Effects of Beef Breed Type, Residual Feed Intake and Collagen Heat Solubility on Meat Quality, Connective Tissue Characteristics and Expression of Collagen-Related Genes of *Semimembranosus* Muscle

by

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Abstract

Cattle breed, residual feed intake and genetics are factors that affect beef quality. Genotype can influence meat toughness and approximately 46% of beef quality variation can be attributed to the variation between animals. Calpain (CAPN1) and calpastatin (CAST) genes are associated with myofibrillar protein degradation early post mortem and are correlated with variation in beef toughness but the relationships between genes controlling collagen synthesis and degradation and beef toughness have not yet been examined. The objectives of this research were to: (1) determine the effect of breed and residual feed intake on beef quality and collagen characteristics; and (2) determine how the expression of genes involved in collagen synthesis and degradation were related to meat quality and collagen characteristics of the *semimembranosus*, a muscle from the inside round. Seventy-one (71) carcasses were harvested from purebred Angus (n = 23) and Charolais (n = 24) and Angus crossbred (Kinsella composite, n = 24) steers of high (n = 35) and low (n = 36) residual feed intake status. The *m. semimembranosus* muscle (inside round) was removed from the right side of each carcass, and steaks from each muscle were aged for 3 and 13 days. Breed and residual feed intake did not affect meat quality and collagen characteristics however post mortem aging decreased Warner-Bratzler shear force and increased collagen heat solubility.

From the data set, twelve steers with low [10.66 $\pm$ 2.99 (standard deviation, SD) %] and twelve with high [20.61 $\pm$ 7.51 (SD)%] intramuscular collagen heat solubility at day 3 post mortem were selected within each breed. The expression of 14 candidate genes in the m. *semimembranosus* was evaluated using quantitative real time polymerase chain reaction (RT-qPCR) using gene specific primer pairs relative to *18S RNA* expression. Breed type significantly ($P<0.05$) affected the mean ΔCt of *CTGF, FOXO1, P4HA1* and *SMAD2*. Collagen heat solubility did not affect the expression level of the genes; however, there was an interaction between breed type and collagen heat solubility in the ΔCt mean for *CTGF*. Sarcomere length was negatively correlated ($P<0.001$) with *COL3A1* and *COL5A2* and *P4HA1* ($P<0.05$) gene expression. Warner-Bratzler shear force at 3 days post-mortem was negative correlated with *COL3A1* ($P<0.05$) and *FOXO1* ($P<0.01$) but was positively correlated with *CTGF, IGF-1* while *SMAD2*. *COL5A2* and *MMP2* were positively correlated ($P<0.05$) with heat-soluble collagen at 3 days post-mortem. The concentration of pyridinoline cross links (mol PYR/mol collagen) was negatively correlated ($P<0.05$) with *COL3A1* and *COL5A2* while positively correlated with *COL5A1*. Expression of *LOX* was positively

correlated ($P<0.01$) with the concentration of pyridinoline (nmol PYR/ g raw meat). Results indicated that expression of genes related to collagen synthesis and degradation were related to increased beef toughness. Overall, this study indicated that selection for low RFI cattle will not compromise m. *semimembranosus* meat quality, and that the contribution of collagen to beef toughness can be influenced through breed type selection.

To my husband Mutaz Mukhtar

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Abbreviations

ADG	Average daily gain
ANOVA	Analysis of variance
BW	Body weight
cDNA	Complementary deoxyribonucleic acid
Ct	Threshold cycle
DMI	Dry matter intake
DOT	Day on test
dpm aging	Day post-mortem aging
EC	Ehrlich Chromogen cross link
FUFAT	Final ultrasound back fat
IMCT	Intramuscular connective tissue
LH	Lysyl hydroxylase
mRNA	Messenger ribonucleic acid
MWT	Metabolic body weight
PCR	Polymerase chain reaction
PYR	Pyridinoline cross -link
RFI	Residual feed intake
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative reverse transcription polymerase chain reaction
SD	Standard deviation
WBSF	Warner- Bratzler shear force

Chapter 1: Introduction and literature review

Red meat, which encompasses beef, pork and lamb, plays an important role in promoting human health by providing proteins, vitamins and minerals to the human body (Xiong et al., 2014). As meat is a source of essential nutrients, beef producers and the meat industry are motivated to ensure that high quality meat products are supplied to consumers. The chemical, microbiological, sensory, and technological characteristics of meat contribute to the definition of its quality. The eating quality attribute tenderness is the most important for consumers as it affects their decisions during meat purchasing (Miller et al., 2001). Meat tenderness is affected by all structural components of muscle, but the two main components affecting meat tenderness are the myofibrillar proteins and collagen, which is the main protein found in connective tissue. Connective tissue plays an important role in terms of meat tenderness as it is responsible for background toughness (Bailey 1989; Young and Dobbie 1994; Calkins and Sullivan, 2007; Sullivan and Calkins, 2011).

Beef tenderness appears to affect the economics of the beef industry. The demand for beef decreased by 23% between 1979 to 1990 due to the lack of quality and consistency in the tenderness of beef (Miller et al., 2001). In Canada, it was estimated that inconsistencies in beef tenderness cost the Canadian beef industry $21 million annually (Beef Cattle Research Council, 2006), especially with regard to the high connective tissue cuts such as round and chuck muscle, as they are considered less utilizable and therefore are normally sold as ground meat. The average price at retail of a high connective tissue primal cut like the round in 2018 in Canada was an estimated $17.85 Canadian per kilogram compared to $22 Canadian per kilogram for sirloin (StatCan, 2018), with sirloin being considered a more tender primal than round.

Tenderness has long been known as a key characteristic used to achieve high eating quality products for beef consumers. Although the literature clearly demonstrates that there is a breed effect on the collagen content and the heat solubility of collagen in beef (Cross et al. 1984; Monsón et al. 2005; Christensen et al .2011; Panea et al. 2018), there is a research gap on how breed contributes to the meat quality of high connective tissue muscle. Young and Dobbie (1994) concluded that collagen concentration and collagen heat solubility of the semimembranosus and biceps femoris muscles were not affected by beef breed; therefore breed did not affect collagen

related tenderness attributes. Furthermore, Girard et al. (2011) concluded that there was no effect of breed cross on collagen content in the semitendinosus or gluteus medius muscles.

There is contradictory evidence regarding the effect of selection for residual feed intake on meat quality. Zorzi et al. (2013) compared low and high residual feed intake groups using fifty-nine Nellore bulls and found that low RFI Nellore bulls had higher mean shear force values at days 0, 7 and 21 post mortem than high RFI bulls. Baker et al. (2006) found no difference in mean shear force between low and high RFI Angus steers. Zorzi et al. (2013) also found there was no difference due to RFI in terms of total collagen content. Also, Baker et al. (2006) and Fidelis et al. (2017) concluded that there was no significant difference between low and high residual feed intake cattle in terms of their intramuscular total collagen content. The effect of RFI status on the heat solubility of collagen is equivocal as Fidelis et al. (2017) found no difference in collagen heat solubility between low and high RFI Nellore bulls, but Zorzi et al. (2013) found that heat soluble collagen was greatest in low RFI Nellore bulls. The variation in the results observed for the effect of RFI on beef quality may be due to an interaction between breed and RFI, with RFI possibly having a greater effect in one breed than another. Therefore, the primary objective of this thesis was to address the research gap of how breed type and selection for residual feed intake contribute to beef quality specifically as affected by connective tissue.

1.1 Beef quality and how it is measured

Meat quality is a culmination of the chemical, technological, microbial and sensory characteristics of meat and how they relate to meeting the expectations of consumers who are demanding tasty and healthful meat (Biswas and Mandal, 2020). Meat producers are challenged to obtain reliable information on meat quality as most techniques are invasive and require the destruction of the very product they seek to sell, although non-invasive techniques such as near infra-red spectroscopy are under development (Damez and Clerjon, 2008).

There are many techniques to predict the quality of the meat and they are usually divided into the categories of technological and sensory methods, as the former uses objective machine measurements while the latter uses subjective (untrained) and objective (trained) human panel measurements.

1.1.1. Meat technological measurements

Muscle composition is an important part of meat quality and includes physical methods that determine the proportions of muscle, fat and bone as well as proximate chemical methods that estimate proportions of protein, fat, water and ash (Shija et al., 2013). The importance of proximate chemical composition analysis of meat comes from the influence these components have on production processes and the end-product eating quality (Myhan et al., 2016). Proximate analysis of meat is performed traditionally using the methods of the Association of Official Analytical Chemists (AOAC, 1995); however near-infrared reflectance (NIR) spectroscopy has been developed as a rapid technique for chemical composition determination (Yang et al.2016).

Meat pH is considered one of the important measurements that indicates quality of the meat (Węglarz, 2010); thus, high quality meat tends to have an ultimate pH ranging from 5.4 to 5.6 (Węglarz, 2010). High pH such as that greater than 5.7 is associated with meat that has a dark colour (dark cutting), reduced flavour, increased water-holding capacity and rapid growth of microorganisms (Silva et al.1999; Węglarz,2010). Glycogen depletion pre-slaughter has been identified as responsible for ultimate pH variation (Mahmood et al. 2018) and is caused by various factors such as animal stress, a low energy diet, extremes in ambient temperature, inclement weather and prolonged or frequent transportation events (Li et al. 2014).

Two methods are used to measure meat pH and include a simple method by litmus paper with pH indicated by paper colour which varies with meat acidity and these colours are then compared with a colour scale associated with pH (Smulders,1986). Potentiometric measurement is a common method used in pH determination and involves using a pH probe that is placed at least 2 cm deep into the muscle that is attached to a meter that measures the electrical difference between the meat exudate and the probe reference electrode (Smulders,1986).

1.1.2. Sensory measurements

Beside the technological measurements, eating quality can be assessed by sensory evaluation that include consumer and trained panels (Kerth and Miller, 2015). Sensory measurement of fresh meat encompasses appearance as well as eating quality, with assessment of

appearance including description or rating of meat colour or structural features and eating quality including assessment of meat odor, texture, tenderness, juiciness and flavour (Smulders,1986).

Consumers consider the bright-red colour desirable in fresh red meat (Kim et al. 2009) because it represents to them the freshness of the meat (Tapp et al., 2012). Meat colour is affected by myoglobin content, and so measuring the concentration of myoglobin can give insight into what is causing the darkness or redness of meat (Smulders,1986). Myoglobin content of meat is affected by animal age with myoglobin content in muscle increasing with animal age, aerobic exercise, diet iron-richness, genetic predisposition toward red muscle fibre type, and the environment (Joo et al., 2013). Very dark red meat is usually a result of a high ultimate pH and low muscle glycogen content and not increased myoglobin, and this is considered a meat quality defect (Mahmood et al., 2016).

Fresh meat colour is normally determined by colorimetric instruments which basically measure differences in reflectance (Smulders,1986) Three colour coordinates, specifically L*, a* and b*, have been identified by the International Commission Illumination (CIE, 1978). The L* value describes meat lightness, a* describes the meat redness to greenness (+ values to – values) and b* describes the blueness (- value) to yellowness (+ value)(CIE, 1978).

Meat juiciness is defined as the amount of moisture released in the mouth during chewing and it is influenced by other meat components such as fat (Juárez et al., 2011). Sensory evaluation is the most reliable way to determine meat juiciness (Winger and Hagyard, 1999) because it is hard to measure juiciness in uncooked meat but it can be predicted by indirect instrumental method like water holding capacity.

