.3. Standard Curve: 2 mg/mL bovine serum albumin was serially diluted to 0.03 mg/mL with distilled water.

Standard (mg/mL)	Standard Volume (uL)	Diluent Volume (uL)
2	200	0
1	100	100
0.5	100	100
0.25	100	100
0.125	100	100
0.06	100	100
0.03	100	100

Concentration Calculation similar to ELISA:

i. Corrected Absorbance:
   Average absorbance (sample or standard) – Average absorbance (blank)
ii. Standard curve formed using corrected absorbance with the standard concentrations on the y-axis. Slope and R^2 value provided.
iii. Wells (mg/mL):
   Corrected Absorbance (sample) x Slope (standard curve)

iv. Pre-dilution (mg/mL):
Wells (mg/mL) x 1 (neat) or
Wells (mg/mL) x 2 (1:2 dilution) or
Wells (mg/mL) x 5 (1:5 dilution)

v. Per sample (mg/g):
Dilution (mg/mL) / Sample weight (g)

vi. Volume correction (mg/g):
(mg/g) x [1 +(sample weight (g) x 1.06)]

vii. Final Concentration (mg/g):
Average dilutions= [Neat (mg/g) + 1:2 (mg/g) + 1:5 (mg/g)] / 3

2.1.4 Hexokinase Glucose Assay

Free glucose concentration within the muscle can be measured using the same process as the glycogen assay but without hydrolysis so that only the glucose present at sampling is quantified (Zhang, 2012). The limitation with this assay is that since samples are frozen quickly after removal, enzyme may reactivate once thawed and continue to break down glycogen to glucose, overestimating the amount of glucose in the sample. Therefore, this was done immediately after homogenisation.

1. Homogenised sample in the homogenisation buffer was centrifuged at 4 000 rpm for 3 minutes at 4 ^{0}C.
2. 40 uL of each sample was aliquoted and pipetted into the corresponding well in a 96-well microtiter plate. The same was done for each standard and blank.
3. 200 uL of hexokinase was added to each well.
4. The plate was incubated at room temperature for 20 minutes and the absorbance read at 340 nm.
5. Pellets were re-suspended for the HSP20 ELISA and BCA assays.

Table 2.4. Hexokinase glucose assay standard curve.

Concentration (µM)	7 mM Glucose solution (µl)	HCl/NaOH mix
1500	214	786
1000	143	857
700	100	900
350	50	950
200	29	971
100	14	986
50	7	993
Blank	0	1000

Concentration Calculation:

i. Corrected absorbance:
   Average absorbance (sample or standard) – Average absorbance (blank)
ii. Standard curve made using corrected absorbance and concentrations converted to ug/mL. Standard concentrations on y-axis.
iii. Wells (ug/mL):
   Corrected absorbance (sample) x Slope
iv. Per sample (ug/g):
   Wells (ug/mL) / Sample weight (g)
v. Volume Correction:
   (ug/g) x [1 +(sample weight (g) x 1.06)]
vi. Convert to uM/g:
   Volume correction / 180.1 (MW glucose)

2.2 Yield Trial

This trial was to examine the relationship between residual glycogen and muscle yield in terms of moisture, protein levels, fat levels, the meat quality, and microbial growth. From a selection of 399 carcasses with normal pH

(originally 400 but one was dropped and removed from the count) in a commercial meat plant. Certain meat quality characteristics are largely influenced by pHu, therefore, by taking only carcasses that had pHu of less than 5.8, this variable was removed and results will only reflect differences in respect to glycogen levels. From the initial samples (n = 399), pH was measured prior to biopsy using core borers which removed approximately 200 mg of meat from the loin. The iodine assay was used to roughly and quickly estimate glycogen levels. Of the 399, 69 samples were chosen which fell in 3 glycogen ranges namely: high (n = 25), medium (n = 21), and low (n = 23) glycogen content. This was done with the assumption that high post-rigor glycogen would mean high pre-rigor glycogen since carcasses were treated under the same conditions in the meat plant. The following day, the striploin was removed and placed on ice. From this, the following analyses were carried out: hexokinase glycogen and glucose assays, drip loss, water binding capacity (WBC), soxhlet fat extraction, Dumas assay for crude protein determination, and microbial levels via aerobic plate count (APC). This was done over three weeks, 100 were done in the first week (Set 1), 150 in the second week (Set 2), and another 150 in the third week (Set 3). Drip, WBC, glycogen, glucose, and APC were repeated eight weeks (Week 8) to simulate the maximum time taken for meat to reach overseas markets, particularly the UK.

2.2.1 Iodine Glycogen Assay

As a colorimetric test, this assay was used to visually estimate the amount of glycogen present in the sample (Krisman, 1962; Bennett et al., 2007b). The original method adapted from Dreiling and colleagues (1987) was as follows:

1. 20 mg of tissue was submerged in 7% perchloric acid (PCA) and homogenised.
2. Sample was centrifuged at 4 000 rpm for 3 minutes at 4 ^{0}C.
3. 30 uL of sample supernatant or standard were aliquoted to each microtiter well in duplicate.
4. 95 uL of the colour reagent (see Appendix 2) was added to each well.

5. Absorbance was measured at 490 nm immediately and was stable for up to 2 hours.

This protocol provided satisfactory colour intensity for pre-rigor samples but showed in poor colour intensity in the post rigor. It was speculated that the this was due to the significantly lower levels of glycogen present in post rigor samples compared to pre-rigor samples (which would have a higher concentration of glycogen). Therefore, the method was altered for these conditions.

2.2.2 Method Development

First, homogenised sample mass (pre-rigor) was increased to see if colour intensity also increased proportionally. The sample weights were increased to 100 mg, 200 mg, and 400 mg. The original protocol was also added onto the plate for comparison. These were all done in duplicate.

Table 2.5. Comparison of average absorbance between 50 mg, 100 mg, 200 mg, and 400 mg samples.

	Average Absorbance	
Sample Weight (mg)	Sample A	Sample B
20	0.1920	0.2030
50	0.2750	0.4700
100	0.6010	0.5340
200	1.4165	1.1150
400	2.7585	2.1245

Results showed a definite increase in colour intensity with increasing sample weight. At this stage, sample weights 20 mg and 50 mg were removed as there was very little colour change observed.

Second, the amount of aliquot pipetted on the plate from the homogenate was also increased to determine at what aliquoted volume showed enough variation between low glycogen, medium, and high glycogen content. From the remaining sample weights, aliquoted volumes increased to 60 uL, 90 uL, and 120 uL. For comparison, 30 uL aliquot was also added. It was decided that 90 uL and 120 uL aliquoted from 400 mg would likely be too saturated.

Table 2.6. Average absorbance read at increasing sample weights and sample volume aliquoted.

		Average Absorbance			
		Sample			
Weight (mg)	Aliquot (uL)	1	2	3	4
100	30	0.0981	0.3878	0.4305	0.0395
	60	0.1568	0.5041	0.5927	0.0506
	90	0.1450	0.5620	0.7118	0.0426
	120	0.1576	0.5806	0.7422	0.0580
200	30	0.1614	0.6590	0.7166	0.0530
	60	0.2386	0.8408	1.0239	0.0728
	90	0.2605	0.9227	1.0597	0.0476
	120	0.2980	0.9717	1.1629	0.0814
400	30	0.1788	1.0709	0.8578	0.03105
	60	0.2390	1.2306	1.1322	0.0406

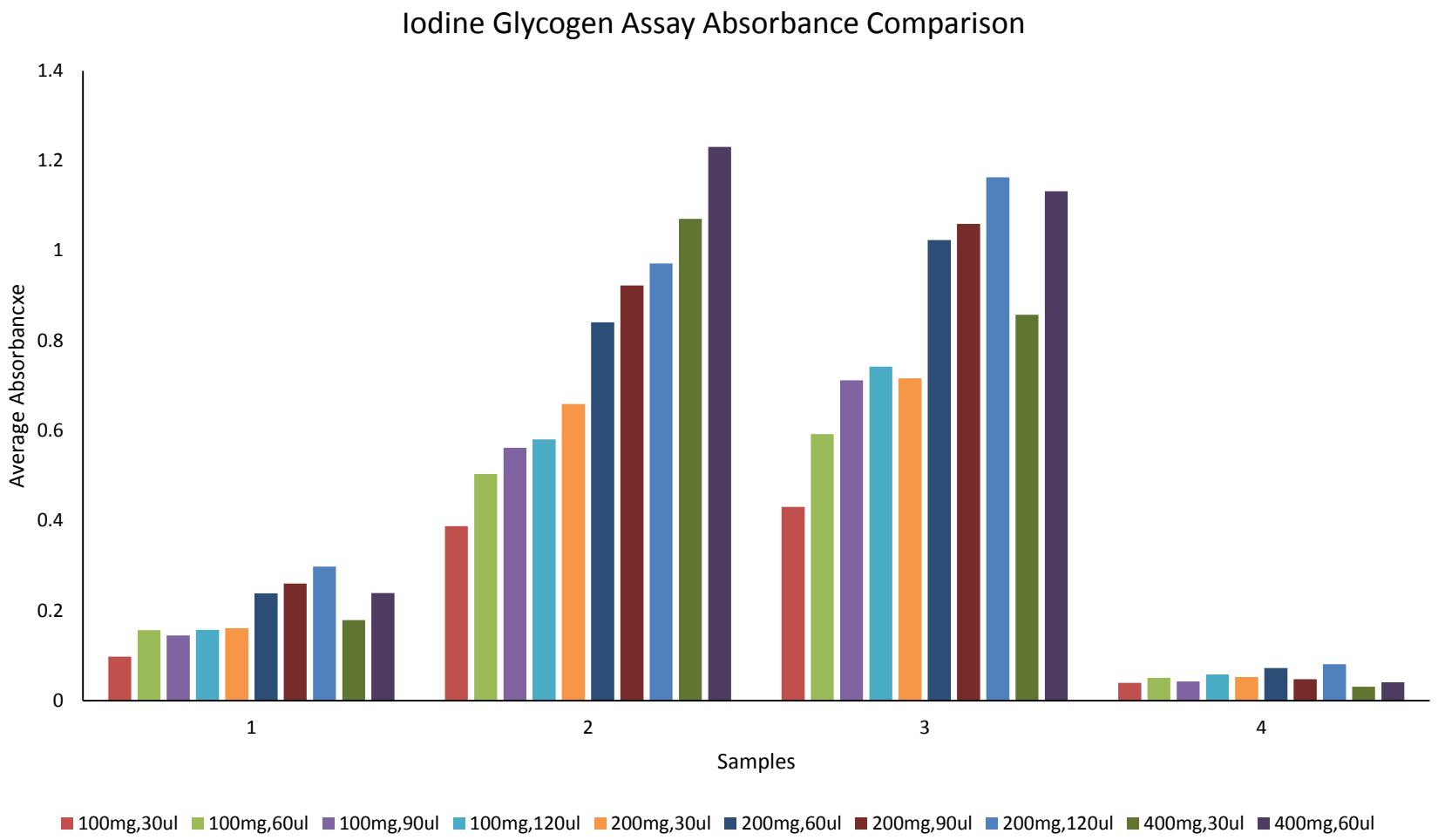

Figure 2.2. Comparison of average iodine absorbance between the different sample weights and sample volumes.

The standard curve had a maximum average absorbance of 1.070 and a minimum of 0.122. It was therefore, decided that 200 mg homogenised sample with 90 uL aliquoted onto the plate gives enough sensitivity and variation in colour intensity that a difference between samples with low, medium, and high glycogen content can be determined even visually.

Samples with the absorbance above 0.500 were chosen as high glycogen, below 0.450 but above 0.200 were chosen as medium, and anything below 0.200 were chosen as low. Criticisms of this assay include: limited detection range and quantification (compared to other methods), concentration-absorbance relationship is affected by the source of standard (rabbit liver vs bovine liver), and that it is a test of whole glycogen concentrations, which may be affected by structural differences in glycogen (Dreiling et al., 1987; Bennett et al., 2007b). Therefore, the hexokinase glycogen assay was used to more accurately determine glycogen concentration.

Concentration Calculation:

i. Corrected Absorbance:
   Average absorbance (sample or standard) – Average absorbance (blank)
ii. Standard curve formed from corrected absorbance of standard. Standard were situated on the y-axis and converted to ug/uL
iii. Wells (mg/mL):
   Corrected absorbance (sample) x Slope (standard curve)
iv. Volume correction in wells:
   Wells (mg/mL) / 3 [30 uL of standard was aliquoted in wells)
v. Per sample (mg/g):
   (mg/mL) x Sample weight (g)
vi. Convert to ug/g:

(mg/g) x 1000

vii. Volume Correction (ug/g):
(ug/g) x [1 + (sample weight (g) x 1.06)]

viii. Convert to uM/g:
(ug/g) / 180.1 (MW glucose)

2.2.3 Hexokinase Glycogen Assay (Straight Acid)

The only difference with this assay and the one previous is that 100 mg of sample was homogenised in 3 mL 2N HCl (instead of taking 300 uL of 20 mg in 1mL homogenisation buffer and adding 74 uL fuming HCl). This was done because more sample was available for analysis. The same principle applies; glycogen is broken down into glucose sub-units and the change in absorbance at 340nm is proportional to the concentration of glycogen in the sample.

Concentration Calculation:

i. Corrected absorbance:
Average absorbance (sample or standard) – Average absorbance (blank)

ii. Standard curve formed using corrected absorbance. Concentrations converted to ug/mL on the y-axis.

iii. Wells (ug/mL):
Corrected absorbance (sample) x Slope (standard curve)

iv. NaOH Dilution (ug/mL):
Wells (ug/mL) x (0.4 / 0.2)

v. Per sample (ug/mg):
(ug/mL) x Sample weight (g)

vi. Volume Correction (ug/mL):
ug/g x [3 + (Sample weight (g) * 1.06)]

vii. Convert to uM/g
(ug/g) / 180.1 (MW glucose)

2.2.4 Hexokinase Glucose Assay

This assay was also done as previous; the only exception is that 100 mg of sample was homogenised in 6 mL of water to equal conditions of the glycogen assay.

Concentration Calculation: Refer to Section 2.3.3 Hexokinase Glycogen Assay (Straight Acid)

2.2.5 Drip Loss

Drip loss was measured by placing a 50 g square sample of meat cut from the core of the striploin an overwrap retail display tray and maintained at 0 ^{0}C at a 15^0 angle vertically. Drip loss was determined as the weight lost after 5 days.

2.2.6 Water Binding Capacity

This measured the “succulence” i.e. the amount of water present in the meat. This was done using the common press method (Irie et al., 1996) whereby exactly 50 mg of sample was placed on a filter paper and pressed for exactly 60 seconds. A photo was taken and, the meat and liquid areas were measured. These were done in duplicates.

Calculations:

i. Outer Area (cm^2) – Inner Area (cm^2) = Liquid Area (cm^2) / 5g sample

2.2.7 Soxhlet Fat Extraction Protocol

This was adapted from the United Stated Department of Agriculture (2009) protocol for fat determination. The theory is that as the solvent heats up and boils, the condenser cools it down, allowing the solvent to trickle into the extractor (where the thimble and sample are). The fat dissolves in the solvent

and, when the extractor is full enough, the solvent and fat siphons back down into the flask (see Figure 1). Ideally, this process occurs for at least 60 cycles which would ensure complete extraction.

Procedure:

1. Samples were homogenised.
2. Using an aluminium dish, 8 g of sample was weighed by difference, in duplicate.
3. Samples were dried overnight at 100 ^{0}C, cooled in a desiccator, and reweighed.
4. Thimbles (Whatman Cellulose extraction thimble 33 mm x 100 mm) were cut by 1 cm at the top to equal the length of the extractor siphon tube.
5. Dried sample was carefully placed inside thimble. If visible fat was present on the dish, thimble was used to scoop up the fat. Thimble was then placed in the extractor
6. 190 mL of petroleum spirit (60 to 80 ^{0}C boiling point) was added to round-bottom flasks with 5 to 6 boiling chips, placed in the heater and covered with the soxhlet extractor and condenser on top. These setups were monitored for at least two extractions to ensure that solvent siphoned back into the flask does not cause already present solvent to go off the boil (which would later overboil causing a fire and explosion hazard).
7. The extractions continued for five hours. A single extraction took approximately 4 minutes, allowing approximately 75 extractions in total.
8. After five hours, heaters were turned off, and flasks allowed to cool.
9. Once the flasks were cool, they were placed in a rotary evaporator (with the water bath temperature set at 45 ^{0}C, the vacuum at max, and the rotation set at 3) to evaporate out the solvent. The rotary evaporator decreases the pressure in the system, hence, the boiling point of the solvent decreases. A condenser cools down the solvent which is collected in a recycling flask (fresh solvent) to be used again.

10. After complete evaporation of the solvent (only a yellow liquid remaining), the flask is removed from the rotary evaporator and the liquid poured into a weighed aluminium dish. A small amount of solvent is added to the flask to ensure all the fat pours into the dish. This was done twice.
11. Dishes are then dried in a drying oven (75 ^{0}C) for 5 minutes, cooled, and reweighed.

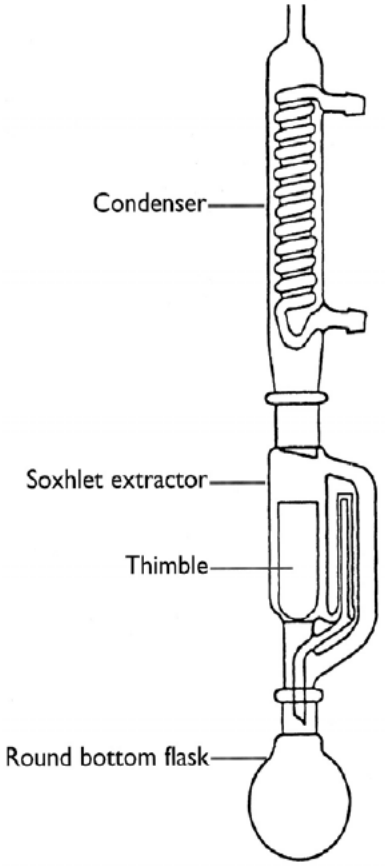

Figure 2.3. Soxhlet setup from Food Science Australia (1998).

Crude Fat Calculation:

% Fat = ((W_2-W_1) / S) * 100

Where:

W_1 = empty aluminium dish

W_2 = dish + fat

S = Wet Sample Weight

Water Content Calculation:

% Water Loss = $[(W_2 – W_1) / W_1] * 100$

Where:

W_1 = Wet Sample Weight

W_2 = Dry Weight

Dish weight was already removed from dry and wet weight.

2.2.8 DUMAS

This was done by Hills Laboratory at Waikato Innovation Park and tests the total nitrogen content of the sample. Crude protein can be calculated using coefficient of 0.94 since this method measures total nitrogen content (including nitrates, nitrites, and other nitrogen-containing compounds) (Simonne et al., 1997). Samples are first homogenised then combusted as per AOAC International Standards 992.15, 19th edition.

2.2.9 Aerobic Plate Count (APC)

This test was used as an indicator of bacterial populations in the sample and were done by AsureQuality Ltd Auckland. Swabs of the loin were taken after boning and delivered to the lab. Units were in colony forming units (CFU).