As meat tenderness is considered the strongest single meat quality attribute based on the survey of meat consumers (Morgan et al. 1991), many studies have been conducted to understand tenderness and how it is measured. The two main muscle components responsible for meat toughness are the myofibrillar and connective tissue proteins. Warner-Bratzler shear force is a traditional method to predict meat toughness by assessing the force required to cut a core of cooked meat (Destefanis et al., 2008). There are many factors influencing the accuracy of the results such as how the muscle in question is sampled and cut, how it is cooked and to what temperature, and how it is cored (round or rectangular), thus it is necessary to follow a Warner-Bratzler shear force measurement protocol to decrease the variation in Warner-Bratzler shear force measurement results (Wheeler et al., 1996).

1.1.3. Collagen quality measurement

Collagen is the most abundant protein in connective tissue and it is considered to influence the background toughness of beef and be resistant to change during post mortem aging (Purslow, 2005). The background toughness of beef can be estimated either by determination of total collagen content, collagen heat solubility or by measurement of trivalent collagen cross-link concentrations (Roy et al. 2015). Total collagen content is measured through the determination of hydroxyproline which uniquely accounts for 14% of collagen (Pearson and Young, 1989). The method of hydroxyproline is based on the three steps with an acid hydrolysis of the meat tissue first followed by quantification of hydroxyproline as the unique amino acid for collagen at 558 nm using a colorimetric method (Stoilov et al. 2018). Finally, the total collagen content is obtained by multiplying the derived hydroxyproline concentration by 7.14.

The heat solubility of collagen is estimated because it provides an indication of the resistance of collagen to degradation during cooking. Commonly collagen heat solubility is measured by cooking dried meat in quarter-strength Ringer's solution (Hill, 1966) as it is more representative of the physiological solution of the muscle environment than water and may contribute to the degradation of the intermolecular bonds within collagen (Latorre et al .2016).

The concentration of the trivalent collagen cross-links is an important factor as they are considered heat stable and thus most likely to influence meat toughness (Roy et al. 2015); therefore, the measurement of trivalent cross-links can be considered an indicator of potential meat toughness (Roy et al. 2015). Ehrlich chromogen (EC) and the pyridinolines (PYR, lysyl and hydroxylysyl) are trivalent collagen cross-links commonly measured. The principle of Ehrlich chromogen determination is based on the cross-links released by tryptic digest forming a chromophore with the reagent p-dimethylaminobenzaldehyde, also known as Ehrlich's reagent, and its absorbance being measured using a spectrophotometer at 572 nm (Horgan et al. 1990). Measurement of pyridinoline cross-links involves protecting the cross-link preparations from light as they are susceptible to degradation by ultraviolet light (Meddah et al. 2000). Measurement of pyridinoline cross-links depends on the fractions containing pyridinoline from hydroxylated tissue being purified using cation-exchange chromatography and then measured by reversed-phase high-performance liquid chromatography (Takahashi et al. 1995). Characterization of collagen heat

solubility and collagen trivalent cross-link concentrations will provide an estimation of the contribution of collagen to the background toughness of meat.

1.2 Factors affecting beef quality

As indicated previously, meat quality is a generic term used to describe the subjective attributes of meat encompassing attributes such as color, texture, juiciness, tenderness, odor and flavor that are most important for consumers as they may influence consumer decisions before and after purchasing of beef (Maltin et al., 2003). These attributes may be affected by production factors such as cattle breed, animal residual feed intake, animal age, pre-slaughter animal management and environmental conditions, intramuscular calpain and calpastatin activities, sarcomere length, collagen heat solubility, density and quantity of collagen cross links and length of post-mortem aging. Animal health is one of the intrinsic factors that can increase the stress on the animal and thus affect the quality of the meat (Xing et al. 2019). These factors may affect meat quality in very specific ways, and their effects can be additive leading to a cumulative effect on meat quality.

1.2.1 Beef breed

Among all animals, cattle are considered one of the most important livestock species (Felius et al., 2011) because they provide meat, milk, hides and historical draught animal capabilities. In this way, cattle are considered multi-purpose livestock (Felius et al., 2011). Most cattle breeds have been assigned to the main two sub-species *Bos taurus* and *Bos indicus* (Haskell et al., 2014). There are differences between the two sub-species in terms of their heat tolerance, as *Bos indicus* is prevalent in hot climates while *Bos taurus* cattle are common in temperate regions (Burrow 2014; Kinghorn et al., 2014). Thus, the main beef cattle sub-species in Canada is *Bos taurus*. Currently there are twenty-six breeds of *Bos taurus* beef cattle breeds recognized under the Animal Pedigree Act (APA) [Canadian Beef Breeds Council (CBBC), 2019b]. There are many studies comparing the two sub-species in term of meat quality and most have revealed that *Bos taurus* produces beef that is more tender and of higher quality than that of *Bos indicus* (Marshall, 1994; Wheeler et al., 1996; Shackelford et al., 1995; Strydom, Frylinck and Smith 2011).

Angus and Charolais belong to the *Bos taurus* sub-species, and are two breeds widely used for beef cattle production in Canada. Angus belongs to the British breeds, which were first imported to Canada from Scotland where they were developed in the 18th century. Although in United States Black and Red Angus are listed as different breeds, in Canada they are considered different colors of the same breed (Wolfger et al. 2016). Angus is the most used beef breed in Canada (Carruthers et al. 2011) and there is increased demand for Angus cattle in Canada due to the breed's high carcass quality (Canadian Beef Breeds Council, 2019a). The Charolais breed belongs to the Continental European breeds and, compared with the Angus breed the Charolais generally is characterized by being larger in size, and producing a carcass with less fat and high yield (Greiner, 2009).

The environment and diet cattle experience during their production lives can affect their rate of growth (Turne, 1984), carcass composition (Park et al. 2018) and the composition and eating quality of their beef (Steen et al.2003; Park et al. 2018). Environment and diet can also interact with breed, for example, with cattle genetically predisposed to depositing increased intramuscular fat doing so when fed high energy diets (Park et al. 2018). When environment and diet are held constant, breed and genetics can affect beef quality due to variation in muscle structure and meat physiology between cattle (Sañudo et al., 2004; Waritthitham et al., 2010). Sañudo et al. (2004) and Waritthitham et al. (2010) agreed that differences existed between individual beef breeds, while Gregory et al. (1994a) concluded that growth and carcass traits were different between breeds. Cross et al. (1984) found variation in carcass traits and meat palatability between the carcasses from Charolais, Simmental, Hereford and Aberdeen Angus steers. Similarly, Barton et al. (2006) found that there were significant differences between breed for growth, slaughter and carcass traits when Aberdeen Angus, Charolais, Hereford and Simmental bulls were compared. In contrast Xie et al. (2012) concluded there was no breed effect on mean Warner-Bratzler shear force, crude intramuscular fat, or fat color when imported Limousin and Simmental were compared to Luxi, Qinchuan and Jinnan cattle (local breeds in China). Numerous authors found that there are significant meat quality differences between *Bos taurus* breeds, as Chambaz et al. (2003) compared the *longissimus dorsi* muscle from Angus, Simmental, Charolais and Limousin steer carcasses and found obvious differences in meat quality between the breeds even with the same marbling score. On the other hand, the study of Bureš et al. (2006), which compared *longissimus thoracis et lumborum* muscle from Aberdeen Angus, Charolais, Simmental, and

Hereford bull carcasses concluded that lipid content was higher in muscle from Angus and Hereford cattle (British breeds) than in carcasses from the Charolais and Simmental breeds; however, Angus had the lowest protein content among all breeds but received the highest mean sensory scores for odour, flavour, texture, and juiciness. Similarly, Wheeler et al. (1996) studied carcass and palatability traits of the *longissimus thoracis* muscle from 888 steers from different breeds and concluded that there were significant differences between breed in terms of gain, fat thickness, marbling scores and shear force.

Based upon these findings, it is clear that breed selection can affect carcass and meat composition, and that substantial differences in meat quality can exist between breeds. There is then the possibility that selection for residual feed intake may affect meat quality more in one breed than another.

1.2.2 Residual feed intake

Residual feed intake (RFI), also known as net feed intake, was first identified by Koch et al. (1963). Residual feed intake is now considered one of the common measures of feed efficiency (Arthur and Herd, 2008). RFI is defined as the actual minus expected feed intake of each animal for a specific period of time based on its size and growth (Koch et al. 1963; Kennedy et al. 1993; Richardson et al.2001; Basarab et al. 2003; Cruz et al, 2006; Chen et al .2011; Fidelis et al. 2017). Cattle are classified based on their feed efficiency into low and high residual feed intake strains (Meyer et al. 2008), where a negative RFI value or lower RFI is considered more efficient than a positive RFI value (Berry and Crowley, 2013). There is strong evidence of genetic variation between RFI groups, and a study by Chen et al. (2011) investigated the variation between low and high RFI in one hundred and sixty-one genes expressed in 44 cattle. The genes identified as affected by RFI were associated with proliferation of cell growth, protein synthesis, and carbohydrate and lipid metabolism.

Many authors agree with the economic and environmental benefits of selection for residual feed intake but selection for more efficient cattle will only be beneficial if carcass and meat quality are not affected. Although the studies available are few, most of the studies found that efficient cattle produced acceptable meat quality, but still the effects of selection for low RFI are inconsistent. A study by Fidelis et al. (2017) on 127 Nellore bulls to examine the relationship

between meat quality and low and high RFI concluded that low RFI Nellore bulls produced carcasses with good quality meat, while Baker et al. (2006) found that there was no relationship between RFI and beef quality in a study with 54 purebred Angus steers, with beef quality estimated using Warner-Bratzler shear force and sensory attributes (tenderness and flavor). Similarly, a study by Gomes et al. (2012) on seventy-two Nellore steer grouped into 12 low, 12 high, and 48 medium feed intake steers, found that selection for low RFI did not affect meat tenderness. Moreover, Zorzi et al. (2013) examined meat quality of longissimus muscle using fifty-nine Nellore bulls that were grouped into low and high residual feed intake, and found that there was no variation between the two groups in terms of carcass weight, muscle composition and sarcomere length but the muscles from the low RFI bulls tended to have higher shear force and increased soluble collagen when compared with muscles from high RFI bulls. Not only is the influence of residual feed intake on meat quality contradictory, but also there is limited literature on how selection for RFI or RFI performance can affect collagen heat solubility and the density or concentration of collagen cross links. Therefore, further studies should be performed to cover the research gap and completely understand the influence of RFI.

1.2 3 Animals age and pre-slaughter conditions

Beef cattle are slaughtered at ages ranging from 14 to 24 month in Canada. Numerous studies have indicated a relationship between meat tenderness and animal age at slaughter (Hiner and Hankins, 1950; Duarte et al.2011; Mir et al. 2017) as tenderness tends to decrease as animal age increases (Wulf et al. 1996; Duarte et al.2011). Furthermore, Purchas (2003) indicated that there were significant correlations between age, body weight and the nutrition of animals with variation in meat quality characteristics. This evidence was supported by the results of Ellies-Oury et al. (2017) who evaluated the meat quality of 40 Charolais heifers that were slaughtered at either 26 or 36 months of age, with two slaughter weights within each age group. These authors reported that age did not affect shear force and meat quality traits, but when heifers were slaughtered at 36 months of age, they found that there was a significant decrease in raw muscle shear force and total collagen content at the higher carcass weight.

To investigate relationships between total collagen, soluble collagen and animal age, a study by Hill (1966) reported that the concentration of intramuscular collagen was higher in very

young (8-9 weeks) and very old (7-15 years) cattle, with large variation in total collagen due to age in cattle aged 16 weeks to 4.5 years. Furthermore, as reported by Bruce and Roy (2019), who investigated the relationship between total collagen and collagen cross links of the *semitendinosus* and *gluteus medius* muscles of beef cattle slaughtered at different ages (12, 20 and 73 months) intramuscular collagen content was not affected by animal age for the *semitendinosus* muscle, while yearling cattle (12 months) showed a higher mean total collagen content than cattle of other ages in the *gluteus medius* muscle. The authors also found that the concentration of pyridinoline increased in the *semitendinosus* and the *gluteus medius* muscles with cattle age, although the Ehrlich's chromogen decreased with animal age in both muscles. Additionally, Moriguchi and Fujimoto (1978) reported that pyridinoline was present in low concentrations in muscle in fetal and newborn calves but increased with the growth and increasing age of the animals.