Chapter 3

Results

3.1 Serial Biopsies

3.1.1 Glycogen

In this experiment, total glucosyl and free glucose were measured to provide the "true" glycogen value (Total Glucosyl – Free Glucose = True Glycogen). Means and 95% confidence limits are shown for all scatter plots. The Green group was introduced at Biopsy B to see whether the first biopsy increased stress levels (Figure 2.1, experimental design). Some values from Farm 2 Red group had to be omitted because of sample contamination and missing post-rigor samples. At Biopsy A, Farm 1 total glucosyl was significantly higher than Farm 2 with medians of 84.31 µmol/g and 70.89 µmol/g, respectively (p = 0.012) with a non-normal distribution (Figure 3.1). In terms of free glucose (also non-normally distributed), medians were 14.32 µmol/g and 13.41 µmol/g, respectively, but surprisingly, there was no significant difference between the two data sets (p =0.435). Moreover, when looking at true glycogen, the difference between the two farms, as indicated by total glucosyl, disappeared (p =0.1914). Farm 1 true glycogen had a mean of 71.06 µmol/g (s^2 =30.0) while Farm 2 mean true glycogen was 59.51 µmol/g (s^2 =20.52) with a normal distribution according to KS normality test. There was also a large variation in the total glucosyl values with a range of 185.59 µmol/g for Farm 1 and 133.79 µmol/g for Farm 2 which translated to true glycogen ranges of 132.9 µmol/g and 113.57 µmol/g, respectively.

What became more surprising was results from Biopsy B (Figure 3.2). Despite animals being regularly introduced to the flock and intact males beginning to exhibit sexual behaviours, there was no significant difference between Farms 1 and 2 in either total glucosyl (p =0.751) with means of 74.85 µmol/g (s^2 = 24.42) and 71.48 µmol/g (s^2 = 26.0), respectively, or true glycogen (p =0.55) with means of 67.95 µmol/g (s^2 = 20.71) and 63.29 µmol/g (s^2 =20.58). One

particular ram in the Red group from Farm 2 was observed who would constantly chase around the females especially during Biopsy B. Focusing on the Red groups of both farms, a significant difference was found in their glycogen levels both at Biopsy A and B (Figure 3.3). True glycogen at Biopsy A was higher for Farm 1 (77.89 µmol/g, s^2 = 21.42) than Farm 2 (45.37 µmol/g, s^2 = 27.45) (p = 0.0007). The same trend was observed at Biopsy B with means of 77.59 µmol/g (s^2 =19.18) and 50.39 µmol/g (s^2 = 21.04), respectively. Admittedly, these sample sizes are rather small with 19 and 14 lambs, respectively.

Another interesting result was the difference in total glucosyl between Biopsies A and B. Focusing on the Red group from Farm 1, since few values from Farm 2 were viable for our purposes, again due to contamination and a lack of post-rigor samples, there was little correlation between the total glucosyl at Biopsies A and B (R^2 = 0.1884), but, when particularly looking at the change in total glucosyl between these two biopsies, a moderate correlation is revealed (R^2 = 0.481) in that lambs with high total glucosyl values at one time-point decreased at the next, and vice versa (Figures 3.4 and 3.5). A preliminary interpretation of this data could be that glycogen tends to fluctuate, rather than remain constant as was originally believed.

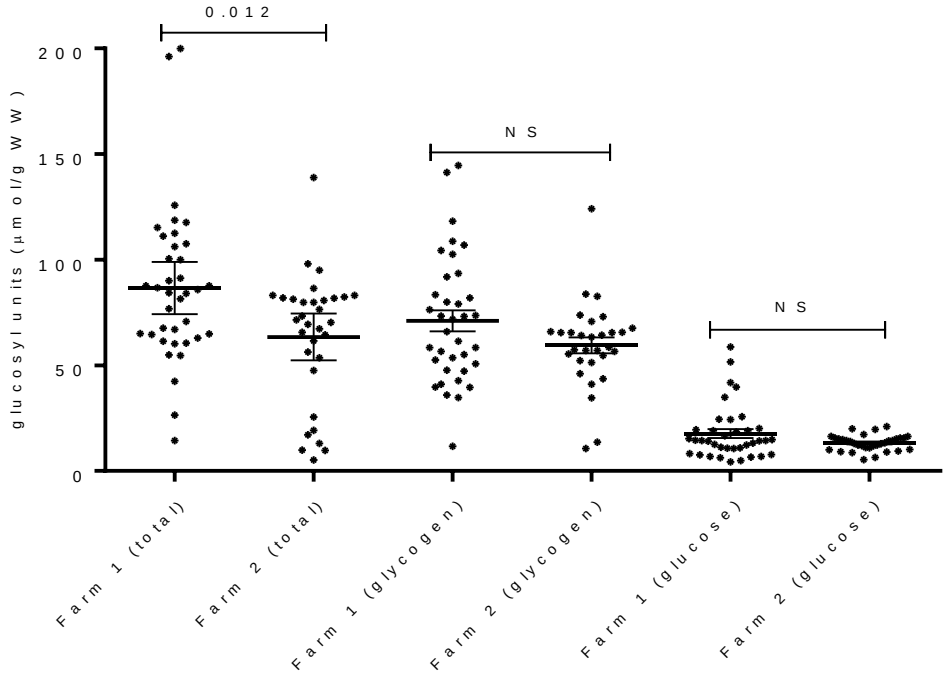

Figure 3.1. Total glucosyl, true glycogen, and free glucose levels at biopsy A in Farm 1 and 2 animals.

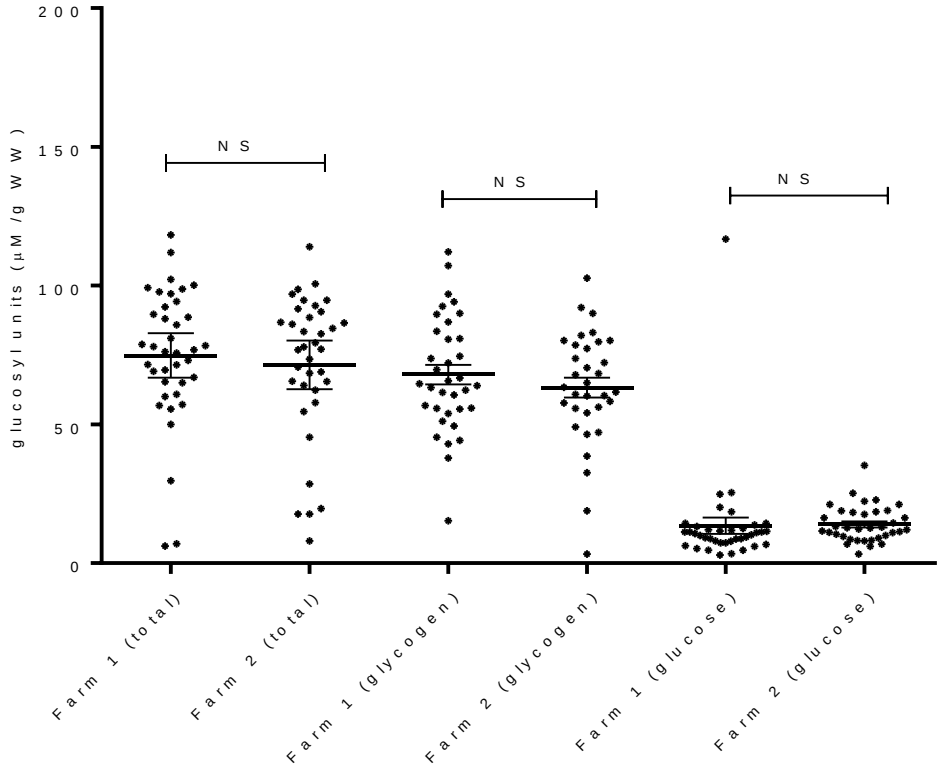

Figure 3.2. Total glucosyl, true glycogen, and free glucose at Biopsy B of animals in Farms 1 and 2.

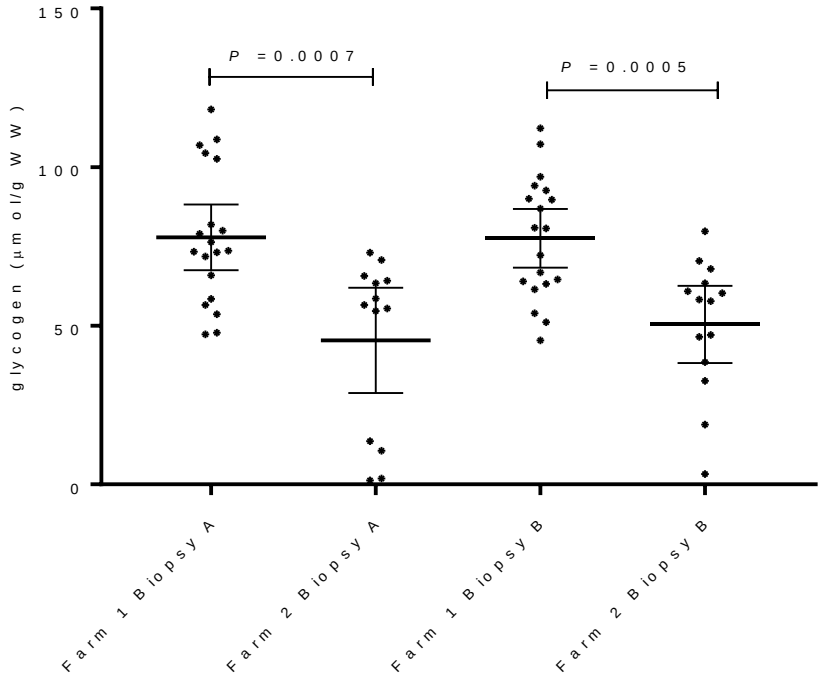

Figure 3.3. True glycogen levels of animals in the Red group at Farms 1 and 2 at Biopsies A and B.

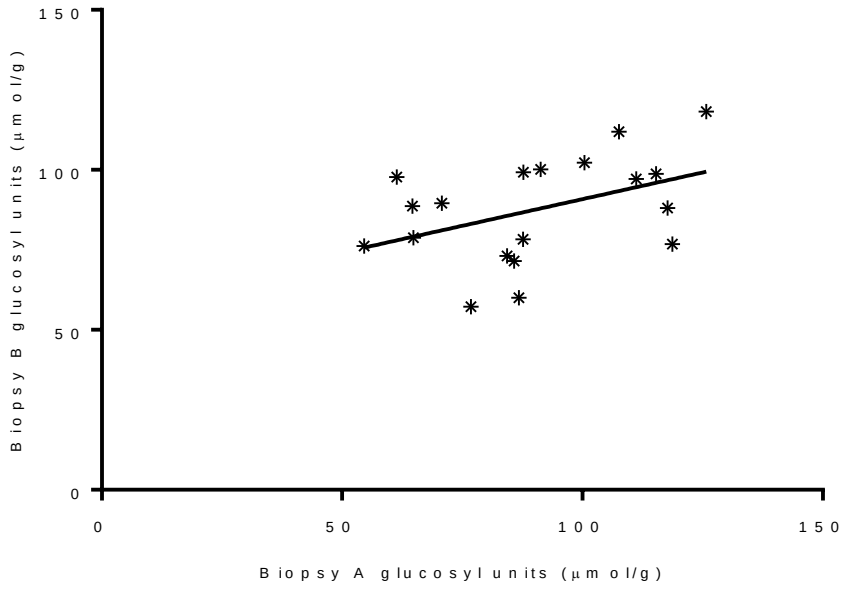

Figure 3.4. Biopsy A and B total glucosyl units at Farm 1. Farm 2 values not included as few were viable. $R^2 = 0.1884$

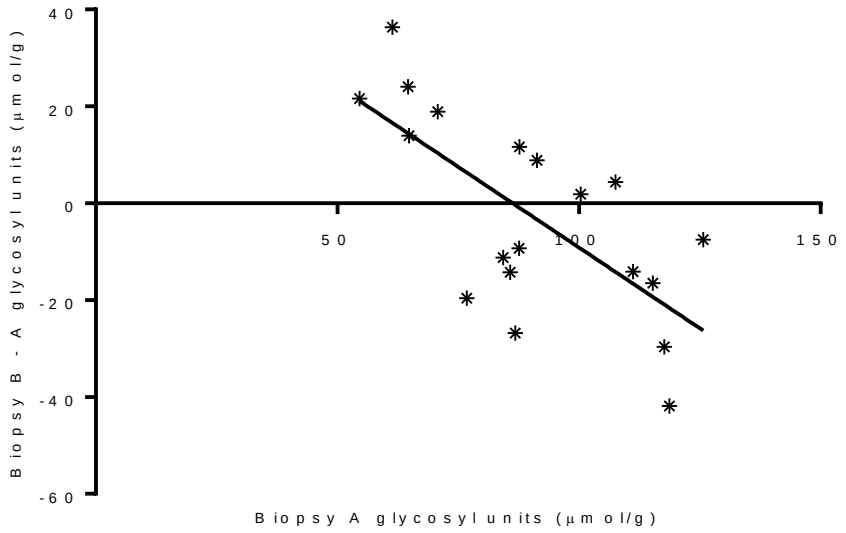

Figure 3.5. Relationship between Biopsy A total glucosyl and subsequent change in total glucosyl at Biopsy B. $R^2 = 0.481$

3.1.2 HSP20

The Green groups were introduced at Biopsy B to determine whether Biopsy A affected the stress levels of the animals. Results showed no significant difference in HSP20 levels in either Farm 1 or 2 between Biopsies A and B (Figure 3.6). Looking at the difference between farms, however, there was a clear difference in HSP20 levels at Biopsy A, with Farm 2 having more HSP20 (median = 4.25 µg/g) than Farm 1 (median = 3.35 µg/g) ($p = 0.0048$) (Figure 3.7). Again surprisingly, there was no significant difference between farms at Biopsy B despite all the presumed stress the animals were under with medians of 2.87 µg/g and 3.44 µg/g, respectively. HSP20 values were not normally distributed and non-parametric tests were done. The values were first normalised to protein by taking the percentage of HSP20 in total protein, then, were log transformed to normalise the distribution. Log transformation again, made the initial difference at Biopsy A more significant from $p = 0.0048$ before log transformation, to $p = 0.0021$ after, then, to $p < 0.0001$ after total protein normalisation (Figure 3.8). At Biopsy B, there was no significant difference in the HSP20 levels of the two farms (Figure 3.9). However, when looking specifically at the Red groups, which were followed throughout, a different trend

emerges. Raw data showed that there was no significant difference between the two farms at Biopsy A but that there was at Biopsy B with Farm 2 having more HSP20 (3.73 µg/g, s^2 = 1.42) than Farm 1 (2.91 µg/g, s^2 = 1.35) (p = 0.0253) (Figure 3.10). Log transformation of data from Biopsy A again showed no significant difference but normalisation to protein did. Calculations showed that Farm 2 had, on average, more HSP20 than Farm 1 (p =0.0373) (Figure 3.11). Whereas, log transformation of HSP20 values at Biopsy B made the difference more significant (p =0.0075) but normalisation to protein decreased the difference in significance (p =0.0246) (Figure 3.12).

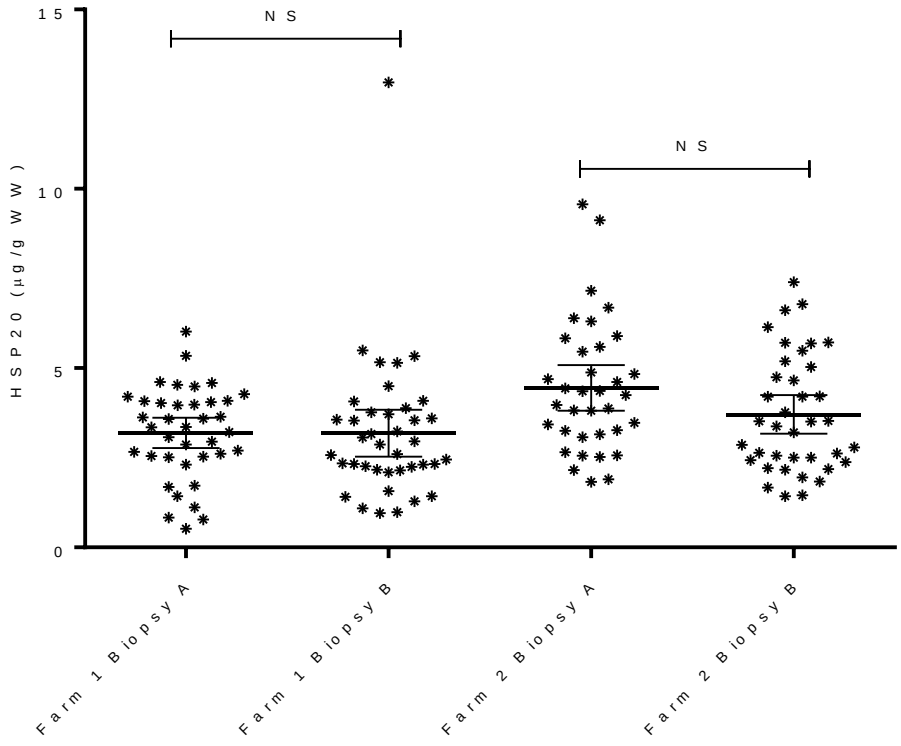

Figure 3.6. HSP20 at Biopsies A and B from Farms 1 and 2, respectively. No significant difference in HSP20 levels within farms at different biopsies.

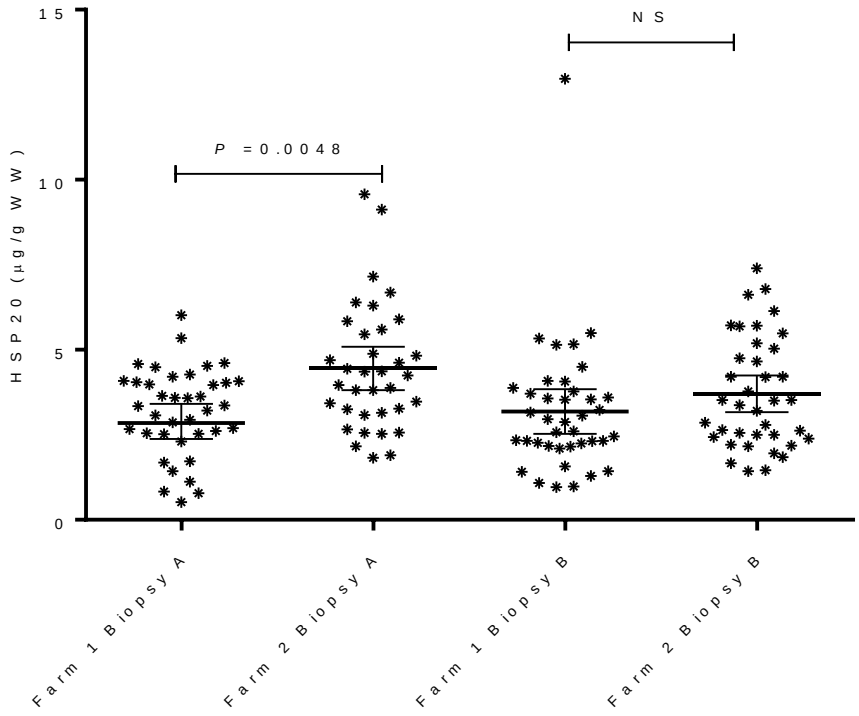

Figure 3.7. Difference in HSP20 between farms at Biopsies A and B.

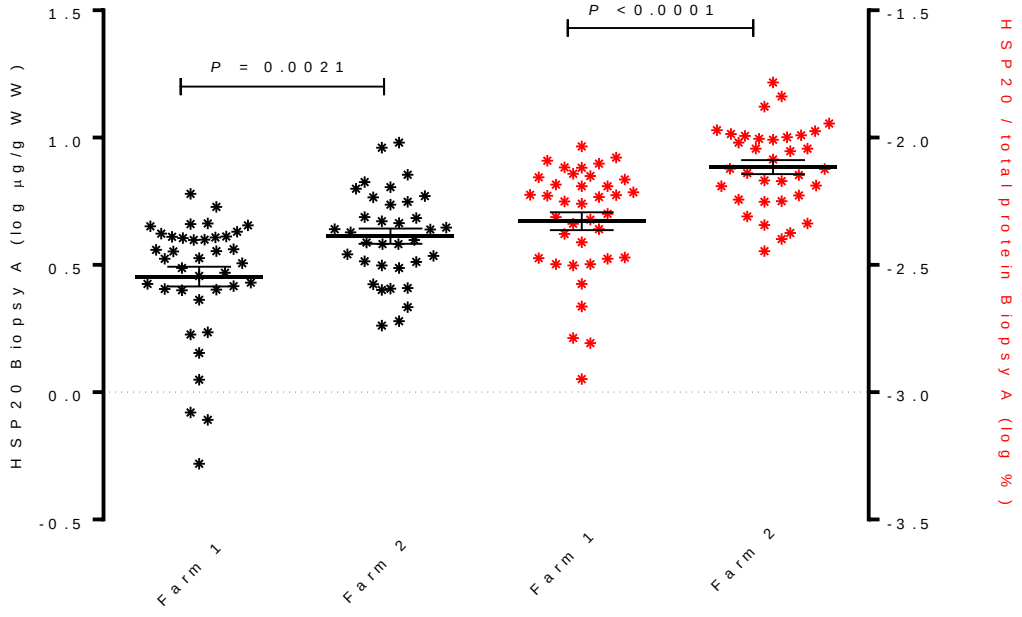

Figure 3.8. Log transformation and total protein normalisation of HSP20 at Biopsy A. Animals from Farm 2 showed higher HSP20 levels.

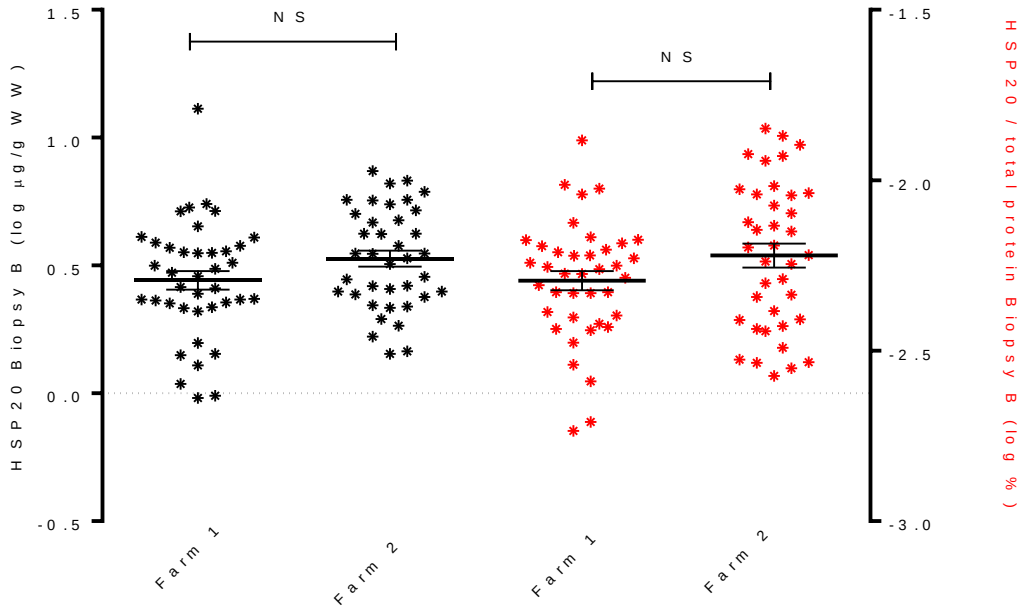

Figure 3.9. Log transformation and total protein normalisation of HSP20 at Biopsy B. No significant difference between farms observed.

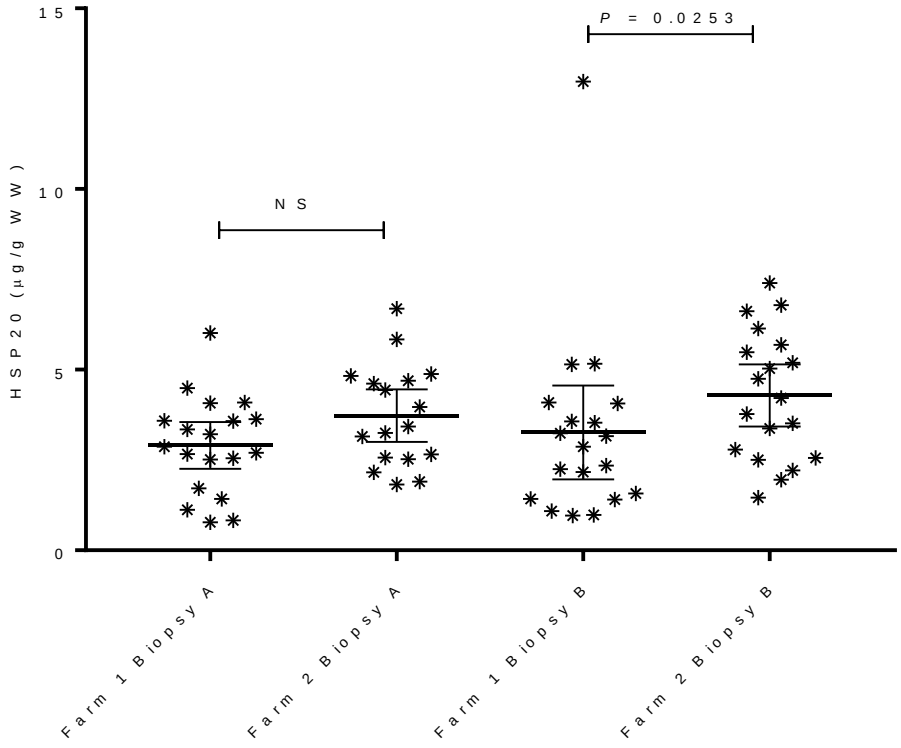

Figure 3.10. HSP20 of lambs in the Red group in Farm 1 and 2.

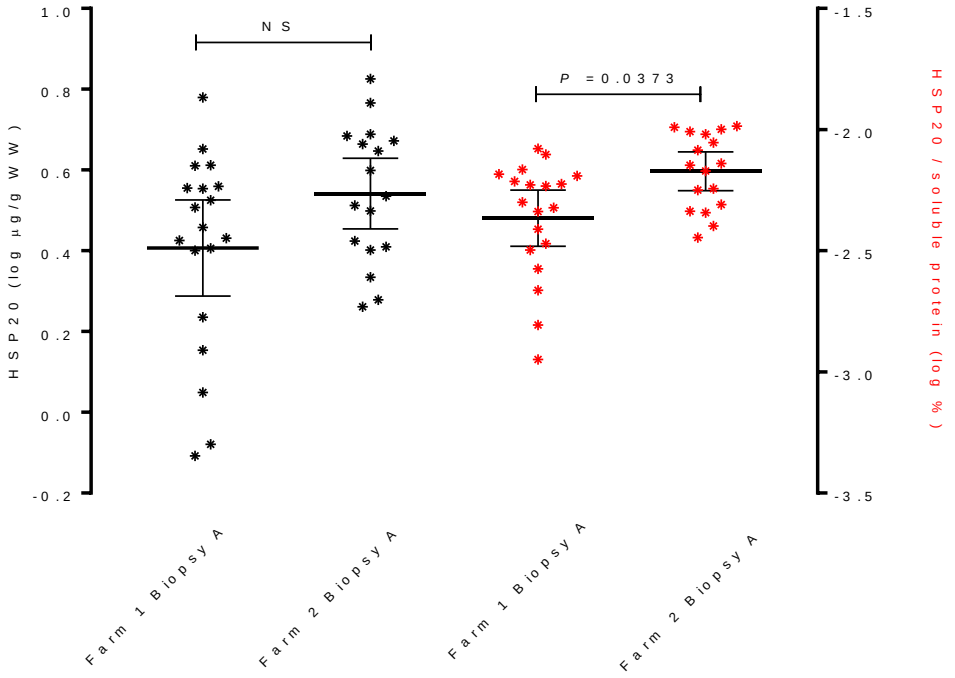

Figure 3.11. Log transformation and normalisation to total protein of HSP20 values at Biopsy A from both farms.

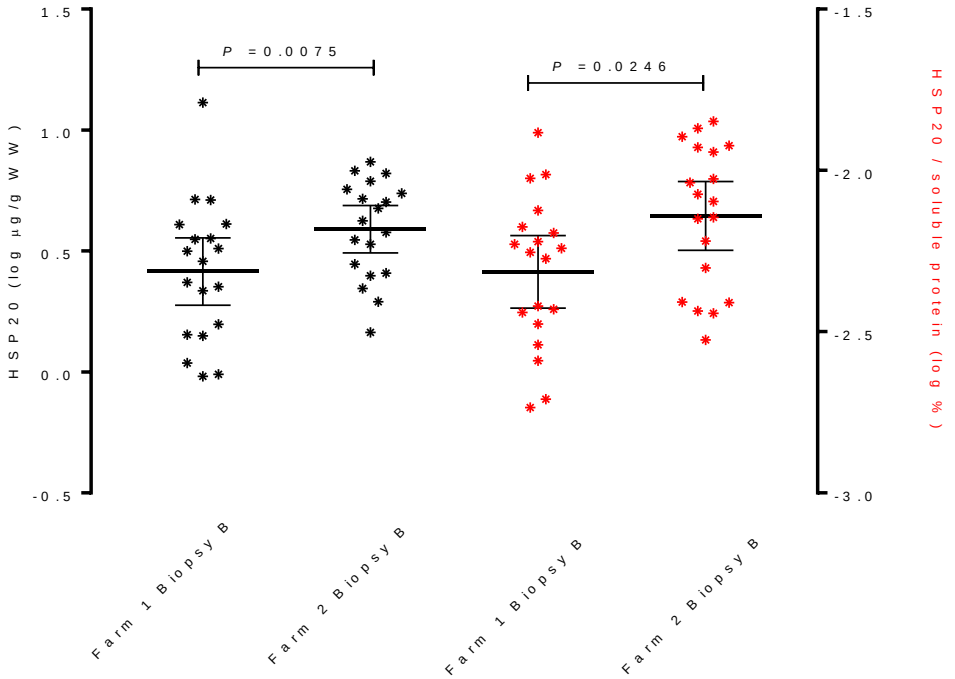

Figure 3.12. Log transformation and normalisation to total protein of HSP20 values at Biopsy B from both farms.

3.1.3 Post-rigor pHu

Post-rigor glycogen levels showed a very significant difference between the two farms (Figure 3.13). Animals from Farm 1 had higher total glycosyl units in their carcasses with a mean of 32.87 µmol/g (s^2 = 9.59) whereas, animals from Farm 2 only had mean total glucosyl of 17.02 µmol/g (s^2 = 10.30) (p = <0.0001). This translated to residual glycogen of 14.92 µmol/g (s^2 = 9.84) and 6.12 µmol/g (s^2 = 4.84) (p = <0.0001), respectively. Furthermore, none of the carcasses from Farm 1 had a pHu of 5.8 or over whereas, 50% of the carcasses from Farm 2 did. High post-rigor true glycogen logically equal high pre-slaughter true glycogen. Since all the animals underwent the same processing (transport, lairage, and slaughter) and storage during rigor, it can be postulated that, Farm 2 lambs had lower pre-slaughter glycogen than Farm 1 which lead to higher pHu (Figures 3.14 and 3.15). Figure 3.18 shows that at high pHu (≥5.8) carcasses had less than 18 µmol/g of residual glycogen.

In terms of HSP20, there appears to be quite a large distribution of HSP20 values both at normal pHu (Figure 3.16) and high pHu (Figure 3.17) but no correlation observed.

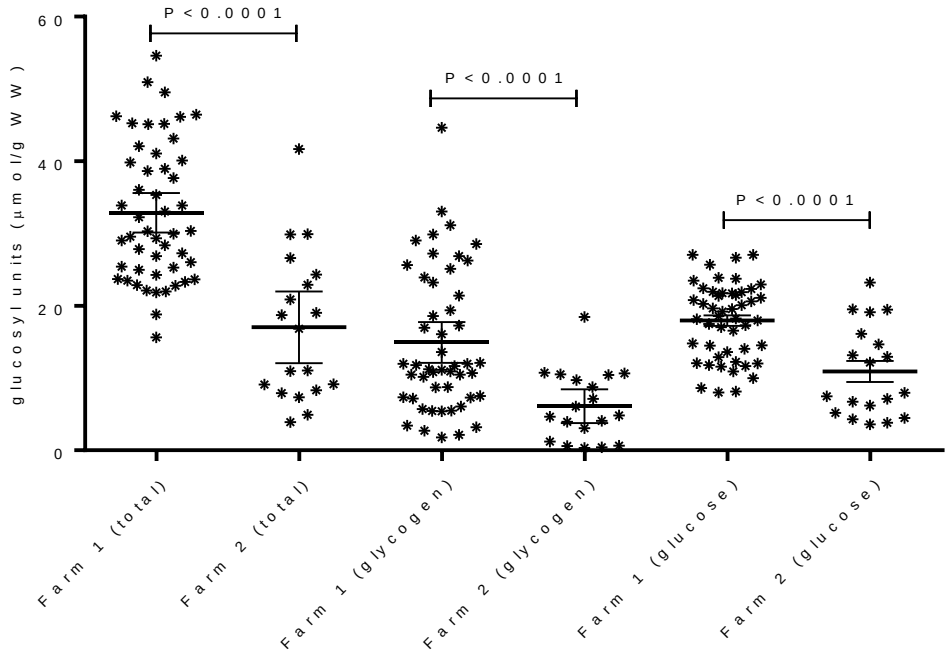

Figure 3.13. Post-rigor total glucosyl, true glycogen, and free glucose levels of all animals in Farms 1 and 2.

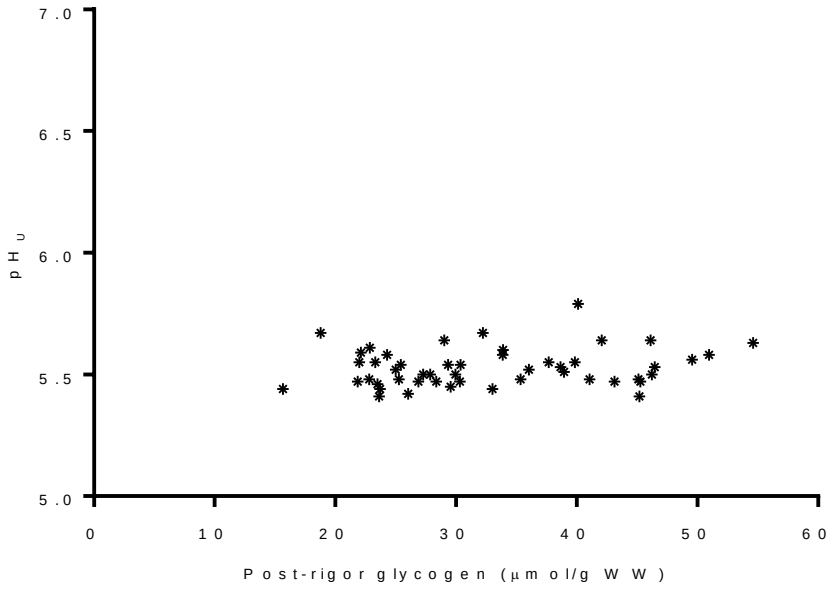

Figure 3.14. Post-rigor residual glycogen of carcasses from Farm 1. None of the carcasses had a pHu over 5.8

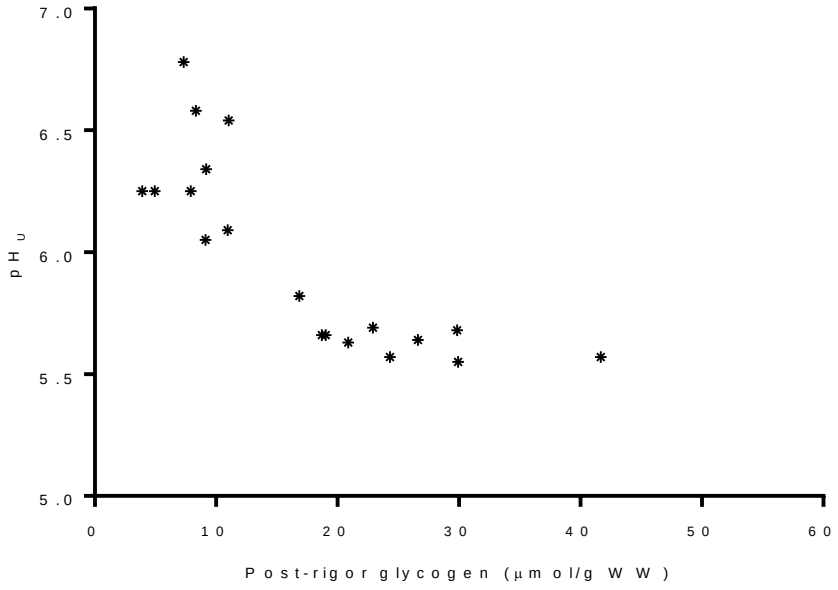

Figure 3.15. Post-rigor residual glycogen of carcasses from Farm 2. Many of the carcasses were high pHu of over 5.8

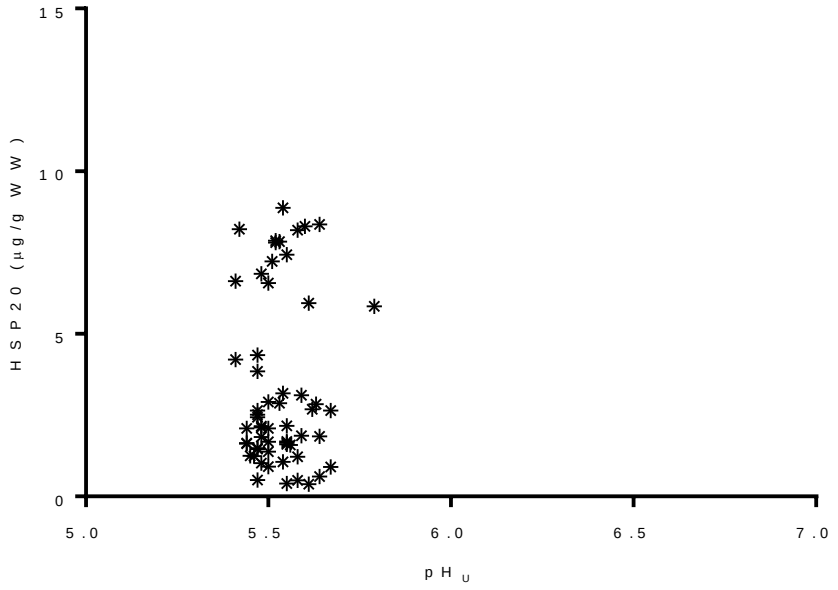

Figure 3.16. pHu and HSP20 levels of carcasses from Farm 1 post-rigor. There appears to be a large variation in the HSP20 levels even at similar pHu.