Pre-slaughter handling is defined as all activities that animals undergo starting from farm to slaughter, and includes husbandry practices such as medication, veterinary inspection, transportation, loading, availability of food and water, animal fatigue, weather and then ultimately the slaughtering process (Adzitey and Nurul 2011; Chulayo et al.2012). Improper handling may stress cattle and thus influence meat quality. Warriss (1990) found that the effect of preslaughter handling occurs mainly through the influence on muscle glycogen stores, with stress reducing glycogen stores and increasing carcass muscle pH, leading to dark muscle color (Apple et al.2005; Mahmood et al.2015). Grandin (1980) reviewed the effect of stress on meat quality and reported that animals that undergo stress prior to slaughter have decreased muscle glycogen, which causes dark, firm and dry meat. Moreover, the author reviewed that calves that were stressed or hoisted while alive would produce meat with decreased tenderness.

1.2.4 Sarcomere length

The sarcomere is considered the smallest contractile unit of the myofibril structure, which is comprised of repeating sarcomeres in series (Pearson and Young, 1989; Ertbjerg and Puolanne, 2017; Guo and Greaser, 2017). A sarcomere is between two Z-lines, and consists of at least thirty different proteins, although actin and myosin are the most abundant (Boland et al., 2019). Under a microscope the light and dark bands of the sarcomere can be clearly observed, the bands alternating as a result of the alternating overlap of thin and thick filaments. The light band

is also called the I-band (I=isotropic), while the dark band is known as the A-band (A= anisotropic), referring to their appearance under polarized light (Pearson and Young, 1989). One sarcomere unit is always comprised of an A-band and on each side of the A-band there is an I-band (Aberle et al., 2012). Thick filaments are composed primarily of myosin and titin proteins, while thin filaments consist of actin along with the regulatory proteins tropomyosin and the troponins (Listrat et al., 2016; Purslow, 2017; Boland et al., 2019). Thick and thin filaments are formed from these myofibrillar proteins, and play an important role in skeletal muscle contraction by facilitating movement of the sarcomeres (Boland et al., 2019).

Sarcomere length is defined as the length between two Z lines in a muscle fiber (Purchas, 2014). The mean length of sarcomeres as assessed during the post-mortem period has a direct and indirect effect on meat quality (Ertbjerg and Puolanne, 2017). During rigor mortis, actin and myosin overlap and bind to each other, thus leading to the formation of the actomyosin complex, which when formed repeatedly in response to calcium release early post-mortem results in sarcomere shortening (Lawrie, 2006). The sarcomere length in rigor muscle has been reported to be about 2 µm and this may be considered short when compared with the sarcomere length in resting muscle, which is about 2.5 µm (Ertbjerg and Puolanne, 2017). Hwang et al. (2004) reported that the shortening of the sarcomere during rigor mortis is considered the main reason that muscle rigidity is observed during early rigor, and for this reason sarcomere length may influence meat tenderness.

Furthermore, there are several studies which confirmed the impact of sarcomere length on meat quality. Starkey et al. (2016) studied many factors and their influence on meat toughness as measured by Warner-Bratzler shear force (WBSF) on the ovine *longissimus*, *semimembranosus* and *biceps femoris* muscle. The authors found that sarcomere length impacted shear force and thus meat tenderness of the *longissimus* muscle. In addition, Lucero-Borja et al., (2014) in their study on beef cattle in Argentina that investigated the factors influencing shear force and meat tenderness concluded that variation in the shear force was associated with a variation on sarcomere length. Additionally, Guzek et al. (2015) showed that sarcomere length was positive correlated with marbling level in the *longissimus lumborum* muscle that was harvested from Limousin bulls. Likewise, Li et al., (2006) reported that Warner Bratzler shear force was strongly negatively correlated with sarcomere length of both raw and cooked *longissimus* that was sampled from purebred Luxi steers. Besides that, Ertbjerg and Puolanne (2017) and Pearce et al. (2011) reviewed

that sarcomere length is associated not only with texture attributes but water holding capacity of both raw and cooked meat as well. Similarly, a study by Fausto et al. (2017) on lamb *biceps femoris* observed that there was increased Warner Bratzler shear force concurrently with sarcomere length that was decreased at day one and ten of post-mortem aging. Contrary to the latter reports, Keith et al., (1985) found poor correlation between sarcomere length and meat toughness. Also, Hall and Hunt (1982) and Johnson et al. (1990) reported that there was no relationship between sarcomere length and meat tenderness. The relationship between sarcomere length and beef toughness as estimated using WBSF is therefore equivocal and warrants continued consideration.

1.2.5 Post-mortem aging

Post-mortem aging has long been known to enhance meat quality, and is defined as the storage of meat under controlled temperature and time. Aging is also called ripening or conditioning (Khan et al., 2016). As muscles are different in type and connective tissue content, the time and temperature that are required to achieve appropriate changing in tenderness are varied (Khan et al., 2016).

Wet and dry aging are considered to be the two types of aging that are used commonly to improve sensory quality attributes such as tenderness and flavour (Campbell et al., 2001; Smith et al., 2008). Dry aging is defined as storage of meat at chilled temperatures without covering, while wet aging is known as aging of packaged meat (usually under vacuum) at a controlled temperature for a specific duration (Smith et al., 2008). Many studies have attempted to compare aging type (wet and dry) and post-mortem storage period on sensory attribute enhancement. A study by Lepper-Blilie et al. (2016), comparing dry and wet aging of beef loins with low levels of intramuscular fat at 14, 21, 28, 35, 42- or 49 days for palatability found that, despite the decrease of Warner-Bratzler shear force during aging, there was no significant difference between aging types on meat tenderness. Furthermore, dry aging did not enhance beefy flavor when compared with wet aging. Additionally, the authors concluded that meat aging up to 28 days did not influence the tenderness. Thus, extended aging to 49 days was considered not beneficial in terms of meat quality. Moreover a study by Kim et al. (2016) comparing two types of aging (wet and dry) for 2 days on *longissimus lumborum* confirmed a higher flavour intensity of dry aged meat compared to wet. Although the sensory panelists did not notice variation between the two aging methods in terms of tenderness and juiciness, dry aging at 3 °C was considered by panelists to contribute to

beef palatability improvement. Jiang et al., (2010) compared wet and dry aging for longissimus and triceps steaks and ground beef. They found there were no differences between the two types of aging for beef palatability for both ground and steaks beef when evaluated by trained panelists.

The mechanism of how post-mortem aging influences meat quality is well understood as proteases, particularly those of the calpain system, degrade myofibrillar cytoskeletal proteins resulting in a noticeable increase in meat tenderness (Lamare et al., 2002; Kim et al., 2018). A study by Phelps et al. (2016) that investigated the effect of extended aging up to 70 days on *M. semitendinosus* beef steaks showed that the toughness of *M. semitendinosus* steaks as estimated using Warner Bratzler shear force decreased through to 70 days of aging. This appeared mainly due to extensive cleavage and degradation of desmin and troponin-T during aging. Most studies suggest that the increase in meat tenderness during aging occurs as a result of degradation of myofibrillar proteins only but a study by Nishimura et al. (1998) reported that the tenderization of beef with post-mortem aging is also due to the weakening of mechanical strength of the intramuscular connective tissue, which occurred slowly and extended over ten to fourteen days of post-mortem aging. Similarly, in a study by Liu et al. (1995) on chicken *semitendinosus* muscle, the authors found that there was weakening of the endomysium and perimysium during post-mortem aging. The authors suggested that extension of aging increased the tenderness of chicken due to intramuscular connective tissue degradation.

Notably, a negative effect of aging on meat quality also has been reported by Spanier et al., (1997) who found that during post-mortem aging some unfavourable tastes such as bitter and sour supplanted previous beefy, brothy, and sweet desirable flavours. Campo et al., (1999) found that aging of beef up to 21 days developed the undesirable liver odour. In addition, Colle et al., (2016) reported that although continued aging had a positive effect on *biceps femoris* and *semimembranosus,* it also had a negative influence on meat quality through decreased steak shelf life and thus might decrease colour stability or compromise microbiological safety. Furthermore Juárez et al., (2010) concluded that extended aging of beef had no influence on meat quality, but in economic terms there was no benefit to extend ageing up to 14 days due to chill storage costs. Therefore, aging beef 14 to 21 days post mortem should produce a product of acceptable tenderness without undesirable flavours.

1.2.6 Calpain and calpastatin activities

Calpains (CAPN) belong to the family of cysteine proteases, which are activated in the presence of Ca^{2+} and degrade myofibrillar proteins, resulting in the increase in meat tenderness observed with time post-mortem (Colle et al.2018). Calpains are divided into two types, μ-calpains (calpain-1) and m-calpains (calpain-2) according to the Ca^{2+} concentration that is required for their respective activities (Lana and Zolla, 2016). Calpain enzymes are suppressed by a specific endogenous protein known as calpastatin (CAST). Calpain and calpastatin are found in all mammalian cells (Murachi, 1983). A study by Geesink and Koohmaraie (1999) indicated that calpastatin plays an important role in decreasing the rate of proteolysis of myofibrillar proteins as well as limiting the extent of destruction by μ calpain. Further, binding calpastatin to inhibit calpain occurs also in the presence of Ca^{2+} but this occurs at higher Ca^{2+} concentration when compared with calpain activation (Ouali and Talmant, 1990; Koohmaraie and Geesink, 2006). The μ- and m-calpains and their specific inhibitor calpastatin are known as the calpain system (Pomponio et al., 2010; Coria et al., 2018).

There is a strong association between calpain concentration and activity and meat tenderization during post-mortem aging (Lian et al. 2013; Bhat et al. 2018). In addition, Koohmaraie (1994) indicated that calpain enzymes are mainly responsible for most post-mortem proteolysis and thus for improving meat tenderness. Furthermore, a study by Koohmaraie et al. (1991) suggested that the variation between lamb, beef and pork in meat tenderness is mainly due to variation in the calpain to calpastatin ratio. Moreover, a study by Colle et al. (2018) concluded that beef *longissimus lumborum* injected with calcium chloride led to activation of calpain-2 and improved tenderness.

The mechanism of how calpains improves meat tenderness during post-mortem aging is well understood and where the calpains cleave titin and desmin, proteins that link myosin and actin to the Z-lines, is known (Taylor et al., 1995; Kemp et al., 2010). Boland et al. (2019) supported that calpains are acting along the Z-line region. Moreover, the calpains have been shown also to degrade protein such as vinculin and nebulin (Tylor et al., 1995) and synemin (Bilak et al., 1998) post-mortem. In addition, Du Toit and Oguttu (2013) in their review indicated that μ-calpains (calpains -1) are responsible for the degradation of desmin, filamin, and nebulin, while m-calpains (calpain-2) degraded the troponins and tropomyosin.