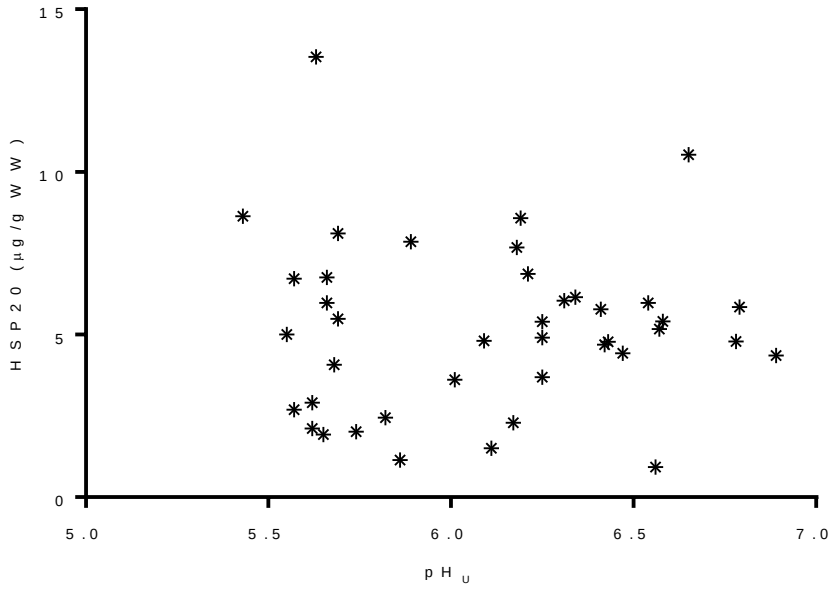

Figure 3.17. pHu and HSP20 levels of carcasses from Farm 2 post-rigor. Large variation in both pHu and HSP20 levels. There appears to be no correlation between the two.

3.1.4 Glycogen and HSP20

From all these experiments, little correlation was observed between true glycogen and HSP20 levels (Figures 3.18, 3.19, and 3.20)

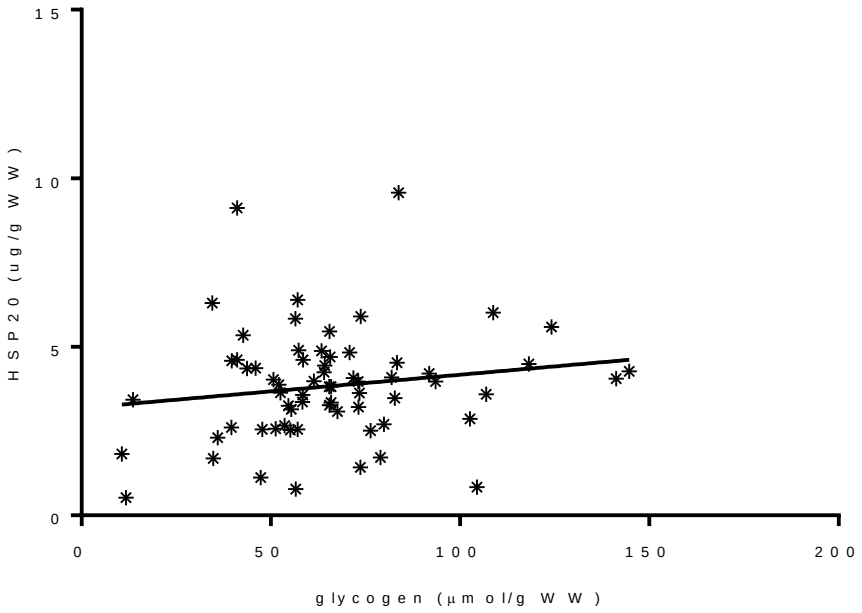

Figure 3.18. Little correlation observed between glycogen and HSP20 at Biopsy A at both Farm 1 and 2. $R^2 = 0.0253$. Negative values were omitted.

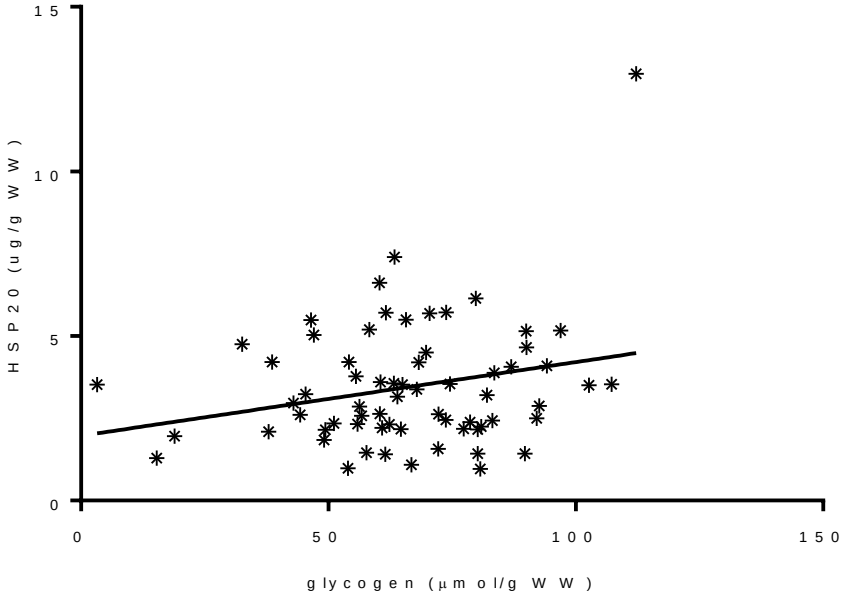

Figure 3.19. Little correlation observed between glycogen and HSP20 at Biopsy B at both Farm 1 and 2. $R^2 = 0.05997$. Negative values were omitted.

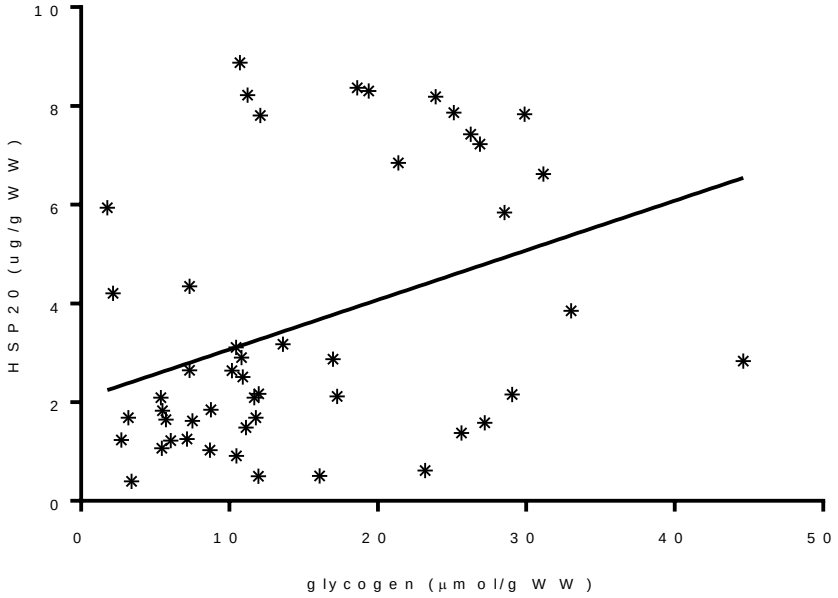

Figure 3.20. Again, little correlation observed between glycogen and HSP20 post-rigor in carcasses from Farm 1. Farm 2 values were not included as few were viable. $R^2 = 0.1323$. Negative values were omitted

3.3 Yield Trial

3.3.1 Residual Glycogen

To show a range of residual glycogen levels, at least 20 carcasses each of high, medium, and low residual glycogen were required. The glycogen iodine assay was used to roughly estimate residual glycogen content. Of the first 100 carcasses sampled (Set 1), only 9 had high glycogen (>0.500 absorbance), most had low residual glycogen (~0.200 absorbance reading). From Set 1, 9 high, 11 medium, and 10 low residual glycogen samples were randomly chosen for the next testing. In the next 150 carcasses (Set 2), only 5 had high glycogen. From Set 2: 5 high, 10 medium, and 10 low samples were chosen. Finally, in Set 3 (n = 149), 11 samples with high residual glycogen were chosen along with 3 samples that had an unusual cloudy orange colouration (normally colour would change to between dark brown and light brown depending on amount of glycogen present), the latter were placed in the low group. In total, samples were: 25 high, 21 medium, and 23 low residual glycogen (n_{total} = 69) which provides a spread of residual glycogen content.

Figure 3.21 shows a moderate positive correlation of glycogen results using the iodine assay and the hexokinase assay (R^2 = 0.4324). There was some noise in the data but overall, a positive trend was observed. Despite the criticisms of the iodine assay, these results showed that the assay does work for a rough estimation of the residual glycogen content of the samples.

The three samples that turned cloudy orange after iodine colour reagent addition were tested with the hexokinase glycogen assay. Results showed that these samples had little glucosyl units, 8.88 µmol/g, 11.80 µmol/g, and 18.86 µmol/g. They were kept for other analyses and placed in the low glycogen group.

There was no significant difference in the residual glycogen levels between Day 1 and Week 8, although there was between total glucosyl units and glucose levels (Figure 3.22). Free glucose did significantly decrease at Week 8 from a median of 18.85 µmol/g to a median of 8.78 µmol/g. This decrease can be

explained by bacterial activity i.e. bacteria using glucose as a food source. The change in total glucosyl units can, therefore, be explained by the decrease in free glucose levels.

In terms of pHu, a similar trend with the Serial Biopsies experiment was observed (Figure 3.23). A large distribution of residual glycogen levels was observed at ideal pHu from 35.92 µmol/g to 2.17 µmol/g, lower than the values observed from t. he previous experiment. It is possible that these carcasses had just enough muscle glycogen to reach ideal pHu. Some samples from Set 1 were not tested for free glucose as not enough sample was taken.

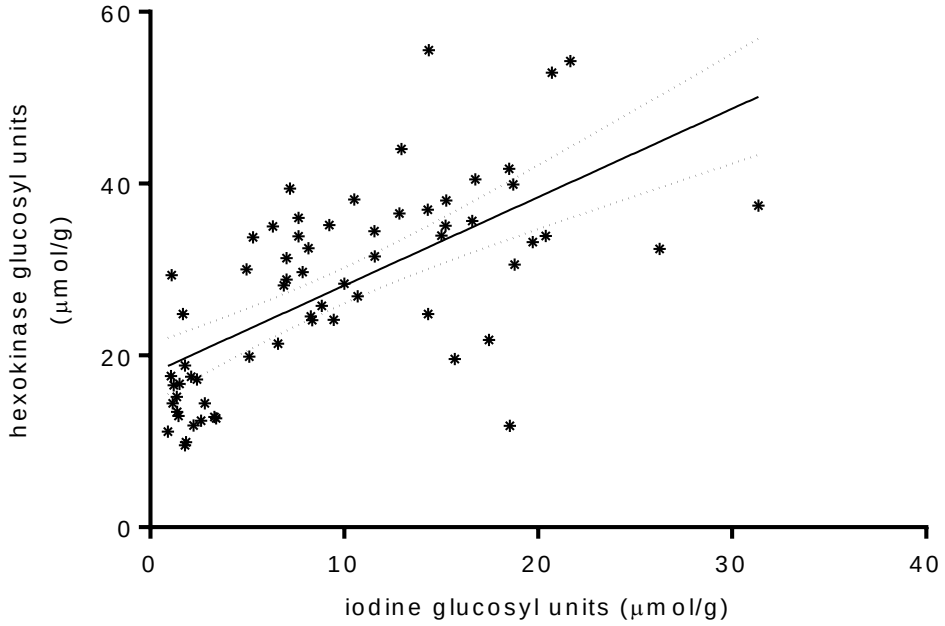

Figure 3.21. Correlation of total glucosyl results using iodine assay and hexokinase assay with mean and error. $R^2 = 0.4324$

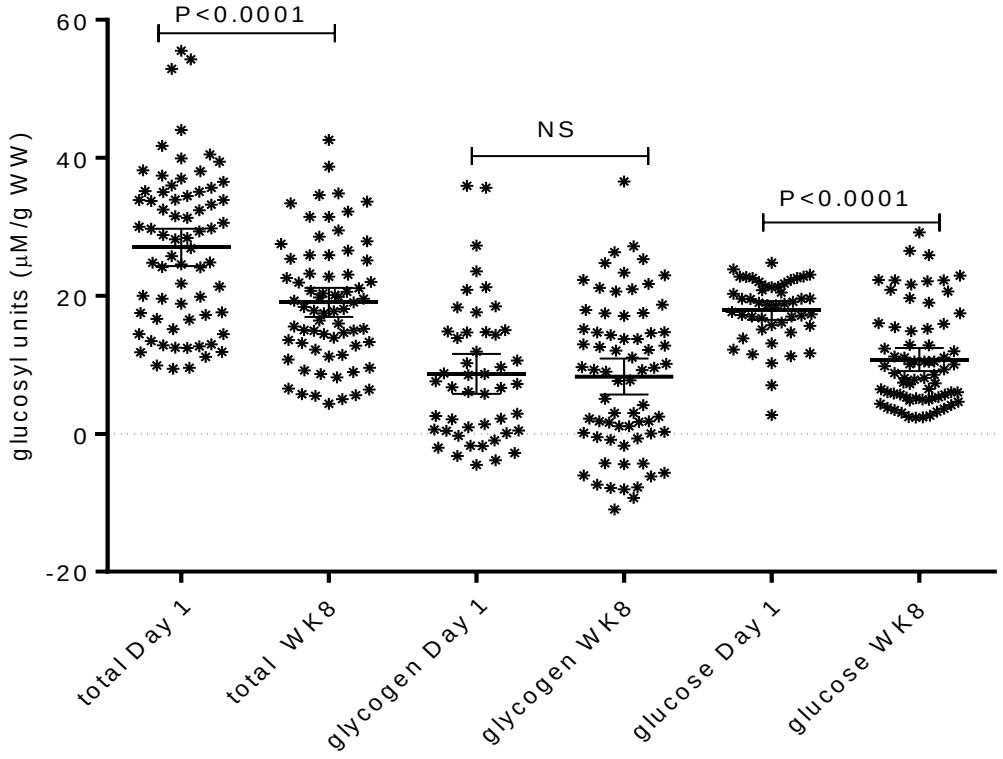

Figure 3.22. Residual total glucosyl, true glycogen, and glucose levels post-rigor at Day 1 and Week 8 with means and 95% confidence limit.

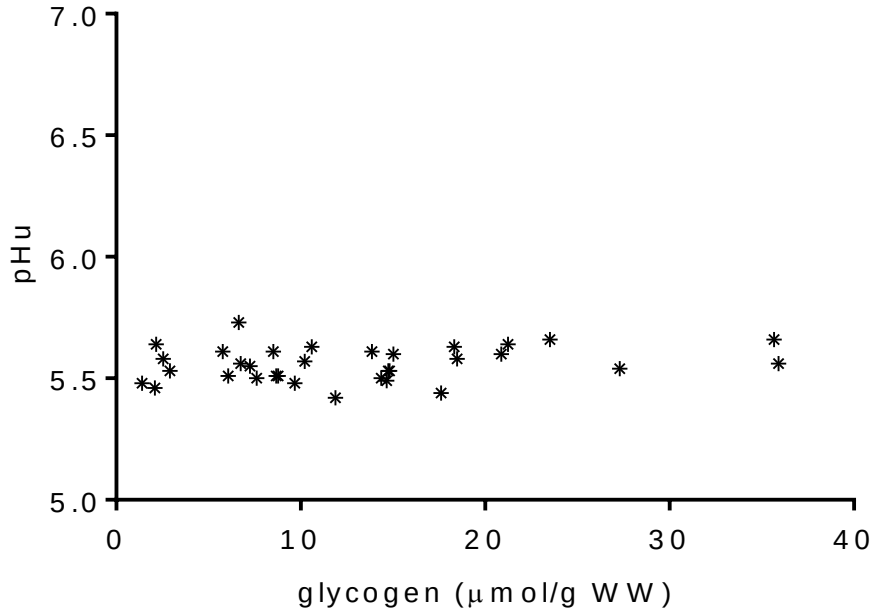

Figure 3.23. Relationship between residual glycogen and pHu. Some values from Set 1 were not included because few of the Set 1 samples were tested for glucose (not enough sample). Large distribution of residual glycogen at ideal pHu, some values lower than 18 µmol/g. pHu does not to fall below 5.5 in these samples due to the high buffering capacity of the *M longissimus dorsi* (loin) which is predominantly made of white muscle fibres.

3.3.2 Meat Quality Attributes: Day 1 and Week 8

Unfortunately, no relationship between residual glycogen and either water binding capacity (WBC), drip loss, water content, fat content, or protein content was observed at either Day 1 or Week 8.

WBC significantly decreased (p <0.0001) from 10.52 $\Delta cm^2/50mg$ (s^2 = 1.57) to 6.65 $\Delta cm^2/50mg$ (s^2 = 1.95). In line with the WBC results, drip loss significantly increased (p <0.0001) from a median of 2.14% to 3.02% (Figure 3.24). There was little variation in WBC and drip loss results between high, medium, and low glycogen groups. Logically, when WBC decreases, drip loss would increase. However, perplexingly, there was little correlation between WBC and drip loss at either Day 1 or Week 8 with R^2 values of 0.0325 and 0.0751, respectively (Figures 3.25 and 3.26). As expected, the microbial count increased significantly (by several orders of magnitude) between Day 1 and Week 8 from

a median of 74 colony forming units (CFU) to 41,000,000 CFU at Week 8 (p <0.0001) (Figure 3.27).

Water content at Day 1 was also measured and had little variation between samples at a median of 73.9% and a range between 68.0% and 77%. Surprisingly, there was no correlation between water content and either WBC and drip loss (Figures 3.28 and 3.29). The only moderate correlation observed was between water content and fat content (R^2 = 0.3035) (Figure 3.30). Fat content was normally distributed with a mean of 3.04% (s^2 = 0.94) while crude protein was also normally distributed with a mean of 3.43 g/100g (s^2 = 0.17). Unsurprisingly, there was little variation in the protein content of the samples with a minimum of 3.20 g/100g and a max of 3.85 g/100g.

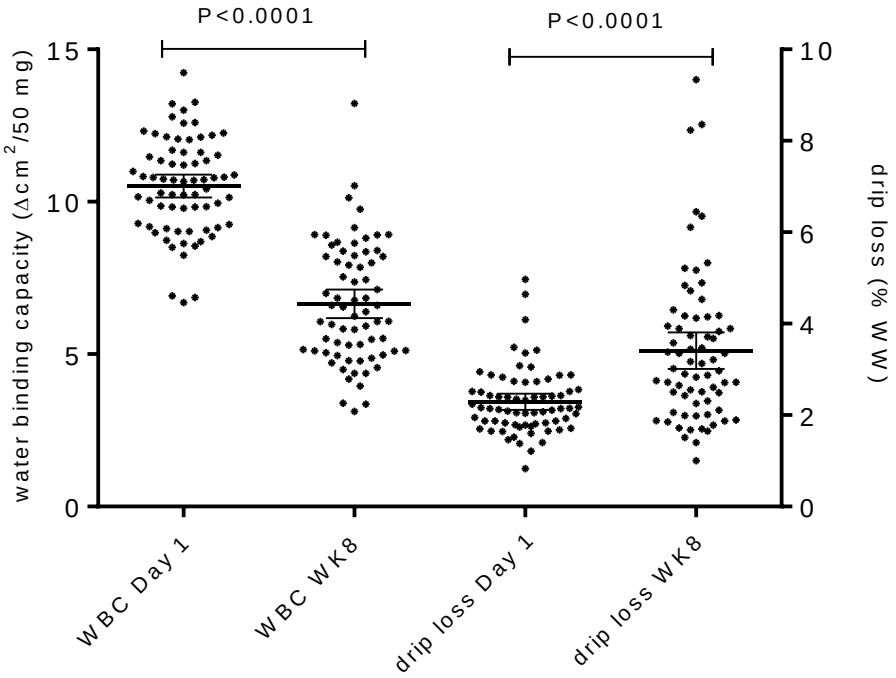

Figure 3.24. WBC and drip loss at Day 1 and Week 8 comparison with means and 95% confidence limits.

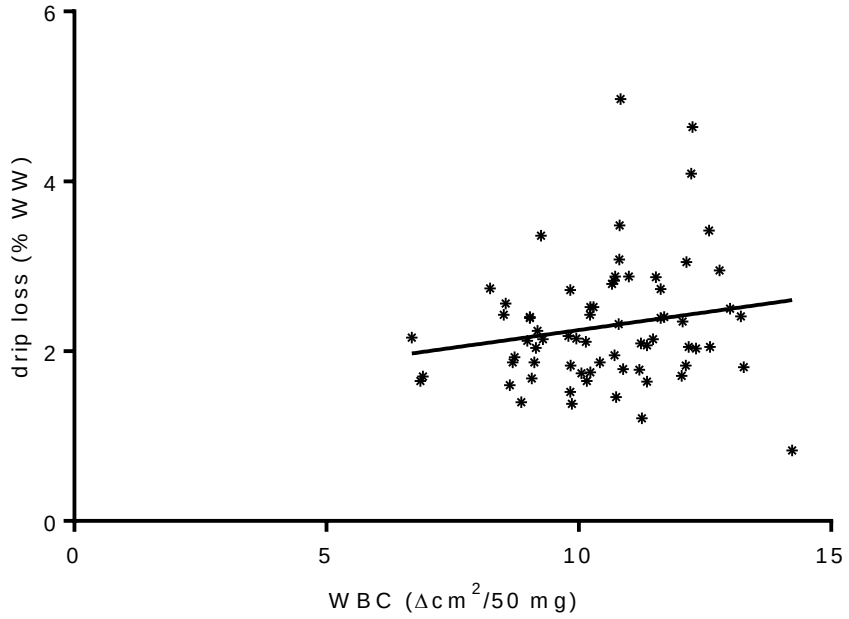

Figure 3.25. Little correlation observed between WBC and drip loss at Day 1. R^2 = 0.0325

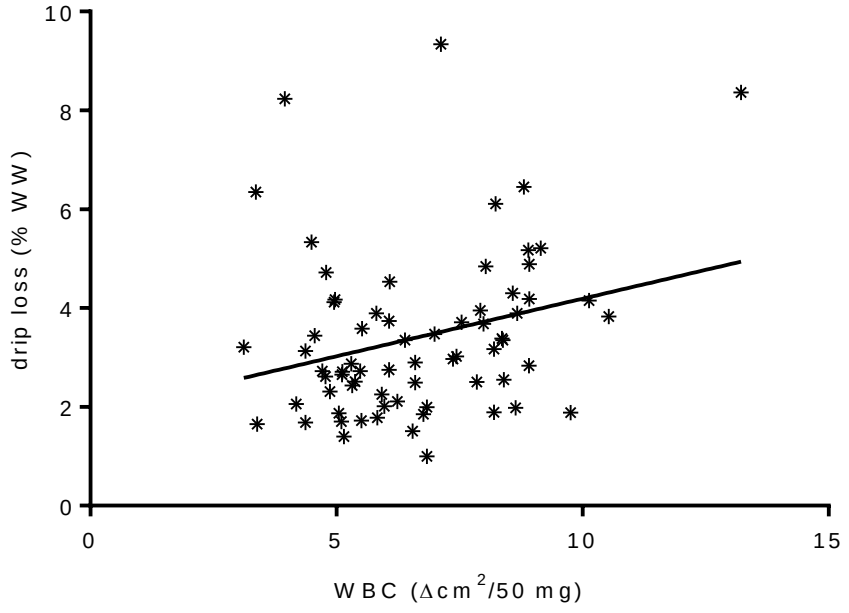

Figure 3.26. Little correlation observed between WBC and drip loss at Week 8. R^2 = 0.0751

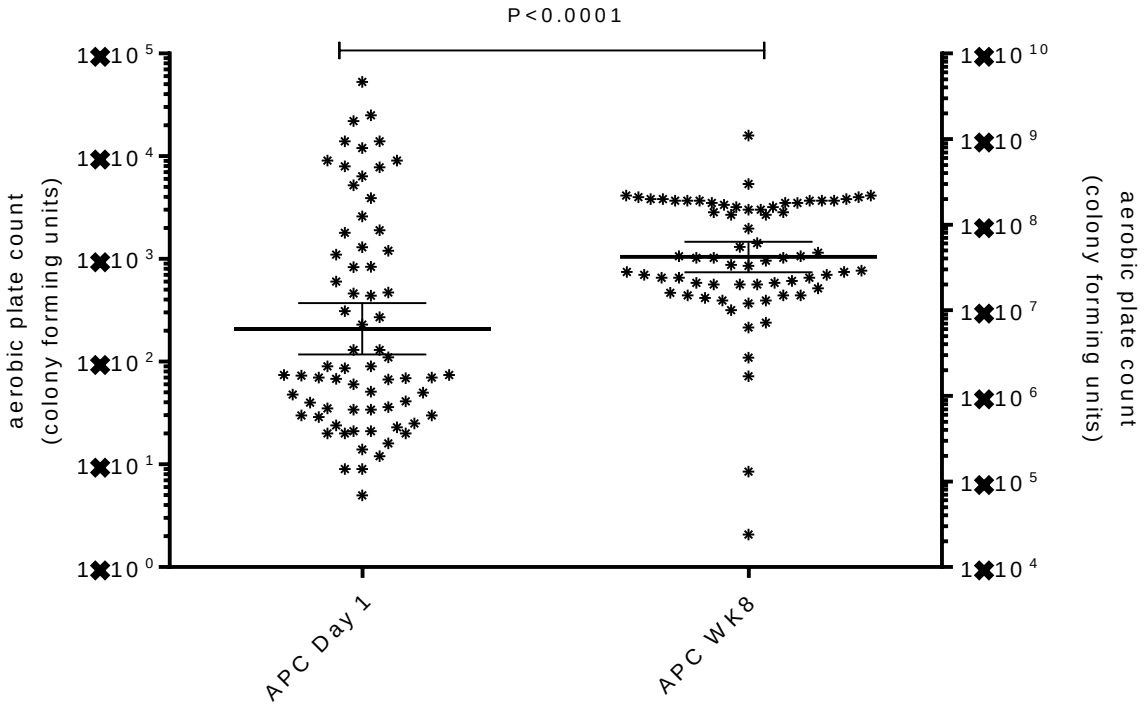

Figure 3.27. Change in microbial count (colony form units) between Day 1 and Week 8. Geometric means and 95% confidence limits shown.

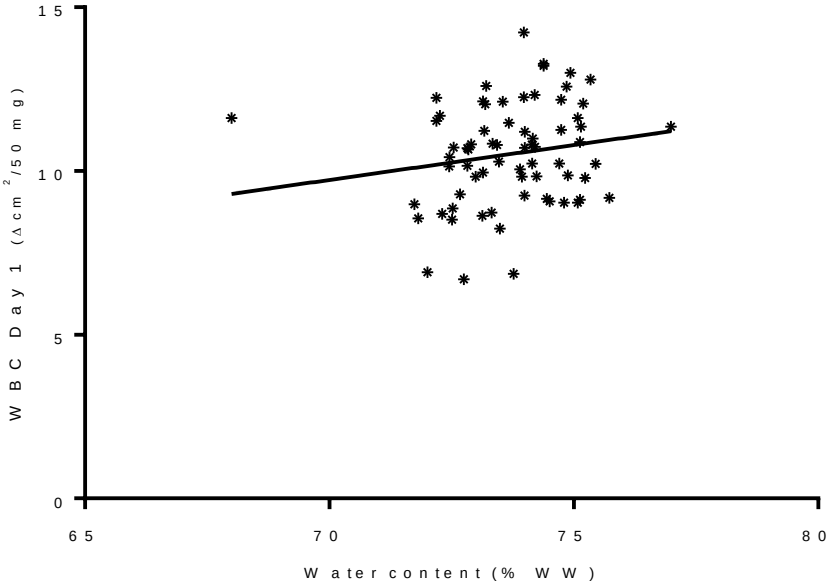

Figure 3.28. Little correlation between water content and WBC at Day 1. $R^2 = 0.0309$

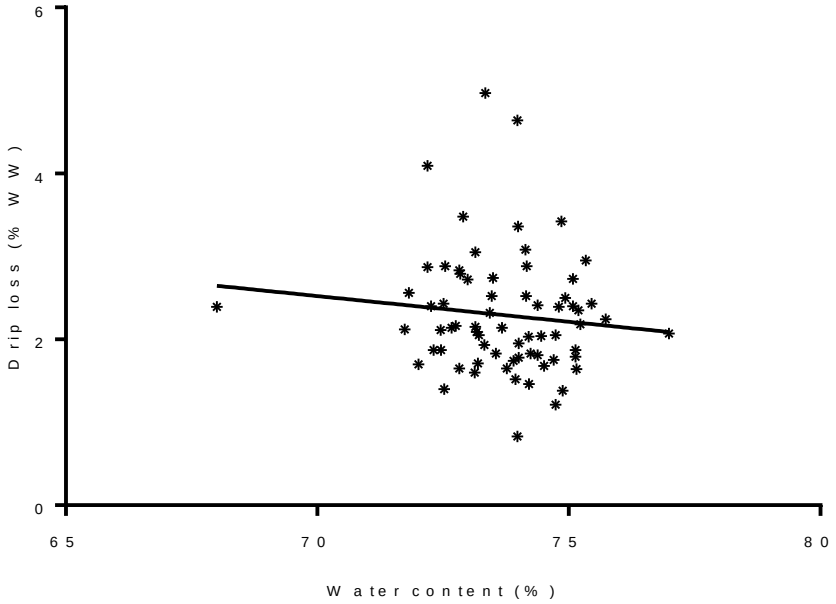

Figure 3.29. Little correlation between water content and drip loss at Day 1. R^2 = 0.0122

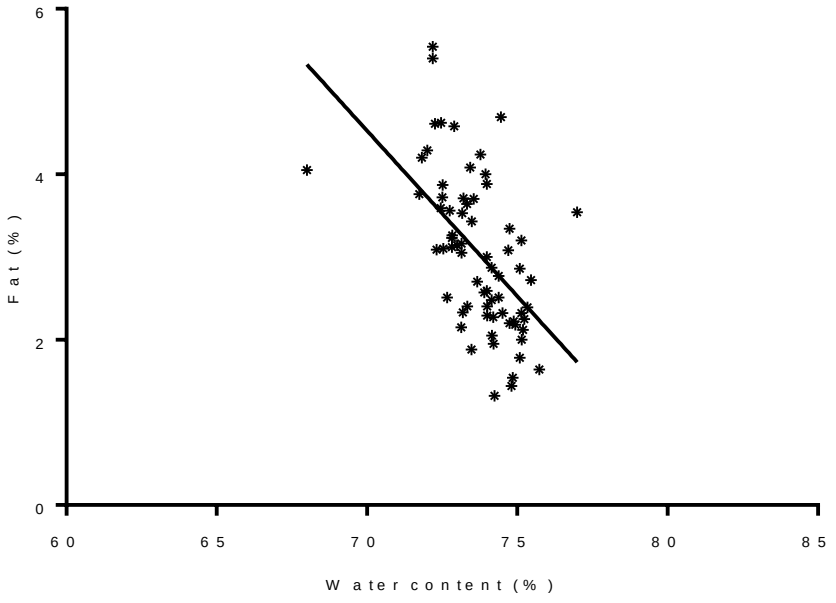

Figure 3.30. Moderate negative correlation between water content and fat content. R^2 = 0.3035

Chapter 4

Discussion

4.1 Biopsy Experiments

Muscle glycogen concentration at time of slaughter is a critical factor affecting meat quality (Immonen et al., 2000b). Glycogen is the metabolic substrate that fuels anaerobic glycolysis that drives pH decline post slaughter (Braden, 2013). Insufficient muscle glycogen at slaughter results in high ultimate pH (pHu) (≥5.8) and results in meat that is a dark colour, has reduced shelf-life, and can tough meat (Newton and Gill, 1981; Calkins and Hodgen, 2007; Farouk et al., 2013). Although, meat with especially high pHu (>6.0) are tender, they also have very dark colour and have low water binding capacity (high drip loss) (Daszkiewicz et al., 2009). The main cause of glycogen depletion in ruminants is stress, either physical, psychological, or a combination of both and any negative effect is generally thought to be a function of the type, duration, and intensity of the individual stressor and the susceptibility of the animal to it (Ferguson et al., 2001). Numerous studies have dealt with stressors such as transport (Puolanne and Aalto, 1980; Wythes et al., 1981; McVeigh et al., 1982; Tarrant et al., 1992; Speer et al., 2001) and lairage (Puolanne and Aalto, 1981; Daly et al., 1996; Daly and Wright, 1996; Warner and Pethick, 2000; Miranda-de la Lama et al., 2009). Significant depletion of muscle glycogen pre-slaughter has a well-documented effect on several key meat quality attributes such as pHu, tenderness, ageing potential, colour, and water binding capacity (Gregory and Grandin, 1998). However, there is limited knowledge available on the significance of on-farm glycogen concentration on the ultimate quality of ruminant meat, particularly lamb. The overall aim of this study was to fill this gap in knowledge.

4.1.1 Serial Biopsies

4.1.1.1 Glycogen

The serial biopsies had two aims: a) to evaluate the stability of glycogen over time and b) assess the relative contribution of on-farm vs off-farm factors on the incidence of high pHu. The two farms were chosen because of their different productions systems: Farm 1 lambs (castrated males and intact females) were bred, reared, and finished on-site under “good” production system, whereas, despite having a breeding and finishing programme, Farm 2 primarily relied on procuring and finishing lambs resulting in a highly mixed mob of rams, wethers (castrated males), cryptorchids (males with undescended testicles), and ewes and frequent introduction of new lambs into the mob. There was high variability in muscle glycogen which typically ranged from 45 uM/g (the minimum amount of glycogen required to lower muscle pH from 7.2 to 5.5) (Tarrant, 1989; Ferguson and Warner, 2008) to close to 200 uM/g. These results coincide with other research that showed resting muscle glycogen ranged from 60 uM/g to 130 uM/g in cattle (Immonen et al., 2000a; Immonen et al., 2000b). There were samples containing less than 45 uM/g at B1 (mostly Farm 2 lambs), but did increase in value by B2. Since this variability was observed in lambs from the same mob, and so, all were exposed to similar environments and feeding regimes, the source of this variability is not immediately obvious.

There was, however, significant difference in muscle glycogen levels between farms 1 and 2 with animals from Farm 1 overall having more muscle glycogen than Farm 2. Considering the near-constant introduction of new store lambs into the mob in Farm 2 behavioural stress may be a contributing factor to the incidence of low muscle glycogen. This ties in with previous research showing that new animals should not be introduced to the mob prior to slaughter (Puolanne and Aalto, 1980; McVeigh et al., 1982; Lacourt and Tarrant, 1985; Warriss, 1990) as these changes in social structure as well as novel and unfamiliar environments increases pre-slaughter stress (Ferguson and Warner, 2008). This may also be compounded by the rise in testosterone levels of the

intact rams in Farm 2 leading to the development of sexual behaviour such as aggression towards other rams and constant chasing of females (which was observed in Farm 2 at biopsy B), further increasing individual stress.

However, no obvious correlation between muscle glycogen at different time points was observed. In fact, there appeared to be a cyclical nature in its expression i.e. high at one point and low at the next, and vice versa. This suggests that muscle glycogen is not a particularly stable metabolite, in contrast with the significantly correlated values for HSP20. Only two time points were done in this experiment, hence, to confirm this assertion, more time points will be required.

In terms of the relative contribution between on-farm vs off-farm factors in determining muscle glycogen at slaughter, there were considerable differences observed between the farms despite the similarities in processing off-farm. Lambs were slaughtered in the same facility and lairaged overnight. The only real difference is the travel time with Farm 1 lambs having travelled six hours compared to three hours for Farm 2 lambs. The current opinion is that short transport times (less than four hours) have little effect on pHu as long as conditions are good and there was no trauma (Grandin, 2000). Therefore, despite the longer travel time of Farm 1 animals, the pHu of their carcasses were within ideal (5.5 to 5.8) whereas, 66% of carcasses from Farm 2 had high pHu (<5.8). A more recent study by Villaroel and colleagues (2003) found no significant difference in pHu of beef when animals are transported at three hours or six hours. Furthermore, behavioural stress at lairage may be a contributing factor to the high pHu of Farm 2 carcasses. Rams high in testosterone are house in closed quarters during lairage, exaggerating on-farm behaviours (e.g. aggression against other rams and chasing ewes), increasing individual stress levels.

4.1.1.2 Glycogen and pHu

Looking at residual glycogen, it was clearly observed that sample with high pHu contained residual glycogen of less than 18 uM/g whereas those with ideal pHu had higher concentrations of residual glycogen. These results suggest that high

muscle glycogen pre-slaughter (indicated by high residual glycogen post-slaughter) leads to ideal pHu, whereas, low muscle glycogen leads to high pHu. This trend is very similar to what Daly and Wright (1996) found in terms of lactate levels i.e. when pHu of lamb carcass is ideal, lactate levels can vary wildly but when pHu is high lactate levels are quite low. No pHu below 5.5 was observed despite high residual glycogen due to the high buffering capacity of white muscle fibres which predominantly make up the loin (*M longissimus dorsi)*.