Many authors supported the evidence of the key role of calpains-1(CAPN1) and calpastatin (CAST) genes on meat tenderness. Furthermore, the authors confirmed that these genes have moderate genetic effects on meat tenderness (Page et al., 2004; Casas et al., 2005; White et al., 2005; Casas et al., 2006; Schenkel et al., 2005). In addition, several proteomic, gene expression and genomic studies indicated that calpains and calpastatin participated in meat tenderization during post-mortem aging. Guillemin et al. (2011) provided a functional analysis of proteins that are associated with tenderness of muscle from Charolais young bulls and steers. They identified 24 proteins marker and found that the CAPN1 sub-network was comprised of large numbers of target proteins that included myofibrillar proteins such as desmin and vimentin and connective tissue proteins such as the collagen alpha chains. Bagatoli et al. (2013) conducted a study examining the gene expression of calpastatin in the *longissimus lumborum* muscle of lamb and suggested that as the calpastatin gene expression increased there was a concomitant lowering in the tenderness of lamb. On other hand, Gandolfi et al. (2011) in their study of calpastatin gene expression and polymorphisms in the muscle of crossbred pigs concluded that calpastatin had a central role in meat quality mainly by affecting tenderness and drip loss, as well as by influencing the activation of calpain post-mortem. Furthermore Corva et al., (2007) investigated the polymorphisms of CAPN1 and CAST gene in *Bos taurus* cattle and their relationship with beef tenderness, and found that there were correlations between genetic markers on two genes and meat tenderness. Moreover, the authors concluded that genetic variation in the CAPN1 gene could contribute to variation in tenderness within the beef production system. The activity of calpains is limited to myofibrillar proteins, however, and therefore not anticipated to affect collagen solubility or its contribution to cooked meat toughness.

1.2 7 Connective tissue characteristics

Connective tissues associated with skeletal muscle include those known as the extracellular matrix (ECM), as well as tendons and ligaments that link muscle to bone to effect movement. The characteristics of the various connective tissues are defined by their structure, their composition and the concentration of the various connective tissue proteins in them.

There are three different layers of connective tissue in muscle. The first layer is the epimysium which surrounds the whole muscle. The second layer is the perimysium which accounts

for about 90% of all connective tissue (Kovanen, 2002) and mainly surrounds the muscle fibre fascicles. The third layer is the endomysium, which surrounds individual muscle fibres. The endomysium is linked directly with the sarcolemma, which is the muscle cell membrane, thus confining each muscle fibre. It is considered a thinner layer than the other two layers (Turrina et al., 2013). The endomysium amounts to between 0.47 and 1.2% of the dry weight of every single muscle fibre (Purslow, 2010), while the perimysium accounts for 0.43 to 4.6% of the muscle on a dry weight basis (Purslow, 2010). The endomysium and perimysium have key roles in meat quality through their influence on meat tenderness. However, the epimysium does not contribute to meat tenderness as it is usually removed when meat is eaten (Roy et al., 2018), but it has a critical role in living animals as it forms a protective wall between muscles as well as between muscles and bones (Astruc, 2014).

One of the main functions of connective tissue in all muscle cells is to give integrity to the skeletal muscle and to bind muscles to each other. Another key role of connective tissue is to facilitate muscle contraction because it has an important role in connecting muscle to bone and enabling the muscular force transmission system (Borg and Caulfield,1980).

The main components of connective tissue are collagen, elastin, proteoglycans and glycoproteins (Nishimura, 2010; Guo and Greaser, 2017). But among these components, collagen is considered the most abundant protein in the body, accounting for approximately 25 to 35% and 1–2% of the entire body and muscle weight, respectively (Astruc, 2014). Total collagen varies in beef muscle based on muscle location and function in the cattle body and it can represent about 1 to 10% of muscle on a dry weight basis (Purslow, 2005).

1.2.7.1 Collagen structure and classification

Approximately 28 types of collagen have been identified in less than 40 years in vertebrates (Khoshnoodi et al., 2006; Hulmes 2008). However, all collagen types consist of a foundation structure of three polypeptide chains in a triple helix (Figure 1-1) that are composed primarily of a three amino acid (Gly-X-Y) repeating sequence (Ramshaw et al., 1998; Gelse et al., 2003). While X and Y can be any amino acid (Figure 1-1) (Hulmes, 2008), they are usually proline and hydroxyproline, respectively, and this triplet and hydroxyproline are distinguishing features of collagen.

Glycine (GLY) is considered the most abundant amino acid in collagen and it dominates collagen composition by accounting for about one-third of total amino acids that are found in a collagen molecule (Pearson and Young, 1989). The second most abundant amino acid is proline, which accounts for 12 % of the total amino acids in collagen, followed by alanine at 11% and hydroxyproline at 10%. Hydroxylysine, a product of post-translational modification of lysine by lysyl hydroxylase, constitutes about 1% of fibrillar collagen. Other amino acids that comprise the collagen structure are tyrosine, histidine, and sulfur-containing amino acids, which constitute less than 1% of the amino acids in collagen (Pearson and Young, 1989). Hydroxyproline and hydroxylysine are considered two unusual amino acids that are found in collagen and they play an important role in the collagen structure. Hydroxyproline has a crucial role in stabilizing the collagen triple helix through intramolecular hydrogen bond formation, while hydroxylysine contributes to the biosynthesis of collagen cross-links (Pearson and Young, 1989; Gelse et al., 2003). The amino acid tryptophan is rare in the collagen structure and its presence is therefore used as an indicator of collagen impurity. Hydroxyproline is used to determine collagen concentration (Pearson and Young, 1989) as hydroxyproline is found almost exclusively in collagen.

All collagen types (I-XVIII) are grouped into four super families based on their structural attributes (Boland et al., 2019). These four super families are:

- Fibril forming including type I, II, III, V and XI
- Non-fibrous including type IV, VII and X
- Filamentous including type VI
- Fibril-associated including type IX and XII

The fibril forming collagen family includes the most abundant collagen types which constitute about 90% of the total collagen in muscle (Gelse et al., 2003). In skeletal muscle those types are distributed throughout all connective tissue layers. The epimysium includes mostly type I collagen while type I, III and V are predominantly found in the perimysium, while the endomysium contains types I, III, IV and V (Kovanen, 2002).

1.2.7.2 Collagen biosynthesis

Beside synthesis of the triple helix, collagen undergoes significant post-translational modifications (Pinnell, 1982; Kovanen, 2002). Like any other protein, collagen biosynthesis starts in the cell nucleus with transcription of DNA of the specific collagen type gene to mRNA specific for each α chain (Figure 1-1). After that, mRNA moves to and penetrates the endoplasmic reticulum where translation takes place, resulting in the formation of the procollagen α chains for the exact collagen type (Hulmes, 2008) (Figure 1-2).

Following this, there is a series of post-translational modifications made to the procollagen α chain that lead to the completion of the formation of the procollagen molecules (Figure 1-2). Proline and lysine in the procollagen α chain in particular undergo extensive modification. These modifications include the hydroxylation of proline and lysine where proline is transformed enzymatically to 4-hydroxyproline and 3-hydroxyproline by prolyl-4-hydroxylase and prolyl-3-hydroxylase, respectively (Piez, 1976; Pearson and Young, 1989; Myllyharju,2005; Hulmes, 2008). Likewise, during the post-translation modification processes, lysine is converted to hydroxylysine by lysyl hydroxylase, and the formation of hydroxylysine is critical as it is necessary for collagen cross-linking biosynthesis to occur (Hulmes, 2008; Yamauchi and Sricholpech, 2012). Following these post-translational modifications, O-linked glycosylation also occurs in the endoplasmic reticulum and results in the formation of galactosyl-hydroxylysine and glucosylgalactosylhydroxylysine from hydroxylysines and this glycosylation is catalyzed by galactosyltransferase and glucosyltransferase, respectively (Yamauchi and Sricholpech, 2012). The enzymes that contribute to the post-translation modification process are activated in the presence of ascorbic acid (Pearson and Young, 1989). However, Pinnell (1982) reported that a deficiency in ascorbate during collagen synthesis may impact collagen production process. Beside ascorbic acid, oxygen and ferrous iron are also required by these enzymes (Pearson and Young, 1989; Hulmes, 2008) as well as a-ketoglutarate (McCormick, 2009) to be active.

Following post-translational modification of proline and lysine, three α chains combine and interchain and intrachain disulphide bonds form to stabilize the alignment of the α chains. The formation of the disulphide bonds is catalyzed by protein disulphide isomerase and this leads to the formation of the triple helix through electrostatic attractions and repulsions between amino acid regions along each α chain (Myllyharju, 2005). Subsequently the triple helix exits the endoplasmic reticulum where it undergoes enzymatic cleavage in the N- and C-terminal regions by N and C proteinases (Myllyharju and Kivirikko, 2004). Following this “trimming”, a

right-handed triple helix or tropocollagen molecule is formed. Thereafter, tropocollagen enters into the Golgi apparatus and is secreted into the extracellular space (Hulmes, 2008), where the tropocollagen molecules self-assemble into microfibrils and initial covalent collagen cross-linking formation catalyzed by lysyl oxidase occurs (McCormick, 2009).

1.2.7.3 Collagen cross-linking formation

The main role of cross-linking is to give stability and strength to the collagen quaternary structure (Pearson and Young, 1989). The intermolecular bonds belong to the cross-linking groups where chains of different molecules are linked by a covalent bond (Light and Bailey, 1982). This type is responsible for stabilization of the collagen (McCormick, 2009) thus the number of these intermolecular cross-links can have a drastic impact on the strength of the overall collagen structure (Pearson and Young, 1989). Intramolecular cross-links also exist (Kang and Gross, 1970) and these cross-links occur between two α chains in the same collagen molecule (Light and Bailey, 1982).

Beside intracellular modifications, lysine also undergoes extracellular modifications. The extracellular modifications occur after tropocollagen is incorporated into collagen microfibrils, and the lysines affected are the lysines and hydroxylysines found in the N- and C-telopeptides (Yamauchi and Sricholpech, 2012). These lysines and hydroxylysines are converted into allysines and hydroxyallysines respectively by lysyl oxidase (Pearson and Young, 1989). This modification is known as oxidative deamination (Yamauchi and Sricholpech, 2012) and lysyl oxidase requires copper ions and oxygen to be active (Pearson and Young, 1989; Kivirikko, 1996). Following that, the reaction between allysine and hydroxyallysine or between allysine and hydroxyallysine and another lysine or hydroxylysine can lead to the formation of two different cross-link pathways (Eyre, 1987; Eyre et al. 1988; Eyre and Wu, 2005), specifically the allysine and hydroxyallysine pathways to yield initial intramolecular and intermolecular cross-links (Pearson and Young, 1989; Kivirikko,1996; Yamauchi and Sricholpech, 2012). Cross-links produced in this process are divalent cross links (Figure 1-3), which are considered reducible and heat labile (McCormick, 2009). The last steps that divalent cross-links undergo is a series of reaction to produce trivalent and in some tissues such as skin even tetravalent cross-links (Kivirikko, 1996). These multi-valent cross links are considered non-reducible and heat stable (McCormick, 2009). Because these

trivalent cross links are considered heat stable their concentration may impact toughness of meat negatively (Roy et al., 2015). Trivalent cross links have been well characterized and they arise mainly from the hydroxyallysine pathway (Figure 1-3) and these include the pyridinolines (PYR, hydroxylysylpyridinoline and lysylpyridinoline), and the Ehrlich chromogen (EC) (Eyre,1987; Kivirikko,1996; McCormick, 2009). Both the pyridinolines (PYR) and Ehrlich chromogen (EC) are trifunctional cross-links; however, the EC has a pyrrolic, five-sided ring while the pyridinolines contain a 3-hydroxypyridinium ring (Kuypers et al., 1994). Both PYR and EC are found in Types I and III collagens, Chemically, the EC cross link connects three different collagen peptide alpha chains in stoichiometric amounts, connecting two C-terminal type I collagen peptides at Hyl(Lys)-16 c and one type I collagen peptide at Hyl-87, suggesting that EC connects three different collagen molecules. The PYR cross link, however, appears to connect three different collagen molecules in type I collagen but only two different collagen molecules in type III collagen (Kuypers et al .1994) The implications of these structural differences on cooked meat toughness is not entirely known, although EC cross link concentrations have been associated with decreased Warner-Bratzler shear force (Bruce and Roy, 2019), suggesting that this cross link may be less heat stable than PYR. This difference in heat stability may be the reason that different methods of isolation and quantification arose and are used for each of these cross links, as PYR is isolated after hydrolysis, while EC can be detected as a chromogen by exploiting the predisposition for p-dimethylaminobenzaldehyde to bind to hydroxylated pyrrole rings (Bergman and Loxley, 1963).

1.2.7.4 Collagen degradation

Like collagen synthesis, the degradation process is important to secure normal muscle growth during animal life (Purslow, 2005). The balance between the biosynthesis and degradation processes is known as collagen turnover. Sprangers and Everts (2019) indicated in their review that collagen degradation has an important role that makes it is essential for tissue homeostasis. Gross and Lapiere (1962) were the first investigators of collagenolytic activity through their study of developing tadpoles in which they identified an unknown enzyme later characterized as matrix metalloproteinases (MMPs) (Rodríguez et al., 2010). The MMPs are a family of zinc-dependent endopeptidases that are mainly responsible for degradation and turnover of collagen (Kovanen, 2002; Purslow, 2005; Purslow et al., 2012) and other components of the extracellular matrix

(Lauer-Fields et al., 2000; Rodríguez et al., 2010). Also, MMPs have a critical role in the regulation of many biological processes, which were reviewed by Christensen and Purslow (2016).

To date, twenty-five MMPs have been investigated (Lauer-Fields et al., 2002) and three groups of MMPs have been identified based upon collagen substrate. The MMP collagenases include MMP-1, MMP-8, and MMP-13 (Bord et al., 1998), which are responsible for the degradation of type I, II, and III collagen (Qi et al., 2016). The MMP gelatinases are comprised of MMP-2 and MMP-9 (Sylvestre et al., 2002) which cleave denatured collagen type IV, VII, and X (Qi et al., 2016) The stromelysins consist of MMP-3 and MMP-10, which play an important role in the degradation of collagen types III, IV, and V as well in the cleavage of other extracellular matrix components such as proteoglycans and fibronectin (Bord et al., 1998). Furthermore, MMPs are inhibited by four types of specific endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMP-1 to TIMP-4) (Bord et al., 1998; Kovanen, 2002; Carrick-Ranson, et al., 2019). TIMPs are secreted along with MMPs by fibroblasts (Purslow, 2005) and play an important role in maintaining the balance between synthesis of the extracellular matrix as well as its degradation by MMPs (Parsons et al., 1997). On the other hand, the TIMPs can be activated by MMP-2 and MMP-9, which are responsible for the cleavage of denatured collagens (Christensen, and Purslow, 2016). Besides MMPs, lysosomal cysteine proteases also contribute to degradation of certain collagens as reviewed by Sprangers and Everts (2019).

Recently, there is evidence that remodelling of the extracellular matrix during the life of an animal has a promising future for the beef industry. Christensen and Purslow (2016) in their review concluded that targeted remodelling of intramuscular connective tissue by controlling the MMPs that are contributing to intramuscular collagen turnover can increase the synthesis of a new collagen and reduce mature cross-link deposition to enhance meat tenderness. In addition, Nishimura (2010) indicated in his review that altering the extracellular matrix by increasing deposition of intramuscular fat causes remodelling of the extracellular matrix and thus may also increase meat tenderness. Moreover, Du et al. (2013) concluded in their review that remodelling progenitor cells by introducing nutrient supplements in the diets of cattle to increase adipogenic cell differentiation may increase marbling, reduce connective tissue synthesis and increase meat tenderness. Furthermore, Purslow (2005) reported in his review that nutrition and physical activities of the animal may modify the amount and structure of intramuscular connective tissue and influence meat toughness. On the other hand, Roy et al. (2015) concluded in their study that

growth promoted by ractopamine hydrochloride in beef steer diets was responsible for the remodelling of muscle that resulted in a decrease in mature collagen cross link concentration. The results of these reviews and studies indicate that expression of genes related to collagen biosynthesis and degradation may provide insight into pathways that may be used to modify the contribution of intramuscular collagen to beef toughness.

1.2.7.5 Total collagen content, heat soluble collagen and meat quality

Nowadays, it is well known that myofibrillar and connective tissue proteins are major contributors to meat toughness. The contribution of myofibrillar proteins to meat toughness can be resolved during the early post-mortem by proteolytic enzymes or management of early post-mortem muscle pH (Li et al. 2014). Whilst the intramuscular connective tissue contribution is known as "background" toughness because of the difficulty in changing it during meat aging (Bailey,1989; Purslow, 2005), the amount, composition and structure of intramuscular connective tissue can have a profound impact on meat toughness (Purslow, 2004; Nishimura, 2010). The contribution of connective tissue to beef toughness can be estimated either by measuring total collagen content, heat soluble collagen content or the maturity of collagen cross-links (Roy et al. 2015). Listrat and Hocquette (2004) reported that both total collagen content and collagen cross link concentrations influence raw and cooked meat tenderness. On other hand, collagen type may play a critical role in variation of meat toughness, especially collagen types III, XII and XIV. Astruc (2014) stated in his review that type III collagen may reduce background toughness as it is more sensitive to proteases; however, type XII and XIV collagens tended to decrease collagen solubility and thus may contribute in increased meat toughness.

Many researchers have studied and reviewed correlations between collagen content, collagen heat solubility, and mature collagen cross-link concentrations with raw or cooked meat quality. Christensen et al. (2011) studied the correlation between collagen characteristics and raw and cooked meat texture of bovine *longissimus thoracis* muscle. The authors concluded that although total collagen and insoluble collagen contents were positively correlated with values of raw meat texture, there was no correlation between total collagen and cooked meat toughness of *longissimus thoracis* muscle. Meat quality attributes including meat tenderness of Angus, Simmental, Charolais and Limousin steers were studied by Chambaz et al. (2003). The authors

found a negative correlation between collagen and collagen solubility with sensory panel tenderness that was only noted for the Charolais, and the authors ascribed this to the Charolais steers showing significant variation in age. Moreover, a study by Torrescano et al. (2003) found a correlation between shear values of fourteen muscles that were harvested from four Swiss Brown bull carcasses and they found that Warner–Bratzler shear force of all raw muscle samples from all muscles were highly positive correlated with either total collagen or insoluble collagen. On the contrary, a study by Keith et al. (1985) reported that the measurement of total collagen did not predict beef toughness as they found a low negative correlation between total collagen and overall tenderness of thirteen different muscles harvested from ten Angus steer carcasses. In contrast, Bailey (1989) reported that the total collagen content can account for meat texture variation. Clearly, the contribution of collagen to meat toughness is still equivocal, and warrants investigation.

1.3 Gene expression and meat quality

The enhancement of meat quality is considered a priority for beef producers in particular because consumers rank meat tenderness one of the most important eating quality (O'Quinn et al., 2018) impacting their purchasing decisions before and after purchasing beef. The genetics of an animal is one of the factors that may influence the biological characteristics of muscle by affecting such characteristics as intramuscular fat and connective tissue (Bernard et al. 2007). As biological characteristics are regulated by genes (Bernard et al. 2007), understanding of which genes and how these genes can control these physiological characteristics can provide help with understanding their influence on meat quality (Hocquette et al. 2012).

Gene expression is considered one method to identify genes that contribute to meat quality (Plastow and Bruce, 2014). Several studies confirmed the correlation between the calpain gene (CAPN1), which encodes the calpain enzyme considered responsible for myofibrillar proteins degradation and beef toughness (Page et al. 2002; Casas et al 2006; Lee et al. 2014). Furthermore, a study by Nattrass et al. (2014) suggested that CAPN1 and CAST gene markers may explain the variation between muscle shear force and consumer panel taste for meat tenderness. It would make sense then that expression of these genes would impact post mortem meat tenderness particularly as it developed during ageing of the meat. Although the genetic variation in beef toughness related

to myofibrillar proteins is somewhat understood, there is limited understanding of the genetic variation and the expression of those genes associated with toughness of high connective tissue muscles in the same animal or between breeds. A study by Piorkowska et al. (2016) investigated the transcriptome of broiler breast muscles and the authors found that *P4HA3 (prolyl-4-hydroxylase A3)*, *LEPREL4 (prolyl 3-hydroxylase family member 4)*, *PCOLCE2 (procollagen C-endopeptidase enhancer 2)*, *COL16A1 (collagen type XVI alpha 1 chain)*, *COL20A1* (*collagen type XX alpha 1 chain*) and *VWA1 (von Willebrand factor A domain containing 1)* genes were related to collagen synthesis, with expression of these genes down-regulated in chickens that produced meat with decreased toughness. Furthermore, Gonzalez et al (2014) found that mRNA levels of lysyl oxidase (*LOX*) from the m. *longissimus lumborum* collected with a biopsy at weaning were negatively correlated with total collagen, insoluble and soluble collagen of the biopsy; however, at slaughter, mRNA levels of *LOX* were negatively correlated with Warner-Bratzler shear force and positively correlated with sensory connective tissue amount. The authors concluded that the activity of LOX enzymes at weaning time may account for variation in meat tenderness at slaughter. In addition, a study by Qi et al. (2015) found that the expression of *MMP-1* and *MMP-2* from the bovine *longissimus dorsi* muscle were correlated negatively with shear force, and that *MMP-1* was negative correlated with cooking loss and positively correlated with water holding capacity. Fonseca et al. (2017) used RNA-Seq to investigate genes that had different expression in *longissimus dorsi* muscle from Nellore male cattle that were grouped as tender and tough. After identification, the genes were validated by real-time quantitative PCR. The authors identified about 40 genes that were differentially expressed in the *longissimus dorsi* muscle, and found that the *PCP4L1* gene (*Purkinje cell protein 4 like 1*) had increased expression in tender meat whilst the *BoLA-DQB* gene (*major histocompatibility complex, class II, DQ beta*) had increased expression in carcasses with tough meat. Clearly, further study of the expression of genes related to collagen biosynthesis pathways and the enzymes involved in the post-translational modification, as well as enzymes related to the degradation of collagen, will provide additional insight into the contribution of collagen to meat toughness.

1.4 Collagen related genes

The following genes are related to collagen synthesis and degradation and may be related to the contribution of collagen to the background toughness of meat.

1.4.1 Collagen Type III Alpha 1 Chain (*COL3A1*)

The collagen type III alpha 1 gene encodes for the α1(III) chains (Maehata et al. 2007). Collagen type III plays an important role in the composition of connective tissues (Chuan-hao et al. 2016). Miskulin et al. (1986) found that the production of type III collagen is dependent upon the growth status of the cells and concluded that the mRNA expression level may be controlled by periods of rapid and slow fibroblast growth. Bao et al. (2007) in their study using 84 castrated pigs found that *COL3A1* mRNA was positively correlated with total and insoluble intramuscular collagen but was negatively correlated with soluble intramuscular collagen. Bao et al. (2007) suggested that collagen characteristics such as cross links were influenced by the mRNA level of *COL3A1* and that may be due to the differences in PYR stoichiometry between the collagen types I and III noted by Kuypers et al. (1994).

1.4.2 Collagen Type V (*COL V A1* and *COLV A2*)

Collagen type V is comprised of alpha 1(V) and alpha 2(V) chains (Birk et al. 1990) and accounts for approximately 20% of the total fibrillar collagen synthesized (McLaughlin el al. 1989). It is found mainly in the pericellular connective tissues of muscle (Sato et al. 1991). Collagen type V plays an important role in the structural arrangement and production of heterotypic fiber such as those of type I collagen as well as controls the diameters of collagen fibers (Giunta and Steinmann, 2000).