4.1.1.3 HSP20

HSP20 also had high inter-individual variability on-farm. Unlike muscle glycogen, however, HSP20 expression was stable with time suggesting that, at least with these concentrations, are not in response to an external stressor, rather it reflects the differences in endogenous protein expression. However, the significant increase for Farm 2 Red group at B2 may somewhat be contributed by stress of ewes being chased by a rowdy ram during biopsy. Furthermore, the increase in concentration at post-rigor compared with the biopsy sample, evident in Farm 2 lamb, may indicate that its expression increases in response to off-farm stressors, similar to findings by Morgan and colleagues (2008) in cattle. There also appeared to be a slight cyclical nature in HSP20 but again, more time points are needed to confirm this.

Unfortunately, no relationship was found between muscle glycogen level and HSP20 concentration i.e. HSP20 concentration was not a good predictor of muscle glycogen depletion. It is perhaps not surprising considering the HSP20 levels at the two biopsy time points may likely be endogenous levels, rather than stress-induced levels. However post-rigor, when HSP20 showed an increase, 93% of high pHu carcasses (≥5.8) also had HSP20 concentrations of more than 4 ug/g whereas, 66% of carcasses with ideal pHu had HSP20 levels less than 4 ug/g. These results support findings from Apple and colleagues (1994) stating that physiological stress, in terms of exercise, had little effect on muscle glycogen. This may be because at intense exercise and glucose requirements, hepatic gluconeogenesis is activated.

4.1.2 Summary

Significant inter-individual variation in muscle glycogen levels of at rest animals on-farm was observed but since this was observed in the same mob exposed to similar environments and feeding regimes, the source of this variability is unclear. Serial biopsies revealed a cyclical nature of muscle glycogen on-farm. These suggest that variability can be an important contributor to risk of high pHu meat i.e. susceptibility to high pHu may be related to when an animal is slaughtered relative to this cycle. Therefore, off-farm stressors are not solely responsible for muscle glycogen depletion and subsequent high pHu. Physiological stress, as measured by HSP20, were not significantly different between the two farms. Furthermore, no correlation was observed between muscle glycogen and HSP20. This lead to the suggestion that, the stress brought on by other individuals within the group (specifically rams) was the cause for the observed difference in muscle glycogen, not environmental stress. It is, therefore, recommended that, although intact rams have higher saleable weight, these rams be castrated before mixing with other rams and ewes. This would be important for both the commercial aspect (decrease risk of high pHu) and animal welfare aspect (decrease in animal stress levels). More biopsy time points are needed to confirm these assertions.

4.2 Yield Trial

Muscle glycogen is the main metabolic substrate that drives post-mortem anaerobic glycolysis and the eventual pH decline (Braden, 2013). Approximately 45 mmol of glycogen is needed to decrease the pH of muscle from 7.2 to 5.5 or equivalent to 45 uM/g (Immonen et al., 2000c). The concentration of glycogen pre-slaughter, is therefore, critical to for this pH decline, but glycogen concentration varies greatly depending on the muscle, species, and nutritional level of each animal. However, it is mostly dependent on pre-slaughter stress (McVeigh and Tarrant, 1982; Crouse et al., 1984; Pethick et al., 1994; Immonen et al., 2000c). Immonen and Puolanne (2000) found that when pHu is below 5.75, residual muscle glycogen varied considerably from 10 uM/g to 80 uM/g in beef because anaerobic glycolysis of bovine muscles cease when pH has reached 5.6 to 5.3 even in the presence of large amounts of glycogen (Greaser, 1986) likely due to proteolysis. A follow-up study by the same authors found “the effects of residual glycogen concentration on the physical and sensory quality of normal-pH beef were quite numerous but moderate in size” (Immonen et al., 2000c). Furthermore, glycogen concentration is a potential contributor to water binding capacity of meat as the glycogen molecule binds three to four times its weight in water (Olson and Saltin, 1970). However, residual glycogen concentration and its consequent differences in pork quality was later proved to be due to genetics (Estrade et al., 1993; Lundstrom et al., 1996). Still, potential effects of residual glycogen on lamb meat quality has not been studied. This section aims to bridge this gap in knowledge.

4.2.1 Residual Glycogen

The first part of the yield trial aimed to quickly select samples with high, medium, and low residual glycogen for comparison. The iodine assay was used to colorimetrically estimate the amount of glycogen in the longissimus dorsi of 399 carcasses, with pHu of <5.8 post-rigor, to obtain at least 60 samples: 20 high, 20 medium, and 20 low residual glycogen to provide a range. The

challenge was finding samples with high residual glycogen. In set 1, 80% of the samples were low glycogen while in set 2, 85% of samples were low. Slaughter of these animals (29 March 2017 and 6 April 2017, respectively) was preceded by extreme stormy weather with flooding and severe thunderstorms throughout the North Island brought about by two storms, the “Tasman Tempest” and cyclone “Debbie” (Lawton, 2017; Morton, 2017; n.a., 2017b; n.a., 2017a). The extreme weather is likely the cause of such low residual glycogen as it is significant source of stress that can deplete glycogen stores pre-slaughter (Lacourt and Tarrant, 1985; Warriss, 1990; Lahucky et al., 1998). This appeared to be supported by the lower rate of low residual glycogen by set 3 (slaughtered 30 April 2017) at 38% meaning more glycogen stores pre-slaughter compared to the previous sets.

The iodine assay, however, is does not provide a precise value of glycogen. The main criticism of this method is that it tests whole glycogen concentrations, therefore, structural differences in glycogen can affect results (Dreiling et al., 1987; Bennett et al., 2007a). The hexokinase assay provides a more accurate value of glycogen in the sample as detailed by Bennett and colleagues (2007a) and Zhang (2012). However, there was a positive correlation in glycogen values as measured by the iodine assay and the hexokinase assay demonstrating that the former delivers a good approximation of glycogen. Using the latter assay, residual glycogen values at pHu below 5.8 were similar to those reported by Immonen and Puolanne (2000) in beef. The trend was also similar to the serial biopsies experiment and to previous experiments by Daly and Wright (1996) for lactic acid.

4.2.2 Meat Quality Characteristics

4.2.2.1 Day 1

Having established a range of residual glycogen values, the samples were tested for crude protein, crude fat, drip loss, water binding capacity (WBC), and microbial count 24 hours post-slaughter. It has been reported that muscle protein is not affected by fattening system (Rowe et al., 1999) but that both fat

(Caballero Garcia De Arevalo et al., 1992; Rowe et al., 1999; Bas and Morand-Fehr, 2000) and muscle glycogen (Lowe et al., 2002; Jacob et al., 2005) are largely influenced by feeding regime. The low variability observed in protein values suggest protein levels are stable. The lack of correlation between fat and residual glycogen suggest that despite both being largely influenced by feed, the latter cannot predict the former. It can be speculated that this is because it is easier to change muscle glycogen concentration pre-slaughter than it is to change fat and this change would then translate to the amount of residual glycogen. Perplexingly, crude protein and crude fat values from these experiments were much lower than reported by the United States Department of Agriculture (1963).

Unlike the moderate correlation between residual glycogen with WBC and drip loss in beef as reported by Immonen and colleagues (2000c), no correlation was observed between these in the lambs tested. Furthermore, no relationship was observed between residual glycogen and microbial count since the latter was extremely variable, rejecting the initial hypothesis that it may affect shelf-life as influenced by bacteria. Although, these results may be compounded by the significantly high microbial count in samples from Set 2 compared to sets 1 and 3. Lastly, it was initially hypothesised that, since glycogen binds a significant amount of water, higher residual glycogen would also equal higher water content and, consequently, increased weight. This relationship, however, did not materialise as little variation in water content was observed. A negative relationship between fat and water content was observed, in line with findings from Rowe and colleagues (Rowe et al., 1999) who reported that lambs with the lowest amount of fat had the highest moisture content.

4.2.2.2 Week 8 (Storage)

Samples were kept for eight (8) weeks to simulate the maximum time taken to arrive at overseas supermarkets, more specifically the UK, by ship during which, lamb is aged and tenderised by enzymatic systems (Herrera-Mendez et al., 2006; Renton, 2011; Taylor, 2013). Samples were again tested for WBC, storage drip loss (water loss over eight weeks), and microbial count. Similar to Day 1 results, no correlation between these characteristics and residual

glycogen was found. It was then hypothesised that residual glycogen at Day 1 may affect microbial count at Week 8, but again, this was rejected. The effect of pHu on microbial population is well established (Grau, 1981; Farouk et al., 2013). However, results showed no correlation between pHu at Day 1 and microbial count at either Day 1 or Week 8.

All samples tested in this experiment had pHu of under 5.8 and the effect of pHu on WBC, drip loss, and microbial populations is well known (Grau, 1981; Francis, 2000; Huff-Lonergan and Lonergan, 2005; Farouk et al., 2013). The high variability of residual glycogen and the lack of correlation between this and either WBC, drip, and microbial count combined with the low variability in both WBC and drip, suggest pHu is the main factor that influences these characteristics and that residual glycogen has no influence on these characteristics. Hence, little difference will be observed in these characteristics as there is also little difference in pHu of samples. Samples with a range of pHu will be required to verify this hypothesis. It would also be interesting to determine the effects of residual glycogen on shear force values, weight loss in thawing and frying, sensory juiciness, and fresh meat redness as Immonen and colleagues (Immonen et al., 2000c) did in beef.

4.2.3 Summary

Residual glycogen was found to be highly variable at ideal pHu in lamb meat, in line with previous research in beef. However, unlike beef, residual glycogen had no influence on water binding capacity and drip loss. Further, it had no correlation with either crude fat, crude protein content, or microbial count. Similar to previous findings, a moderate negative relationship was found between fat content and water content in meat. It is proposed that, since residual glycogen is highly variable at ideal pHu and variability in water binding and drip loss was low, pHu is the main influencer of these characteristics, not residual glycogen.

4.3 Limitations

One major limitation of the biopsies was that, since samples were immediately frozen in liquid nitrogen once taken, there was a possibility that, once thawed, enzymes will resume breakdown of muscle glycogen. This was minimised by ensuring samples did not thaw before homogenisation, immediate addition of sample to acid (serial biopsies of pre-rigor samples), and keeping samples on ice. A second major limitation of the biopsy experiment was that lambs were not followed from when they left the farm to slaughter. It was known that they were transported, lairaged, and slaughtered the morning of the following day, however, this length of time may have introduced another external stressor that was not factored in this experiment. Furthermore, there was some confusion during the tagging of carcasses immediately after slaughter, which was why some samples were missing, mislabelled, or lost. Additional, yet minor, limitations include: the use of the muscle density 1.0597 g/cm3 (or 1.06 g/mL) which, as mentioned by Mendez and colleagues (1960), can be quite species-specific; and that the residual glycogen values used were in actual fact, residual glycogen-free glucose post-rigor.

In the yield trial, the process by which samples were categorised as “high, medium, and low” glycogen had some overlap after correcting for blanks in the absorbance reading and did not take into account the sample weight. Perhaps it would have been more appropriate to have calculated the glycogen concentration from the corrected absorbance reading before choosing the categories for each sample or, for more accuracy, the hexokinase assay could have been used. That was, however, too time-consuming.

4.4 Conclusion

Significant inter-individual variation in muscle glycogen on-farm despite similarities in environmental stimuli and feeding regimes need more careful scrutiny. This, combined with the observed cyclical nature in muscle glycogen over time suggest on-farm factors can be an important contributor to risk of

high pHu post-slaughter and that off-farm factors cannot be exclusively responsible for high pHu. More time points are needed to confirm this assertion. Muscle glycogen differences and heat shock protein 20 similarities between farms suggest that behavioural stress, rather than physiological, may be the cause for the variation in muscle glycogen. Residual glycogen levels were found to be highly variable at ideal pHu in both sets of experiments and in line with previous research. However, it had no correlation with protein, fat, water binding capacity, drip loss, or microbial count at either the day after slaughter or after storage. This may be because pHu has a higher influence on the latter three. More research is needed to verify this hypothesis. Further, it would be interesting to elucidate the effect of residual glycogen on shear force, thawing and frying water loss, and sensory juiciness.

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Appendices

Appendix 1: HSP20 Solutions

Coating Solution

Monoclonal mouse anti-HSP20 (HyTest 4HSP20)	10 uL
Carbonate-bicarbonate buffer, pH 9.4	10 mL

Phosphate-buffer Saline (PBS)

Phosphate-buffer saline (Thermo Scientific 28372)	1 packet
Distilled Water	1 L

PBS-Tween

PBS	500 mL
Tween20	250 uL

Blocking Buffer (PBS-milk)

PBS	50 mL
Skim non-fat milk	2.5 g

Homogenisation Buffer

1 M Tris HCL, pH 7.5	1 mL
1 M KCl	2 mL
100 mM Phenylmethylsulfonyl Fluoride (PMSF)	50 uL
Protease Inhibitor Cocktail (Sigma P834- 1 mL)	50 uL
Distilled water	16.9 mL

Detection Antibody

HSP20 polyclonal antibody (Stressgen #SPA-796)	5 uL
PBS-milk	10 mL

Secondary Antibody

Mouse anti-rabbit-HRP/50% glycerol (Peirce 31464)	4 uL
PBS-milk	10 mL

OPD Solution

10 X Stable peroxide buffer	1 mL
Distilled water	9 mL
OPD Tablet (Sigma P9187)	1 tablet

NB: Toxic and light sensitive. Gloves always worn when handling and stored in dark.

BCA Reagent

Reagent A	10 mL
Reagent B (blue liquid)	200 uL

Appendix 2: Iodine Assay Reagents

Iodine-Potassium Iodide (I-KI) Solution

Iodine (Sigma 207772- 5g)	2.6 mg
Potassium Iodide (Thermo-Fisher BSP PL7739.5000)	26 mg
Distilled Water	1 mL

Colour Reagent

Calcium chloride	7.5 g
Iodine-Potassium Iodide Solution	130 uL
Distilled water	10 mL

NB: Calcium chloride was dissolved completely before I-KI solution was added

Appendix 3: Serial Biopsies

Appendix 4.1: Farm 1

Table of collated results from On Farm Biopsies with comments for each sample. * Negative values removed.

Group	Sample #	pHu	Kill Weight (kg)	Glyco A (uM/g)	Glyco B (uM/g)	Glyco C (uM/g)	Glu A (uM/g)	Glu B (uM/g)	Glu C (uM/g)	HSP20 A (ug/g)	HSP20 B (ug/g)	HSP20 C (ug/g)	Protein A (mg/g)	Protein B (mg/g)	Protein C (mg/g)	Comments	A Sample Weight (mg)	B Sample Weight (mg)	C Sample Weight (mg)
Red	1	5.48	18.2	107.57	111.93	45.12	4.94	4.68	23.73	2.867	3.536	6.847	48.5	58.81	42.76		16.4	11.1	22.2
Red	2	5.51	18.4	115.28	98.77	38.94	6.54	8.79	12.06	6.016	5.144	7.225	72.06	53.04	47.21		10.2	12.2	25.1
Red	3	5.79	16	64.6	88.68	40.1	10.99	25.47	11.56	2.665	3.566	5.841	68.58	55.7	44.1		13.3	12.1	23.8
Red	4	5.58	19.4	91.31	100.19	50.96	19.47	13.23	27.06	4.078	4.067	8.186	63.27	54.17	48.87		15.8	14.5	17.4
Red	5	5.55	17	87.68	99.3	39.84	14.29	6.7	13.58	3.629	2.871	7.429	60.88	53.96	53.39		12.4	18.4	28.4
Red	6															Lost			
Red	7	5.6	19.8	100.39	102.22	33.88	18.52	5.25	14.5	4.089	5.166	8.304	59.77	54.69	53.75		9.7	24.1	24.3
Red	8	5.53	19.8	125.75	118.25	46.45	7.54	6.04	16.57	4.488	12.97	7.829	73.52	99.16	60.93		9	5.3	23.9
Red	9	5.64	19.8	111.21	97.11	42.08	4.31	2.96	23.48	3.588	4.089	8.364	71.58	71.37	59.6		9.8	13.6	22.1
Red	10	5.67	19	70.75	89.62	32.23	12.29	8.7	21.75	3.577	2.249	0.911	112.33	59.3	32.63		5.1	9.1	22.7
Red	11	5.64	19.2	118.71	76.83	46.13	39.73	4.64	22.94	1.719	1.574	0.615	64.49	47.12	32.15		9.8	26.7	21.6
Red	12	5.47	20	117.68	88.03	43.13	13.26	7.3	27.05	0.833	0.96	0.506	73.97	52.22	30.78		6.8	15.4	25.5
Red	13	5.58	18.4	61.37	97.71	33.87	14.08	8	21.92	1.119	1.426	0.499	51.46	49.53	28.85		7.8	13.7	27.1
Red	14	5.54	16.6	85.78	71.51	29.33	12.59	10.03	23.88	3.214	1.409	1.064	49.12	38.81	33.04		8.3	17.9	20.9
Red	15	5.58	18.4	86.8	60	24.29	6.87	8.88	18.24	2.698	2.344	1.22	46.16	39.73	23.37		13.9	12.1	28.3
Red	16	5.55	18.4	84.31	73.1	21.98	10.62	6.34	18.58	1.426	1.088	0.397	42.11	42.35	31.28		15	21.4	21.3
Red	17	5.61	18.2	64.83	78.79	24.8	8.25	24.85	28.82	0.78	0.979	0.379	50	50.12	27.44		11.9	13.8	28.9
Red	18	5.48	17.2	54.57	76.2	35.34	6.8	11.56	26.65	2.547	2.169	1.028	55.51	58.4	28.86		14.5	12.8	25.1
Red	19	5.44	18	76.78	57.19	23.7	10.84	11.78	17.97	3.348	3.236	1.644	42.37	48.46	33.37		15	17.5	22.8
Red	20	5.55	18	87.63	78.32	23.3	11.25	14.35	20.1	2.515	3.159	1.686	52.82	56.72	33.72		16.1	14.2	23.6
Yellow	1	5.47	18.4	60.5		21.4	24.51		28.92	2.306		2.437	52.68		57.05		8.5		22.9
Yellow	2	5.61	19.2	60.15		22.86	19.01		21.08	4.609		5.939	60.47		52.3		12.3		22.6
Yellow	3	5.41	18	54.96		23.63	15.26		21.47	4.584		4.205	56.46		47.55		20.7		32.6
Yellow	4	5.47	18.2	67		26.87	14.49		19.56	3.642		2.645	61.22		42.53		11.1		38.6