1.4.3 Connective tissue growth factor (*CTGF*)

CTGF plays a crucial role in angiogenesis, migration, and cell adhesion and an important role in wound healing (Holbourn et al. 2008). CTGF is induced in fibroblasts by transforming growth factor beta 1 (TGFβ1)(Perbal et al. 2001) where it has been linked to the development of fibrosis through increased synthesis of collagen (Leask and Abrahams 2004).

1.4.4 Insulin-like growth factor-1 (*IGF-1*)

The IGFs are members of a family of insulin-related peptides. Salmon and Daughaday identified IGF-1 in 1957 (Laron, 2001). It is produced in the liver where it acts as an insulin endocrine activator (Aguirre et al. 2016). Svegliati-Baroni et al. (1991) reported that IGF-1 acts myogenically on fibroblasts thus it plays an important role in increased proliferation of fibroblasts and collagen synthesis through phosphatidylinositol 3-kinase and ERK (extracellular-signal regulated kinase) pathways.

1.4.5 Lysyl Oxidase (*LOX*)

Lysyl oxidase is a member of the oxido-deaminase family and is a required coenzyme in the preparation of lysine hydroxylase for the formation of collagen cross-links (Herchenhan et al. 2015). Through these cross-links, it plays an important role in collagen stabilization and is considered a precursor to covalent collagen cross-link formation (Kagan and Li 2003). Herchenhan et al. (2015) concluded that lysyl oxidase is essential for collagen cross-links formation and play a crucial role in collagen fibril shape correction.

1.4.6 Prolyl 4-Hydroxylase Subunit Alpha (*P4HA1*)

Collagen prolyl 4-hydroxylase (P4H) is dioxygenase-dependent and is responsible for the hydroxylation of the amino acid proline which contributes to stabilization of the collagen triple helix (Xiong et al. 2018). The enzyme is comprised of 2 α and 2 β isoforms, and is crucial for collagen maturation and secretion (Myllyharju and Kivirikko, 2004). The α subunit has α(I), α(II), and α(III) isoforms (Gjaltema et al. 2015).

1.4.7 Matrix metalloproteinase 2 (*MMP2*)

Matrix metalloproteinase2 is a member of the family of matrix metalloproteinases (MMPs) which are endopeptidases. MMP_S contain zinc, hence their classification as metalloproteinases, are they are activated by proteolytic cleavage of their amino-terminal domain (Po-Yin et al. 2000). *MMP2* is involved in the degradation of collagen type IV

and plays an important role in extracellular matrix remodeling (Po-Yin et al. 2000). A study by (Qi et al. 2016) concluded that the level of *MMP2* mRNA was negatively correlated with shear force, indicating that a high level of *MMP2* mRNA was associated with decreased meat toughness.

1.4.8 Mothers against decapentaplegic homolog (*SMAD2* AND *SMAD4*)

SMAD2 and SMAD4 are members of the SMAD family that are related to intracellular proteins and play an important role in protein synthesis and gene activation by sending signals from transforming growth factor-β (TGFβ) at the cell surface to the cell nucleus (Attisano and Lee-Hoeflich, 2001). SMAD family members are categorized according to their function. The receptor SMADs (R- SMADs) include SMAD 2, and the common-SMADS (Co-SMADs) include SMAD 4 and the inhibitory SMADs (I-SMADs) (Malhotra and Kang, 2013; Zhang and Ta, 2017). Lee et al. (2006) reported that the SMAD pathway has a key role in the activation of Type I collagen gene expression.

1.4.9 Forkhead Box O1 (*FOXO1*)

Forkhead transcription factor (FOXO1) is a member of the FOXO proteins, which play an important role in various biological functions such as metabolism and cell differentiation, survival, and proliferation (Adachi et al. 2007). Matsuzaki et al. (2003) reported that FOXOs protect cells against oxidative stress through regulation of catalase and manganese superoxide dismutase gene expression. Oxidative stress is associated with decreased new collagen synthesis by intramuscular fibroblasts in some muscles, and thus may lead to increase meat toughness through decreased collagen solubility (Archile-Contreras and Purslow, 2011).

1.4.10 Phosphoinositide-3-kinase (*PI3K*)

PI3K a member of a family of enzymes that is comprised of 15 kinases, all of which play an important role in growth, proliferation, and differentiation of mammalian cells.

Based on its structure and substrate specificity, PI3K is divided into three classes I, II and III (Yano et al. 2007). PI3K contributes to collagen synthesis through the combined PI3K/Akt, Smad3 pathway with TGF-β1 signaling, and therefore plays an important role in catalyzing the expression and activity of lysyl oxidase (Voloshenyuk et al. 2011).

1.4.11 Glycogen synthase kinase 3 beta (*GSK3B*)

Glycogen synthase kinase 3 beta is considered a serine/threonine kinase and has been related to many physiological processes such as diabetes, inflammation and aging (Wang et al. 2019). GSK3B plays an important role in phosphorylating a variety of nuclear and cytoplasmic proteins (Kwok et al .2005). Wang et al. (2019) reported that GSK3B has a crucial role in skeletal muscle through its effects on muscle mass and protein synthesis, as inhibition of GSK3B in muscle satellite cells decreased lipid accumulation through the AMPK (adenosine monophosphate-activated protein kinase) pathway. Decreasing lipid accumulation in muscle of meat species may influence meat quality characteristics such as the flavor, juiciness and tenderness of the cooked product (Troy et al .2016; Guo and Dalrymple, 2017).

1.4.12 Nuclear Factor of Activated T Cells 1 (NFATC1)

NFATC1 is member of the NFAT gene family, which is a group of transcription factors that was identified 25 years ago. NFATC1 is a promoter protein that activates T cells through binding to interleukin 2 (IL-2) (Kim and Kim, 2014). Herum et al. (2013) found that NFAT contributed to the regulation of collagen type I synthesis in tendon fibroblasts, and so may be involved in the synthesis of intramuscular collagen.

1.5 Conclusion

This literature review addressed the factors contributing to variation in meat quality as well as in connective tissue characteristics and included discussion regarding the influence of

breed, selection for residual feed intake, preslaughter conditions, age of animal at slaughter, calpain and calpstatin activities, sarcomere length, post-mortem aging, collagen heat solubility, the density and quantity of collagen cross-links, and the impact of mRNA levels of some genes that contribute to either collagen synthesis or degradation. Clearly the literature has limited information as to how breed can interact with selection for residual feed intake and influence the connective tissue characteristics particularly for high connective tissue muscle such as *m*. *semimembranosus* muscle (inside round).

Several researchers have studied the associations between differentially expressed genes and meat toughness levels, but none has studied the relationship between collagen heat solubility and mRNA levels for genes that are involved in collagen synthesis and degradation. Indeed, for beef producers, such information will inform them if this variation in collagen characteristics is genetic, and potentially collagen heat solubility may be used to select for tender beef to increase the value of high connective muscles. Therefore, the hypotheses tested in this thesis were:

1 That meat quality and connective tissue characteristics of the m. *semimembranosus* muscle are not affected by selection for low residual feed intake status; and

2 That genes associated with collagen synthesis and degradation are differentially expressed within breed type between cattle that produce m. *semimembranosus* muscles that have low and high collagen heat solubility.

Thus, the objectives of the studies employed to test these hypotheses were to:

1. Determine the effect of breed type and residual feed intake on the meat quality and connective tissue characteristics of the semimembranosus; and

2. Determine the effect of breed and selection for low and high collagen head solubility on the expression of genes related to collagen synthesis and degradation and related the expression of these genes to meat quality characteristics of the semimembranosus.