Yellow	5	5.53	19.6	62.9		38.66	20.16		21.69	5.345		2.87	57.84		52.83		13.3		30
Yellow	6	5.42	18.4	112.54		26.02	19.04		14.8	3.967		8.22	61.56		55.09		10.7		16.7
Yellow	7	5.52	16.6	199.98		24.99	58.73		12.91	4.052		7.808	72.21		41.86		5.9		17.5
Yellow	8	5.5	18.8	81.41		20.56	41.84		28.69	2.61		6.561	77.57		58.01		9.5		15.4
Yellow	9	5.41	18.2	14.41		45.18	24.36		14.03	2.945		6.618	70.29		57.55		9.8		19.5
Yellow	10	5.54	18.4	90.03		30.36	34.87		19.66	2.53		8.875	79.36		63.4		8.4		17.2
Yellow	11	5.52	16.8	196.19		36.04	51.55		10.92	4.272		7.867	77.66		57.46		7.6		28.5
Yellow	12	5.44	17	26.52		15.65	14.82		8.14	0.524		1.621	32.06		38.84		9.4		30
Yellow	13	5.48	17.8	100.07		25.27	16.7		8.02	4.53		2.118	64.02		42.12		5.9		28.8
Yellow	14	5.47	16.8	65.03		45.27	14.33		12.24	4.028		3.85	52.78		53.38		8.2		16.8
Yellow	15	5.48	18	84.03		41.07	25.68		12.03	3.36		2.157	68.81		50.8		9.7		24.8
Yellow	16	5.54	17.4	67.57		25.42	6.22		11.81	3.98		3.173	55.11		46.85		18		27.4
Yellow	17	5.63	19.2	106.15		54.61	14.32		9.99	4.204		2.833	60.21		49.26		13.7		25.3
Yellow	18	5.5	17.6	42.47		26.55	7.72		52.57	1.686		0.918	53.48		32.14		4.5		29.9
Yellow	19	5.47	18.6	-3.28*		30.33	19.96		19.22	3.073		1.488	91.92		27.83		8.2		27.3
Yellow	20															pHu = 5.46, kill weight = 19.6 kg. Lost			
Green	1	5.55	19		29.67	16.14		14.34	17.29		1.288	1.606		27.43	28.85			11.7	32.6
Green	2	5.46	19		50	23.49		12.1	20.78		2.093	1.237		40.42	31.55			19.6	35
Green	3	5.64	17.8		92.29	29.02		18.53	20.27		2.454	1.847		61.96	31.99			9.5	33.4
Green	4	5.47	18.4		75.71	28.37		20.13	17.47		3.772	2.51		71.08	29.63			8.1	21.1
Green	5	5.47	18.6		60.79	21.85		11.42	14.53		2.151	4.343		52.35	43.95			19.1	18.1
Green	6	5.5	18.6		55.48	29.94		11.19	18.15		2.602	1.684		55.76	36.21			17.3	27.7
Green	7	5.55	17.8		66.96	37.67		10.21	25.69		2.572	2.171		64.1	32.52			9.4	28.9
Green	8	5.67	18.4		85.84	18.79		11.23	8.63		3.545	2.639		52.95	37.16			22.4	32.4
Green	9	5.47	16		6.13	14.74		12.56	18.62		3.712	1.455		67.61	29.08			12.3	25
Green	10	5.5	19.4		69.62	27.28		7.31	21.89		2.31	2.091		49.24	37.49			20.6	23.4
Green	11	5.56	19.2		7.01	49.55		7.85	22.34		2.266	1.584		34.66	32.85			28	26.9
Green	12	5.55	19.4		71.54	1.71		10.98	20.67		3.599	1.649		58.48	38.76			9.6	26.3
Green	13	5.5	19		-5.13*	46.23		10.24	20.6		3.066	1.375		65.76	32.82			11.1	28.9
Green	14	5.45	19		65.29	29.55		9.37	22.4		2.326	1.251		63.47	32.71			9.4	28.4

Green	15	5.62	17.4		56.75	5.07		13.83	10.4		2.961	2.677		52.67	33.23			11.3	26.2	
Green	16	5.5	19.4		80.98	27.85		11.22	17.04		4.496	2.904		74.64	41.56			9.6	21.9	
Green	17	5.44	19.2		94.24	33.03		10.7	21.35		3.88	2.094		56.94	39.26			18.5	23.1	
Green	18	5.59	19.4		78.03	22.12		116.81	11.67		5.334	3.107		58.64	42.42			11.1	25.4	
Green	19	5.59	17.6		69.11	8.6		3.39	25.28		5.496	1.862		87.84	43.83			7.8	25.5	
Green	20	5.48	17.6		64.96	22.79		9.2	17.31		2.325	1.829		47.25	31.11			18.3	29.5	

Appendix 4.2: Farm 2

Table of collated results from On Farm Biopsies with comments for each sample. * Negative values removed. High pHu in red.

Group	Sample #	pHu	Kill Weight (kg)	Glyco A (uM/g)	Glyco B (uM/g)	Glyco C (uM/g)	Glu A (uM/g)	Glu B (uM/g)	Glu C (uM/g)	HSP20 A (ug/g)	HSP20 B (ug/g)	HSP20 C (ug/g)	Protein A (mg/g)	Protein B (mg/g)	Protein C (mg/g)	Comments	A Sample Weight (mg)	B Sample Weight (mg)	C Sample Weight (mg)
Red	1	5.68	21.6	79.86	91.61		9.09	21.18		4.831	5.687		49.26	60.38		C confused w/ another sample, wasn't done	19.6	5.4	
Red	2	6.18	21	81.37	79.43		15.66	21.15		4.696	5.193		45.38	43.54			28.1	21.6	
Red	3	6.89	19	64.45	54.61	4.74	9.01	8.14	8.67	3.151	5.481	7.675	46.7	46.56	46.51		10.1	16	28.4
Red	4	5.83	18.6	5.21	8.05	3.27	11.05	10.96	7.49	2.524	3.772	4.356	51.53	44.76	33.33		16.1	16.8	20.6
Red	5	5.43	20.8	81.73	86.07		8.69	22.73		3.968	7.394		41.44	104.4			18.5	18.8	
Red	6	6.58	20.8	76.55	17.65	8.33	20.03	17.64	7.12	5.836	6.783	8.638	58.16	47.78	46.06		10.1	11.2	25.8
Red	7			83.11	76.93		19.7	9.04		4.879	3.376		47.77	47.16		R7B was tagged R17B. R7 condemned after slaughter- had arthritis	13.2	12.7	
Red	8	5.69	20.8	25.55	17.73	5.76	11.96	14.46	19.07	3.428	3.519	2.248	60.06	58.32	43.11		17.1	10.1	27.9
Red	9	6.27	22	13.1	28.52	3.98	11.8	9.63	4.15	1.9	1.953	5.484	47.46	54.15	50.45		11.6	23	27.2
Red	10	5.67	17.8	19.18	19.63		17.28	35.23		6.685	2.56		93.67	65.51			6.1	6.9	
Red	11	5.86	18.4	70.25	73.54		15.57	13.22		3.253	6.614		44.85	49.01			20.6	14.8	
Red	12	5.68	22	17.11	70.7	29.85	6.48	12.99	19.11	1.825	1.458	1.139	50.89	48.94	32.56		19.9	15.5	27
Red	13	5.89	23.4		98.67	6.52		18.9	8.16		6.139	4.072		48.29	28.02	R13c lost?		12.9	30.8

Red	14	6.05	20.4	71.52	57.82	6.76	12.96	25.23	7.07	4.617	4.748	7.851	52.25	41.61	47.2	was lost in farm after first biopsy, unsure why we have B. will need to be removed from data set	7	10	18
Red	15			68.51	95.87		11.18	16.48		4.902	5.56		46.15	49.75			12.9	8.3	
Red	16	6.09	21		65.58	10.94		18.49	3.8		5.034	6.122		62.89	35.1	R16a lost?		6.9	29.8
Red	17	5.66	19.2	9.73	68.91	19.01	12.22	8.06	12.93	2.158	2.212	4.809	47.59	56.85	32.76		17.5	17.2	25.4
Red	18	6.55	24.2	9.93	45.44		10.99	6.84		2.655	4.211		47.13	45.95			21.2	10.9	
Red	19	5.85	16.8	62.15	8.58		10.89	13.22		2.57	2.501		55.76	68.28		Glyco A likely contaminated, water got in during time in water bath. Glyco B looked slightly off	14.5	9.1	
Red	20	6.01	21.2	75.28	69.7		11.05	15.82		4.439	2.791		54.01	55.97		Glyco B likely contaminated, water got in during time in water bath	12.4	9.9	
Yellow	1	6.62	21.8	94.98		0.39	12.24		4.19	3.478		3.613	53.9		39.26		12.1		31.7
Yellow	2															Lost samples. pHu= 6.18, kill weight = 21.6 kg			
Yellow	3	5.74	19	65.57		10.55	13.41		12.87	3.878		2.494	51.34		28.58		15.8		30.7
Yellow	4	5.62	17.4	83.17		17.41	9.45		17.42	5.898		2.012	55.06		27.8		11.6		36.4
Yellow	5	5.62	19.2	138.95		19.75	14.79		21.56	5.592		2.908	61.8		32.49		7.7		25.8
Yellow	6	5.66	22	69.41		25.45	5.35		27.56	4.249		2.116	46.94		30.32		14.5		33.6
Yellow	7	5.69	19.6	67.27		22.9	10.18		13.17	2.554		5.967	60.46		27.77		18.2		27.8
Yellow	8	6.57	20.3	86.47		5.12	20.96		6.63	5.457		8.105	47.96		37.11		8.2		31.1
Yellow	9	5.63	18.4	73.37		20.88	16.28		12.13	6.396		5.17	48.25		26.97		12.6		25.9
Yellow	10	6.42	22.6	98.08		4.61	14.34		8.27	9.572		13.532	58.1		41.52		8.8		21.3
Yellow	11	6.47	19.4	82.35		-4.92*	16.4		7.25	3.816		4.685	64.4		45.83		5.5		21.4
Yellow	12	6.65	22	53.65		0.54	9.97		6.87	4.354		4.428	44		40.89		17.7		31.1
Yellow	13	6.78	21.2	79.85		7.32	14.41		6.73	3.818		10.527	56.2		47.83		13.5		23.5
Yellow	14	6.31	21.4			3.52	13.95		5.91	7.155		4.789	70.51		38.71	Glycogen A boiled over, cannot redo	6		28.6
Yellow	15	6.58	19	80.7		1.48	15.16		8.42	3.272		6.038	43.43		47.03		4.7		20.9

Yellow	16	6.19	18.4	81.92		0.48	14.31		7.1	3.078		5.402	54.99		41.07	C sample dilution was originally off-redone	8.6		27.7
Yellow	17	6.11	21.6	56.33		3.96	15.2		6.59	9.122		8.58	62.84		46.63		10.1		23.3
Yellow	18	5.66	19.2	61.5		18.7	15.53		14.64	4.366		1.507	67.29		28.64		7		21.6
Yellow	19	6.25	20.4	47.62		7.9	13.07		7.49	6.301		6.759	59.32		46.32		15.1		27.5
Yellow	20															pHu=5.59, kill weight = 21.2			
Green	1	5.65	21.4		90.58	2.28		10.39	19.4		1.666	1.18		56.91	33.18			16.3	29.3
Green	2	5.57	19.2		77.92	24.32		16.32	19.48		2.162	1.925		74.14	31.43			8.8	22.4
Green	3	6.25	21.6		100.67	4.93		8.55	4.3		5.705	6.719		63.23	42.21			12.7	23.2
Green	4	6.56	23.4		83.5	3.25		3.3	3.84		2.5	3.685		66.89	42.88			8.1	34.4
Green	5	5.55	21		62.32	29.91		6.06	19.5		1.427	0.922		53.46	30.69			22.4	32
Green	6	6.43	17.6		96.92	0.45		6.87	2.86		2.859	5.004		69.13	43.6			8.5	29.5
Green	7	6.25	22		113.99	3.91		11.31	3.58		4.654	4.774		63.28	40.59			10.9	36.9
Green	8	6.79	26		64.07	1.28		9.92	3.67		3.508	4.903		60.73	50.74			12.1	27.7
Green	9	6.34	20.4		84.58	9.16		12.33	4.49		4.205	5.848		46.26	37.86			12.8	26.3
Green	10	6.05	23.6		82.69	9.12		22.31	5.17		2.623	6.147		46.24	36.16			10.6	22.1
Green	11				92.76			18.98			2.637			51.45		No ear tag at slaughter		16.6	
Green	12	6.54	21.4		65.41	11.03		16.29	7.95		5.715	6.043		59.43	43.52			9.8	22.5
Green	13	6.41	21.4		86.57	0.30		18.28	5.68		1.839	5.975		65.49	39.62	C sample was cloudy after homog- redone		14.9	30
Green	14	6.25	19.2		77.07	3		12.08	6.45		4.201	5.778		55.72	40.66			13.5	24.4
Green	15	6.21	24.4		94.78	5.5		12.7	6.75		3.526	5.394		55.49	40.46			9.8	34.2
Green	16	6.17	19.4		94.79	5.01		11.63	5.26		3.205	6.864		49.78	40.09			19.1	22
Green	17	5.57	19		86.82	41.67		8.21	23.21		2.434	2.287		53.53	39.42			18.1	25
Green	18	5.82	22.6		88.47	16.84		11.15	6.18		2.384	2.685		51.68	42.99			19.3	18.8
Green	19	5.64	18.8		68.45	26.62		12.66	16.11		2.182	2.449		67.72	31.76			16.1	30.2
Green	20															not enough lambs to slaughter			

Appendix 4: Yield Trial

Appendix 5.1: Iodine Assay Results

Set 1		Set 2		Set 3	
Sample #	Corrected absorbance	Sample #	Corrected Absorbance	Sample #	Corrected Absorbance
1	0.1200	1	0.0203	1	0.0718
2	0.3016	2	0.0492	2	0.2885
3	0.7891	3	0.1318	3	0.3094
4	0.0365	4	0.0553	4	0.3413
5	0.1406	5	0.0653	5	0.8276
6	0.0477	6	0.1813	6	0.5314
7	0.0349	7	0.6371	7	1.3592
8	0.0549	8	0.0863	8	0.5587
9	0.1160	9	0.2140	9	0.5439
10	0.1869	10	0.1382	10	0.4293
11	0.0616	11	0.3142	11	0.4445
12	0.0295	12	0.0451	12	0.5041
13	0.0562	13	0.1021	13	0.6969
14	0.2906	14	0.1592	14	0.6714
15	0.1644	15	0.1075	15	0.2729
16	0.0395	16	0.4395	16	0.7205
17	0.0047	17	0.1588	17	0.3056
18	0.0698	18	0.0594	18	0.3506
19	0.1341	19	0.0730	19	0.2918
20	0.2223	20	0.1661	20	0.3189
21	0.0217	21	0.2466	21	0.3609
22	0.0585	22	0.0434	22	0.3825
23	0.0383	23	0.0833	23	0.3155
24	0.0660	24	0.1630	24	0.2734
25	0.0599	25	0.0647	25	0.1377
26	0.0400	26	0.2348	26	0.3499
27	0.0338	27	0.0553	27	0.6055
28	0.4286	28	0.0572	28	1.2283
29	0.3545	29	0.0484	29	0.7010
30	0.2070	30	0.0989	30	0.4122

31	0.0952	31	0.0635	31	0.4833
32	0.2112	32	0.0431	32	0.4130
33	0.0447	33	0.0801	33	0.8933
34	0.6466	34	0.1462	34	0.7245
35	0.0293	35	0.0965	35	0.5192
36	0.1905	36	0.0791	36	0.2088
37	0.0617	37	0.0455	37	0.3322
38	0.2393	38	0.1497	38	0.6353
39	0.0643	39	0.0759	39	0.2082
40	0.0663	40	0.0419	40	0.1298
41	0.1058	41	0.0433	41	0.1792
42	0.5882	42	0.0919	42	0.3254
43	0.0945	43	0.0442	43	0.3547
44	0.2664	44	0.0687	44	0.1556
45	0.0401	45	0.1222	45	0.1263
46	0.0833	46	0.1584	46	0.0888
47	0.1470	47	0.1368	47	0.0990
48	0.0786	48	0.1041	48	0.1256
49	0.8327	49	0.0583	49	0.5290
50	0.1387	50	0.0517	50	0.5344
51	0.1652	51	0.1379	51	0.8620
52	0.1490	52	0.0519	52	0.2992
53	0.0711	53	0.0649	53	0.1111
54	0.1598	54	0.3699	54	0.0847
55	0.1109	55	0.1374	55	0.0874
56	0.0361	56	0.2743	56	0.2395
57	0.0468	57	0.0528	57	0.3682
58	0.1386	58	0.0666	58	0.0911
59	0.0605	59	0.0666	59	0.6488
60	0.4507	60	0.0405	60	0.2062
61	0.0573	61	0.2146	61	0.1386
62	0.5055	62	0.0477	62	0.4494
63	0.2780	63	0.2091	63	0.1052
64	0.1151	64	0.0666	64	0.2203
65	0.0522	65	0.1327	65	0.1009
66	0.0904	66	0.0759	66	0.0909

67	0.0856	67	0.0680	67	0.0943
68	0.0660	68	0.1822	68	0.6614
69	0.0862	69	0.1021	69	0.0957
70	0.0655	70	0.0758	70	0.4500
71	0.3371	71	0.0387	71	0.3652
72	0.0736	72	0.0907	72	0.3337
73	0.0428	73	0.0458	73	0.4632
74	0.0606	74	0.2097	74	0.0817
75	0.0879	75	0.2003	75	0.4137
76	0.1444	76	0.0628	76	0.4536
77	0.0899	77	0.0736	77	0.1914
78	0.0842	78	0.0491	78	0.3653
79	0.1242	79	0.0947	79	0.3440
80	0.0564	80	0.2606	80	0.9397
81	0.2664	81	0.1941	81	0.4409
82	0.0533	82	0.0536	82	0.2601
83	0.0358	83	0.2976	83	0.0867
84	0.5057	84	0.0549	84	0.0883
85	0.0758	85	0.0415	85	0.0803
86	0.0418	86	0.0490	86	0.0399
87	0.1466	87	0.0614	87	0.0539
88	0.0886	88	0.1092	88	0.2415
89	0.0924	89	0.0608	89	0.2225
90	0.1490	90	0.0808	90	0.1008
91	0.1519	91	0.1041	91	0.1401
92	0.1245	92	0.3234	92	0.0911
93	0.0652	93	0.0705	93	0.1412
94	0.0664	94	0.0498	94	0.1022
95	0.0625	95	0.0530	95	0.3995
96	0.0482	96	0.0433	96	0.8309
97	0.0856	97	0.0430	97	0.4400
98	0.2769	98	0.0385	98	0.4357
99	0.0736	99	0.0513	99	0.3588
100	0.0452	100	0.0948	100	0.3309
		101	0.0483	101	0.1015
		102	0.0534	102	0.0478

		103	0.0444	103	0.0529
		104	0.0389	104	0.2948
		105	0.4425	105	0.1180
		106	0.2675	106	0.0796
		107	0.0920	107	0.0990
		108	0.0800	108	0.1007
		109	0.4801	109	0.3163
		110	0.0496	110	0.0693
		111	0.1478	111	0.2571
		112	0.4963	112	0.0793
		113	0.0611	113	0.3699
		114	0.0914	114	0.1749
		115	0.2086	115	0.0870
		116	0.0475	116	0.2551
		117	0.1461	117	0.0755
		118	0.0914	118	0.1470
		119	0.0412	119	0.1911
		120	0.0633	120	0.1191
		121	0.2623		
		122	0.0477		
		123	0.1607		
		124	0.0596		
		125	0.0956		
		126	0.0609		
		127	0.1543		
		128	0.0466		
		129	0.0428		
		130	0.0818		
		131	0.0598		
		132	0.3507		
		133	0.0442		
		134	0.0839		
		135	0.0638		
		136	0.0667		
		137	0.0477		
		138	0.0502		