Figure 1- 1:Collagen structure formation

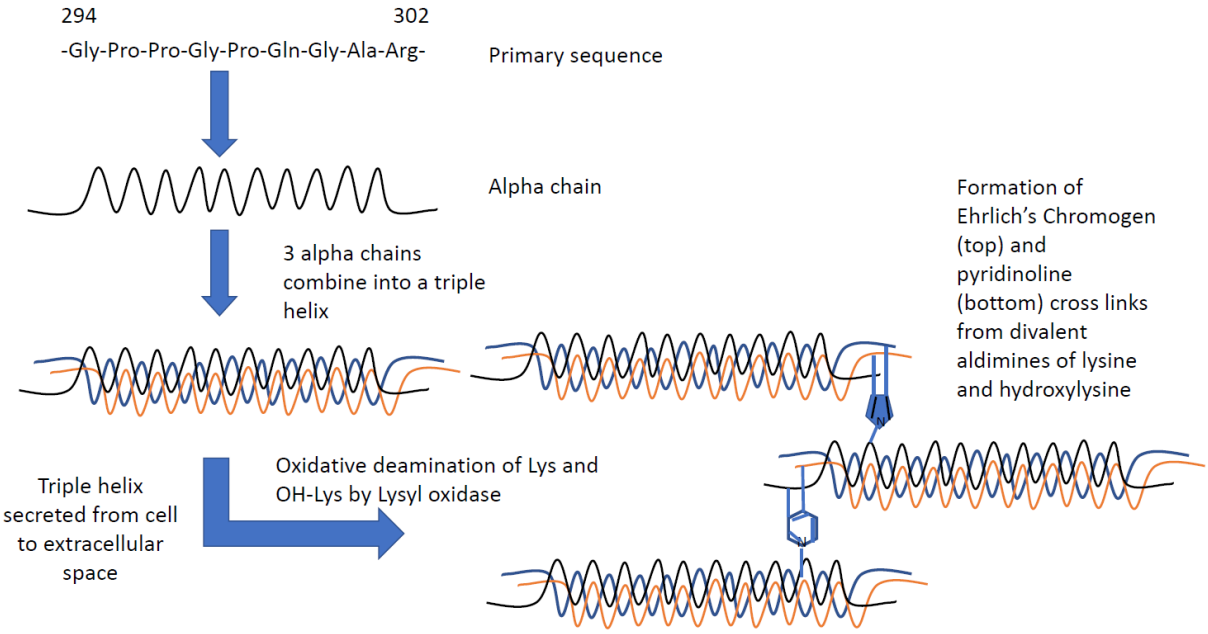


Figure 1- 2: Collagen biosynthesis within a fibroblast or muscle cell

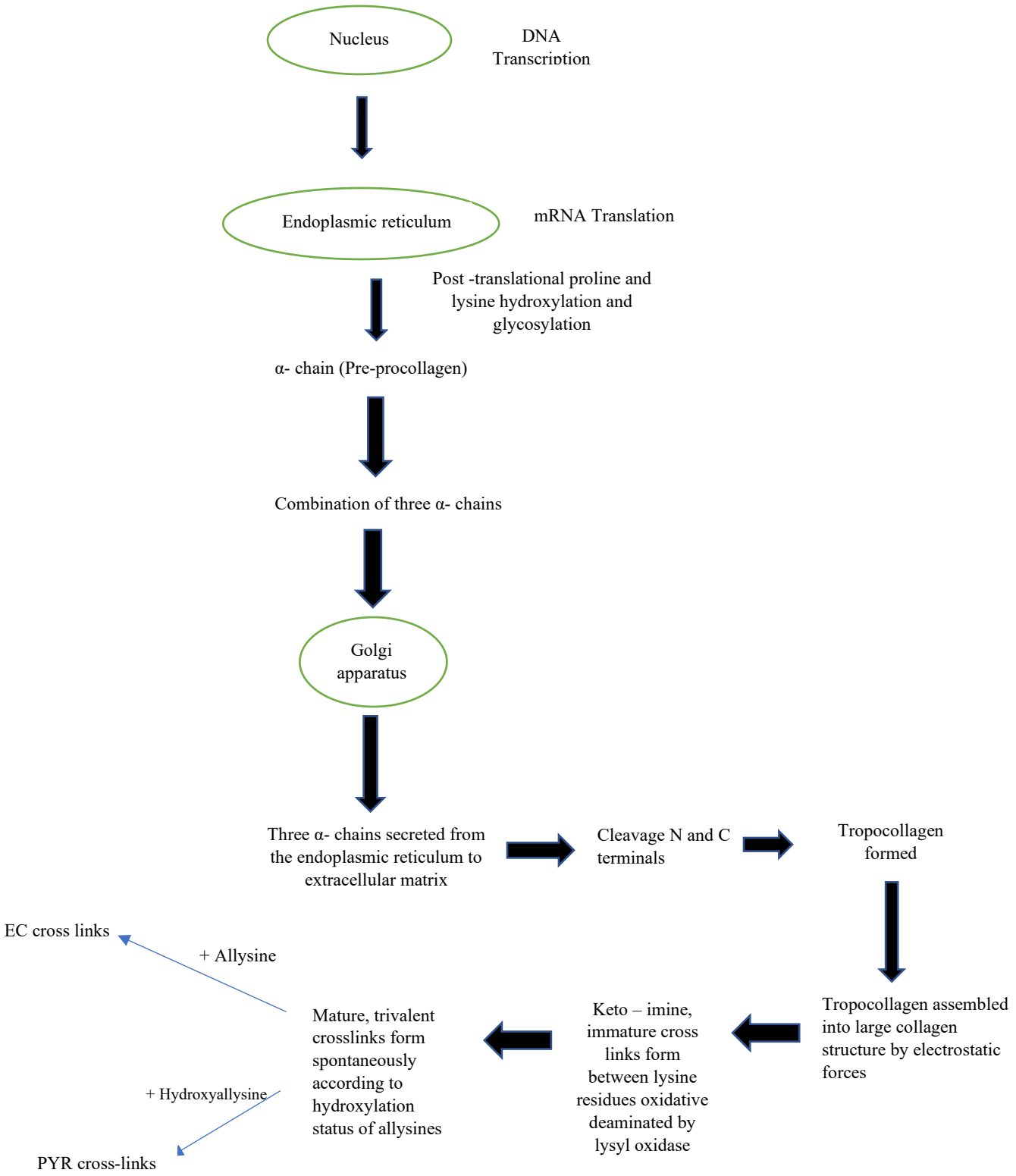
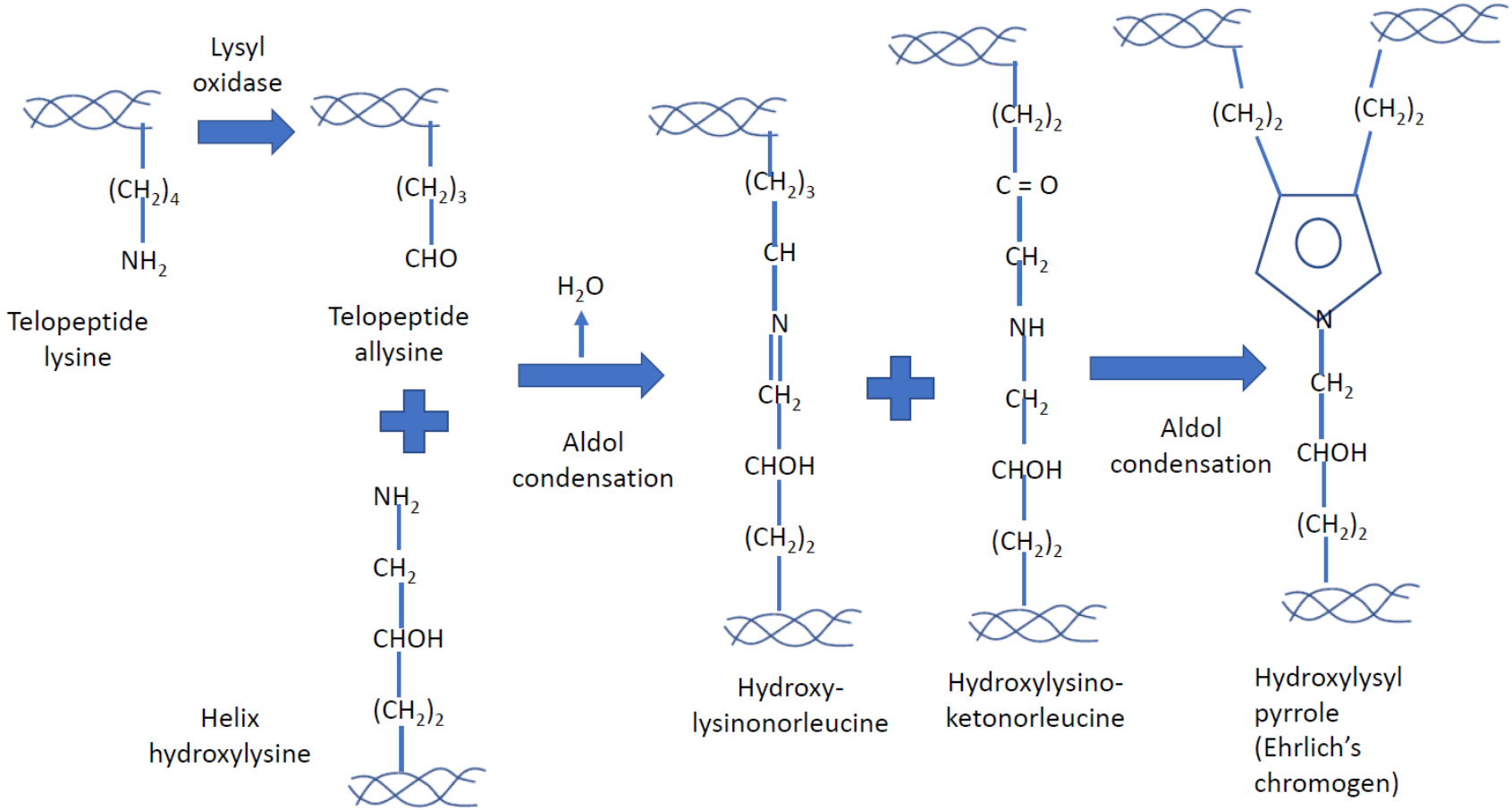
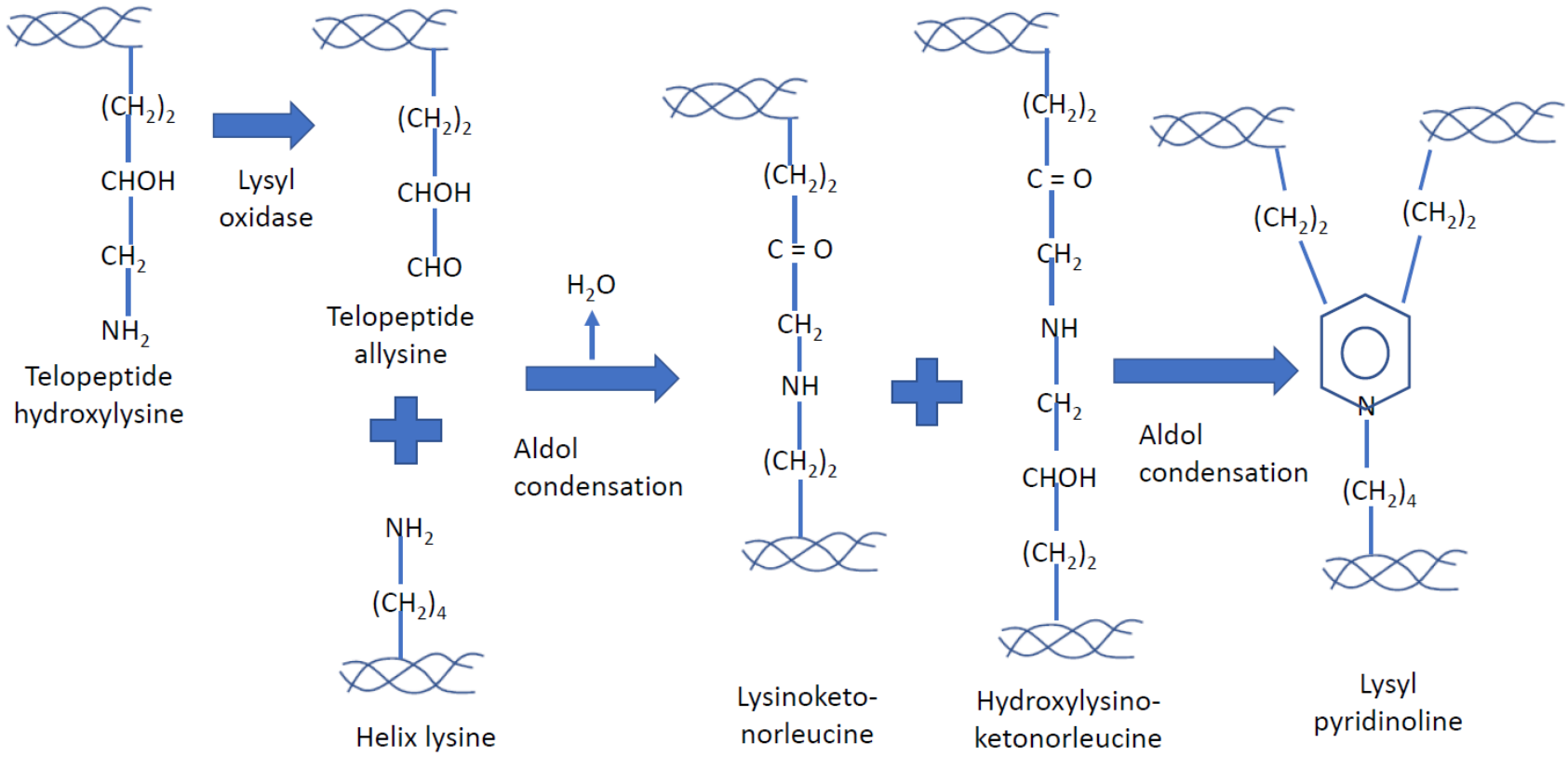


Figure 1- 3 Extracellular formation of a) Ehrlich's Chromogen (hydroxylysylpyrrole) and b) lysyl pyridinoline from aldol condensation of lysines and hydroxylysines in collagen alpha-chains to produce hydroxy-lysinonorleucine and lysinoketonorleucine which combine with hydroxylysinoketonorleucine (formed similarly from helix hydroxylysine and telopeptide hydroxylysine).

a)



b)


(CH2)2
CHOH
CH2
NH2
Telopeptide hydroxylysine
Lysyl oxidase
(CH2)2
CHOH
CHO
Telopeptide allysine
NH2
(CH2)4
Helix lysine
H2O
Aldol condensation
(CH2)2
C = O
CH2
NH
CH2
(CH2)2
Lysinoketo-norleucine
(CH2)2
C = O
CH2
NH
CH2
CHOH
(CH2)2
Hydroxylysino-ketonorleucine
Aldol condensation
(CH2)2
(CH2)2
N
(CH2)4
Lysyl pyridinoline

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Chapter 2: Effects of cattle breed type and residual feed intake on meat quality and connective tissue characteristics of the m. *semimembranosus*

2.1 Introduction

Meat quality is a term used to describe the attributes, properties and concepts of the meat and includes product composition, eating quality and safety (Maltin et al.2003). Elmasry et al. (2012) defined meat quality as "the measurement of attributes that ensure the suitability of meat for consumption as a fresh product or a product for a particular time without spoilage". Generally, these attributes include instrumental characteristics (pH, water-holding capability, shear force), chemical attributes (moisture, fat, protein), eating attributes (tenderness, juiciness) and safety attributes (spoilage) (Xiong et al. 2014). Variation in meat quality is vast, and many intrinsic and extrinsic factors can influence the quality of meat.