		139	0.1210		
		140	0.0776		
		141	0.0600		
		142	0.0843		
		143	0.0570		
		144	0.0490		
		145	0.0551		
		146	0.2080		
		147	0.0612		
		148	0.0487		
		149	0.0557		

Appendix 5.2: Glycogen and glucose assay results using hexokinase

Set	Sample	Glycogen weight (mg)	Glycogen (uM/g)	Glucose weight (mg)	Glucose (uM/g)
1	1	107.8	33.88	152.4	18.85
1	2	133.9	54.27	142	18.61
1	3	146.2	11.14		
1	4	142.3	16.53		
1	5	133.7	30.00	106	2.70
1	6	130.9	12.95	121.6	14.68
1	7	113.8	36.01		
1	8	132.1	38.17		
1	9	121.9	35.17		
1	10	125.1	33.74		
1	11	148.4	14.44		
1	12	136	40.50	115.8	16.98
1	13	165.6	19.88		
1	14	116.6	35.02		
1	15	117.4	55.52		
1	16	129.3	28.17		
1	17	104.6	17.60		

1	18	140.4	52.91	147.5	16.99
1	19	117.3	31.53		
1	20	113	44.04		
1	21	140.1	28.83		
1	22	139.2	13.44		
1	23	135.5	25.76	115.9	19.01
1	24	112.2	16.67		
1	25	118.2	39.44		
1	26	159	36.53		
1	27	109.3	29.34	128.6	19.13
1	28	103.7	18.83		
1	29	141.1	31.32		
1	30	141.1	15.17		
2	1	143.5	24.84	136.9	17.23
2	2	104.5	39.91	120.2	22.30
2	3	107.6	24.12	125.9	22.03
2	4	140.7	24.81	137.3	15.14
2	5	142.9	11.85	115.6	11.25
2	6	122.9	21.36	118.7	21.28
2	7	125.9	14.43	121.4	11.52
2	8	115.7	12.66	139.5	17.15
2	9	109.6	12.83	131.7	13.11
2	10	127.5	28.37	109.7	19.60
2	11	125.4	29.71	113	23.07
2	12	120.1	17.53	111.7	21.37
2	13	123.7	12.42	100.4	10.25
2	14	102.3	29.76	101.9	21.25
2	15	105.5	24.15	112.7	22.75
2	16	143	34.47	118	22.58
2	17	116.9	36.98	127.5	16.10
2	18	133.2	24.54	134.7	17.30
2	19	119.2	17.20	128.6	16.74
2	20	107.3	38.03	106.5	19.55

2	21	106	35.10	124.6	20.28
2	22	145.3	9.89	128.2	11.68
2	23	141.9	32.48	124.3	23.81
2	24	129.2	26.90	120.9	20.83
2	25	144.5	9.55	131	7.01
3	1	113.1	21.79	123.3	22.76
3	2	116	37.45	123.9	22.78
3	3	110.2	33.95	128.1	15.63
3	4	140	35.65	116.9	21.79
3	5	106.3	32.40	122.3	17.65
3	6	152.9	19.59	110.9	18.66
3	7	144.5	30.57	118	24.80
3	8	103.1	33.89	117.5	19.53
3	9	104	33.19	149.1	22.59
3	10	131.5	41.72	153.5	20.47
3	11	156.1	11.81	134.6	13.83
3	12	112	9.41	136	12.19
3	13	148.7	12.50	151.3	15.73
3	14	133.2	19.99	101.3	19.55

Appendix 5.3: Meat quality characteristics results for high glycogen samples.

Set	Sample	Crude Protein (g/100g)	Crude Fat (%)	Tray Drip (%)	Water Binding Capacity (cm^2/5g)	Aerobic Plate Count (CFU)	Water Content (%)
1	2	3.572	2.57	1.74	10.05	68	73.90
1	8	3.666	3.64	1.93	8.73	34	73.32
1	9	3.666	3.12	2.83	10.70	24	72.82
1	12	3.29	3.10	2.88	10.72	21	72.54
1	15	3.29	4.24	1.65	6.86	25	73.77
1	18	3.478	2.40	4.97	10.83	48	73.34
1	19	3.572	3.26	2.79	10.66	74	72.84

1	20	3.478	4.29	1.70	6.91	5	72.01
1	26	3.384	4.58	3.48	10.81	34	72.90
2	2	3.666	2.48	3.08	10.80	7800	74.14
2	4	3.196	5.40	4.09	12.23	12000	72.19
2	17	3.572	3.70	1.83	12.12	1100	73.55
2	20	3.384	2.12	2.35	12.06	840	75.19
2	21	3.384	4.08	2.32	10.79	1300	73.43
3	1	3.384	1.78	2.40	9.03	90	75.08
3	2	3.196	2.32	1.87	9.12	110	75.13
3	3	3.196	2.27	2.03	12.32	40	74.20
3	4	3.384	2.18	2.50	13.00	230	74.93
3	5	3.478	1.64	2.24	9.18	50	75.73
3	6	3.478	2.20	2.05	12.18	70	74.74
3	7	3.384	1.32	1.83	9.84	90	74.24
3	8	3.384	1.44	2.39	9.03	30	74.80
3	9	3.196	3.54	2.07	11.35	270	76.99
3	10	3.384	2.00	1.64	11.35	70	75.15
3	11	3.384	1.88	2.52	10.29	20	73.47

Appendix 5.4: Meat quality characteristics results for samples with medium levels of glycogen.

Set	Sample	Crude Protein (g/100g)	Crude Fat (%)	Tray Drip (%)	Water Binding Capacity (cm^2/5g)	Aerobic Plate Count (CFU)	Water Content (%)
1	23	3.478	3.43	2.74	8.24	67	73.49
1	25	3.29	3.76	2.12	8.98	9	71.74
1	29	3.572	3.56	2.16	6.69	60	72.75
2	3	3.384	1.54	3.42	12.58	3900	74.85
2	6	3.478	2.59	4.64	12.25	1200	73.98
2	10	3.572	4.61	2.40	11.69	130	72.26
2	11	3.384	2.05	2.88	10.99	310	74.16

2	14	3.384	2.77	2.41	13.21	14000	74.38
2	15	3.572	4.05	2.39	11.62	9100	68.00
2	16	3.666	3.14	2.72	9.83	53000	72.99
2	18	3.384	1.95	1.46	10.74	9100	74.21
2	23	3.854	2.39	2.95	12.79	5200	75.34
2	24	3.384	2.86	2.73	11.62	22000	75.08

Appendix 5.5: Meat quality characteristics results for low glycogen samples.

Set	Sample	Crude Protein (g/100g)	Crude Fat (%)	Tray Drip (%)	Water Binding Capacity (cm^2/5g)	Aerobic Plate Count (CFU)	Water Content (%)
1	3	3.384	4.00	1.52	9.83	9	73.94
1	4	3.384	4.69	2.04	9.15	30	74.45
1	6	3.384	2.32	1.68	9.07	12	74.51
1	11	3.478	2.40	1.95	10.71	14	74.00
1	17	3.384	2.29	1.78	11.20	73	74.00
1	22	3.666	4.20	2.56	8.55	41	71.82
1	24	3.572	3.09	1.87	8.69	130	72.31
1	27	3.76	2.51	2.14	9.29	35	72.67
1	28	3.384	3.72	2.43	8.51	69	72.51
1	30	3.478	3.16	2.15	9.95	23	73.14
2	1	3.196	2.25	2.18	9.79	6400	75.23
2	5	3.196	2.22	1.38	9.86	1800	74.88
2	7	3.478	3.08	1.75	10.23	830	74.70
2	8	3.384	3.00	3.36	9.25	2600	73.99
2	9	3.384	3.05	3.05	12.13	600	73.14
2	12	3.384	2.33	1.71	12.04	8000	73.19
2	13	3.76	3.23	1.65	10.16	440	72.82
2	19	3.666	5.54	2.87	11.53	14000	72.19
2	22	3.76	3.34	1.21	11.25	1900	74.74
2	25	3.384	3.20	1.79	10.88	25000	75.13

3	12	3.196	3.88	0.83	14.23	20	73.98
3	13	3.384	2.72	2.43	10.22	20	75.45
3	14	3.196	2.51	1.81	13.27	460	74.38

Appendix 5.6: Sets 1, 2, and 3 APC.

Set	Sample	APC (CFU) day 1
1	1	51
1	2	68
1	3	9
1	4	30
1	5	29
1	6	12
1	7	16
1	8	34
1	9	24
1	10	74
1	11	14
1	12	21
1	13	21
1	14	36
1	15	25
1	16	470
1	17	73
1	18	48
1	19	74
1	20	5
1	21	86
1	22	41
1	23	67
1	24	130
1	25	9
1	26	34
1	27	35
1	28	69
1	29	60
1	30	23
	Average	**56**

	s	**83**

Set	Sample	APC (CFU) day 1
2	1	6400
2	2	7800
2	3	3900
2	4	12000
2	5	1800
2	6	1200
2	7	830
2	8	2600
2	9	600
2	10	130
2	11	310
2	12	8000
2	13	440
2	14	14000
2	15	9100
2	16	53000
2	17	1100
2	18	9100
2	19	14000
2	20	840
2	21	1300
2	22	1900
2	23	5200
2	24	22000
2	25	25000
	Average	**8102**
	s	**11 559**

Set	Sample	APC (CFU) day 1

3	1	90
3	2	110
3	3	40
3	4	230
3	5	50
3	6	70
3	7	90
3	8	30
3	9	270
3	10	70
3	11	20
3	12	20
3	13	20
3	14	460
	Average	**112**
	s	**126**

Appendix 5.7: Week 8 storage results for high glycogen

Set	Sample	Original #	pHu	Estimated Glycogen Level	Glycogen (uM/g)	Glucose (uM/g)	APC (CFU)	WBC (cm^2/5g)	Storage Drip Loss (%)
1	2	3	5.66	High	31.44	22.23	220,000,000.00	6.55	1.51
1	8	28	5.58	High	20.32	26.53	15,000,000	4.87	2.31
1	9	29	5.56	High	19.02	19.68	43,000,000	5.97	2.01
1	12	34	5.66	High	23.05	20.85	13,000,000	4.37	1.68
1	15	42	5.54	High	23.21	22.13	55,000,000	4.18	2.06
1	18	49	5.56	High	25.37	22.91	12,000,000	7.12	9.34
1	19	60	5.5	High	19.27	17.47	20,000,000	5.30	2.87
1	20	62	5.54	High	22.00	19.00	18,000,000	4.71	2.72
1	26	84	5.53	High	20.57	22.31	24,000,000	6.77	1.85
2	2	7	5.44	High	29.48	6.52	140,000,000	6.07	3.74
2	4	16	5.48	High	13.32	3.65	15,000,000	13.22	8.36
2	17	105	5.6	High	25.91	5.31	200,000,000	8.36	3.38
2	20	109	5.58	High	34.59	7.43	190,000,000	7.85	2.50
2	21	112	5.53	High	25.90	4.95	210,000,000	8.64	1.98
3	1	5	5.51	High	19.77	6.02	61,000,000	8.38	3.35
3	2	7	5.49	High	38.70	12.36	16,000,000	8.67	3.89
3	3	13	5.63	High	32.22	11.07	33,000,000	8.2	1.89

3	4	16	5.61	High	33.39	8.08	24,000	8.81	6.45
3	5	28	5.53	High	33.66	11.96	6,300,000	5.52	3.58
3	6	29	5.53	High	18.09	10.43	20,000,000	5.48	2.72
3	7	33	5.61	High	27.92	10.48	10,000,000	6.24	2.11
3	8	51	5.5	High	28.56	11.05	7,200,000	6.08	4.53
3	9	80	5.63	High	34.85	10.11	130,000	5.32	2.43
3	10	96	5.64	High	42.59	6.04	1,700,000	5.15	1.40
3	11	150	5.57	High	22.59	9.82	41,000,000	7.44	3.02

Appendix 5.8: Week 8 storage results for medium glycogen

Set	Sample	Original #	pHu	Estimated Glycogen Level	Glycogen (uM/g)	Glucose (uM/g)	APC (CFU)	WBC (cm2/5g)	Storage Drip Loss (%)
1	1	2	5.6	Medium	19.88	29.18	180,000,000.00	5.51	1.72
1	5	10	5.54	Medium	14.45	22.23	26,000,000.00	4.79	4.72
1	7	14	5.61	Medium	16.00	15.95	34,000,000.00	5.10	1.70
1	10	32	5.64	Medium	14.97	15.47	21,000,000	3.39	1.65
1	13	36	5.57	Medium	8.66	16.05	26,000,000	5.12	2.71
1	14	38	5.49	Medium	15.04	14.89	15,000,000	5.05	1.87
1	16	44	5.56	Medium	9.16	15.19	14,000,000	6.60	2.49
1	21	63	5.54	Medium	14.64	12.82	20,000,000	5.11	2.65

1	23	71	5.56	Medium	5.51	11.19	1,100,000,000	6.07	2.75
1	25	81	5.48	Medium	13.57	21.64	24,000,000	4.49	5.33
1	29	98	5.5	Medium	14.93	25.91	47,000,000	4.78	2.61
2	3	11	5.46	Medium	20.10	5.81	180,000,000	8.92	4.89
2	6	26	5.48	Medium	17.86	5.70	130,000,000	8.23	6.11
2	10	54	5.51	Medium	31.43	8.07	160,000,000	8.20	3.17
2	11	56	5.73	Medium	11.19	3.43	170,000,000	7.92	3.95
2	14	80	5.61	Medium	16.42	4.36	200,000,000	9.15	5.21
2	15	83	5.48	Medium	22.78	5.68	90,000,000	5.81	3.89
2	16	92	5.42	Medium	20.74	6.02	200,000,000	8.90	5.17
2	18	106	5.55	Medium	27.50	5.18	190,000,000	6.84	1.99
2	23	121	5.51	Medium	25.14	7.23	300,000,000	9.76	1.88
2	24	132	5.51	Medium	19.59	4.93	140,000,000	8.03	4.84

Appendix 5.9: Week 8 storage results for low glycogen

Set	Sample	original #	pH	Estimated Glycogen Level	Glyco Hexokinase (uM/g)	Glucose (uM/g)	APC	WBC	Storage Drip Loss (%)
1	3	4	5.57	Low	6.38	5.27	41,000,000.00	6.60	2.90
1	4	6	5.6	Low	9.56	7.85	38,000,000.00	3.12	3.21
1	6	13	5.61	Low	8.17	5.16	29,000,000.00	3.36	6.35

1	11	33	5.66	Low	10.75	10.49	28,000,000	6.39	3.35
1	17	45	5.57	Low	5.74	10.05	13,000,000	3.95	8.23
1	22	65	5.59	Low	5.58	6.43	43,000,000	4.56	3.44
1	24	74	5.58	Low	4.35	8.78	22,000,000	5.83	1.78
1	27	86	5.57	Low	12.75	20.63	21,000,000	4.37	3.13
1	28	94	5.51	Low	5.02	9.31	24,000,000	4.95	4.12
1	30	100	5.58	Low	8.95	7.32	28,000,000	4.97	4.17
2	1	4	5.5	Low	13.16	3.04	190,000,000	7.54	3.71
2	5	23	5.67	Low	6.53	2.39	220,000,000	8.58	4.30
2	7	42	5.53	Low	15.21	4.15	210,000,000	6.84	1.00
2	8	45	5.5	Low	17.77	4.82	190,000,000	7.37	2.97
2	9	48	5.57	Low	12.21	2.63	150,000,000	8.92	4.18
2	12	58	5.63	Low	11.42	2.45	150,000,000	6.99	3.47
2	13	72	5.64	Low	13.91	4.62	190,000,000	5.38	2.51
2	19	108	5.63	Low	18.37	3.76	190,000,000	7.99	3.68
2	22	113	5.61	Low	17.48	2.33	160,000,000	8.91	2.83
2	25	147	5.58	Low	21.91	3.20	180,000,000	10.53	3.83
3	12	140	5.93	Cloudy Orange	15.54	10.47	130,000,000	5.92	2.25
3	13	141	5.62	Cloudy Orange	21.10	8.55	41,000,000	10.13	4.15
3	14	149	5.67	Cloudy Orange	26.58	12.87	2,800,000	8.4	2.55

Appendix 5.10: APC results after 8 weeks of storage by sets

Set	Sample	Original #	APC (CFU)
1	1	2	180,000,000
1	2	3	220,000,000
1	3	4	41,000,000
1	4	6	38,000,000
1	5	10	26,000,000
1	6	13	29,000,000
1	7	14	34,000,000
1	8	28	15,000,000
1	9	29	43,000,000
1	10	32	21,000,000
1	11	33	28,000,000
1	12	34	13,000,000
1	13	36	26,000,000
1	14	38	15,000,000
1	15	42	55,000,000
1	16	44	14,000,000
1	17	45	13,000,000
1	18	49	12,000,000
1	19	60	20,000,000
1	20	62	18,000,000
1	21	63	20,000,000
1	22	65	43,000,000
1	23	71	1,100,000,000
1	24	74	22,000,000
1	25	81	24,000,000
1	26	84	24,000,000
1	27	86	21,000,000
1	28	94	24,000,000
1	29	98	47,000,000
1	30	100	28,000,000
		Mean	73,800,000
		Standard Dev	199,112,410

		Min	12 000 000
		Max	1 100 000 000

Set	Sample	Original #	APC (CFU)
2	1	4	190,000,000
2	2	7	140,000,000
2	3	11	180,000,000
2	4	16	15,000,000
2	5	23	220,000,000
2	6	26	130,000,000
2	7	42	210,000,000
2	8	45	190,000,000
2	9	48	150,000,000
2	10	54	160,000,000
2	11	56	170,000,000
2	12	58	150,000,000
2	13	72	190,000,000
2	14	80	200,000,000
2	15	83	90,000,000
2	16	92	200,000,000
2	17	105	200,000,000
2	18	106	190,000,000
2	19	108	190,000,000
2	20	109	190,000,000
2	21	112	210,000,000
2	22	113	160,000,000
2	23	121	300,000,000
2	24	132	140,000,000
2	25	147	180,000,000
		Mean	173,800,000
		Standard Dev	50,975,484
		Min	15 000 000

		Max	300 000 000

Set	**Sample**	**Original #**	**APC (CFU)**
3	1	5	61,000,000
3	2	7	16,000,000
3	3	13	33,000,000
3	4	16	24,000
3	5	28	6,300,000
3	6	29	20,000,000
3	7	33	10,000,000
3	8	51	7,200,000
3	9	80	130,000
3	10	96	1,700,000
3	11	150	41,000,000
3	12	140	130,000,000
3	13	141	41,000,000
3	14	149	2,800,000
		Mean	26,439,571
		Standard Dev	35,239,362
		Min	24 000
		Max	130 000 000