Cattle breed or genetic group is considered one of the most critical intrinsic production factors that can influence beef quality (Campo et al. 1999; Monsón et al. 2004; Bureš et al. 2006; Christensen et al. 2011; Xie et al. 2012; Guerrero et al. 2013). The literature extensively details the variation of chemical composition and meat quality attributes among the different breeds (Campo et al.1999; Chambaz et al. 2003) where there is clear variation between breeds for beef quality. Although the literature clearly demonstrates that there is a breed effect on collagen content and solubility of collagen (Cross et al. 1984; Monsón et al. 2005; Christensen et al. 2011; Panea et al. 2018), there are no studies of how breed could affect collagen cross-links. The density and types of cross-links present in collagen are considered factors that may contribute to meat toughness; therefore, given that breed is one of the factors that can influence the quality of meat, understanding the influence of breed on this aspect of meat is imperative to ensure the selection of the appropriate breed for production of beef with high-quality attributes. For example, recently in the beef cattle industry, producers have incorporated Angus genetics into their terminal crosses because there is a perception that the Angus breed confers a carcass with high quality traits and will produce generations of cattle with high meat quality.

Feed efficiency is one of the most important factors affecting the profitability of beef production and is a trait for which there is active genetic selection. Most recently, it has been measured as residual feed intake (RFI). RFI is considered as a measure of feed efficiency (Fidelis

et al. 2017) and is defined as the differential between what an animal exactly eats and its expected feed intake over a specific period (Koch et al. 1963; Do et al. 2014; Gilbert et al. 2017). Consequently, animals selected as low or negative RFI are consuming less feed than what they are predicted to consume. Nowadays a beef producer selects for low residual feed intake for profitability and environmental issues, and selection for low RFI cattle would be most beneficial if beef quality remained unaffected or was improved. A review of the literature indicated that there is controversy on whether selection for low RFI animals influences meat quality attributes or not, as Basarab et al. (2003) and Zorzi et al. (2013) found there was a slight influence of RFI on meat quality while McDonagh et al. (2001) revealed that selection of cattle for low RFI was negatively correlated with meat tenderness. Moreover, there are no studies on how selection for RFI influences collagen characteristics, especially collagen cross-links concentrations.

This experiment therefore investigated the interaction of breed type (purebred Charolais, purebred Angus, and Kinsella Composite Angus crossbred) and residual feed intake status (low, high) on the quality of meat and intramuscular connective tissue (IMCT) from the *semimembranosus* (inside round).

2.2 Materials and methods

2.2.1 Experimental design and animal management

This study was reviewed and approved by an Animal Ethics Committee at the University of Alberta (Animal Use Protocol 00000777). A total of 71 castrate males (steers), specifically cross-bred Kinsella Composite (n=24), purebred Angus (n=23), and Charolais (n=24) steers that were either high (n = 35) and low(n=36) RFI were used in a 3 × 2 factorial design to investigate the effect of breed and RFI on proximate, meat quality and connective tissue characteristics of the inside round (m. *semimembranosus*). The Kinsella composite steers were produced by crossing Angus bulls and a hybrid dam line (Kinsella Composite), with the hybrid dam line consisting of approximately 33% Angus, 33% Charolais and 20% Galloway, and the remainder consisting of other beef breeds as described in detail by Goonewardene et al. (2003) and Nkrumah et al. (2007). All calves were born in April or May of 2013 on the Roy Berg Kinsella Research Ranch, University of Alberta, and remained there under similar management conditions for the duration of the study.

Calves in this study were identified with a unique ear tag for each calf and castrated within days of birth. Steer calves remained on pasture with their dams until weaned at approximately 190 days of age. At weaning, steers were gradually alimented onto a background diet of 65.1% oats and 34.9% hay. Steers received this background diet until approximately 11 months of age, after which they were then gradually alimented over approximately 3 weeks onto a finishing diet of 75% barley grain, 20% barley silage, and 5% pellet supplement that included Rumensin^{TM} (as fed, 76.5% dry matter; 14.7 % crude protein, 18.3% acid detergent fibre, 32% neutral detergent fibre, 1.2% calcium, 0.45% phosphorus, and 70% total digestible nutrients on a dry matter basis; 0.24 ppm Mg, 0.93 ppm K, 0.28 ppm Na, 604 ppm Fe, 128 ppm Mn, 182 ppm Zn, and 29.4 ppm Cu). Steers received this finishing diet before, during and after RFI testing.

For RFI testing, steers were placed by breed group into feedlot pens (4 for Angus crossbreds, 1 for purebred Angus and 1 for purebred Charolais) fitted with the GrowSafe system (GrowSafe Systems Inc., Airdrie, AB, Canada). Within the GrowSafe system, individual feed intake was monitored daily. Angus and Charolais steers were tested for RFI from June to August and Angus crossbred steers from April to June. Testing for RFI was conducted at different times due to differences in anticipated finishing/slaughter dates between the breed types thus accommodating differences in physiological age (Loyd et al. 2011) between the breeds, and the GrowSafe feeding system was used as described by Basarab et al. (2003).

Body weight, ultrasound and rib eye area measurements were collected on the live cattle as described by Mao et al. (2013). Briefly, body weight (BW) of the steers was measured twice at the start of the feedlot test, once every 14 days, and twice at the end of the test. Ultrasound measurement of back fat thickness and *longissimus thoracis* (LT, rib eye) area at the 12^{th} – 13^{th} rib was performed at the end of the feedlot test period using an Aloka 500V diagnostic real-time ultrasound with a 17 cm 3.5 MHz linear array transducer (Overseas Monitor Corporation Ltd., Richmond, BC, Canada).

Individual steer RFI values were calculated as the difference between a steer's actual and predicted dry matter intake (DMI). The equation RFI = DMI actual – DMI predicted was used, and RFI values were calculated for all calves within each breed. DMI predicted was calculated using animal average daily gain (ADG) and metabolic BW (MWT) calculated as midpoint $BW^{0.75}$, where midpoint BW was the sum of initial BW and ADG multiplied by half of the days on test (DOT) (Equation 1). In addition to this calculation of RFI, RFI was also adjusted for back fat

thickness (RFIf), where RFIf = DMI actual – DMI predicted based on ADG, MWT and final ultrasound back fat (FUFAT) measured at the end of the test (Equation 2), to remove effects on RFI of differences in animal physiological age (Basarab et al. 2011). The models used to predict expected DMI and expected DMI adjusted for ultrasound back fat thickness were originally described by Mao et al. (2013) as:

$$Y_i = \beta_0 + \beta_1 ADG_i + \beta_2 MWT_i + e_i \quad \text{(Equation 1)}$$

$$Y_i = \beta_0 + \beta_1 ADG_i + \beta_2 MWT_i + \beta 3 FUFAT_i + e_i \quad \text{(Equation 2)}$$

Where β_0 is the intercept; β_1 β_2 and $\beta 3$ are the coefficients on ADG, MWT and FUFAT, respectively; e_i is the residual. The DMI adjusted for ultrasound back fat thickness in model (2) was used to calculate RFIf.

From the RFI test values, steers were classified as negative (low) and positive (high) RFI. Twenty-four steers from each breed (one Angus steer died prior to slaughter) were selected based on RFI and balanced for body weight, with 12 steers having an RFI less than or equal to -0.13 kg (negative RFI) from the RFI mean for the cohort and 12 having an RFI greater than 0.13 from the mean (positive RFI) from the cohort within each breed.

Steers were finished to a back fat of between 8 and 10 mm as estimated using ultrasound; therefore, breeds were finished and slaughtered at different times to accommodate the differences in physiological age and were slaughtered within approximately one month of completing RFI testing. Within each breed, steers were slaughtered over two days with 12 steers killed each day from July to September in 2015, with the exception of Angus steers, which had 8 killed over 3 slaughter days. On each slaughter day, 6 steers from each RFI treatment (high, low) were processed, and for Angus 4 steers from each RFI (high, low) were processed. Mean kill ages were 452, 533, and 511 days for Angus crossbred, Angus, and Charolais, steers, respectively. Each animal was finished to greater than 2 mm back fat prior to slaughter. The animals were received at the abattoir and rested with *ad libitum* access to water for approximately 2 h prior to slaughter. Animals were slaughtered at the Meat Research Laboratory abattoir at Agriculture and Agri-Food Canada, Lacombe, Alberta, a federally inspected abattoir. Both animals and carcasses were inspected by trained personnel from the Canadian Food Inspection Agency and each carcass was graded by trained personnel.

2.2.2 Slaughter and carcass sampling

Steers were slaughtered randomly by breed group from July to October 2014 at the research abattoir located at Agriculture and Agri-food Canada, Science and Technology Branch, Lacombe, Alberta, according to federal regulations. Cattle were received at the abattoir and rested under shelter with *ad libitum* access to water. Immediately before slaughter, calves were weighed, and the live weight recorded before each was stunned with a captive bolt pistol and then humanely slaughtered. At 72-hours post mortem, the carcasses were fabricated and the m. *semimembranosus* (inside round) from the right side of each carcass was removed and fabricated into steaks. Steaks were aged for either 3 or 13 days post mortem (dpm) and then frozen at about -20 °C until shipped frozen to the University of Alberta for analysis. The steaks for day 3 and day 13 were individually vacuum-packaged and aged at about 4 °C and then frozen at about -20 °C until analysis.

2.2.3 Proximate analysis measurement

All proximate measurements (intramuscular fat, protein and moisture) were performed at Agriculture and Agri-Food Canada Meat Research Laboratory at Lacombe, Alberta. For proximate analyses, a 2.5 cm steak was trimmed of epimysium and surface fat, ground (Robot Coupe Blixir BX3; Robot Coupe USA Inc., Ridgeland, MS, USA) and analyzed for protein using a CEM Rapid Analyzer System (Sprint Protein Analyzer Model 558000, CEM Corporation, Matthews, NC, USA), for moisture (Smart Turbo Moisture Analyzer Model 907990, CEM Corporation, Matthews, NC, USA) and fat (Smart Trac Fat Analyzer Model 907955, CEM Corporation, Matthews, NC, USA) contents. Compositional contents were expressed as a percentage of sample weight.

2.2.4 Meat quality traits measurement

After 72 hours post mortem, meat quality measurements were performed as described by Girard et al. (2012) and Holdstock et al. (2014). Temperature and ultimate pH were measured using Fisher Scientific Accumet AP72 pH meter (Fisher Scientific, Mississauga, ON) equipped with an Orion Ingold electrode (Udorf, Switzerland). Three readings for each sample were recorded and then the average of those readings was used for data analysis. Prior to measurements,

the temperature probe was calibrated against ice water, and the pH meter calibrated against commercial buffer standards for pH 4 and 7.

To account for the effect of muscle shortening on meat toughness and drip loss, sarcomere length was measured. For the measurement of sarcomere length, 2 g of each muscle trimmed of connective tissue and large deposits of fat were hand-minced, immersed in 20 mL of 0.02 M EGTA/0.25 M sucrose solution in a 50 mL centrifuge tube, and homogenized for 10 s at 8064 x g (Polytron Homogenizer PT3100 and a 2 cm generator; Brinkmann Instruments Inc., Mississauga ON) at room temperature. A drop of sample was placed on a microscope slide and covered with a cover slip to prevent dehydration. A phase contrast microscope (Axioscope, Zeiss, West Germany) equipped with a Sony DXC 930 Color Video Camera (Sony Corporation, Japan) and Image Pro-Plus software V4.0 (Mediacybernetics, Silver Spring, MD) was used to capture 12 images of sarcomeres per steak sample. Mean sarcomere length value of each steak was calculated from the best ten images where the average length of 3 sarcomeres in each image was calculated using the Image Pro-Plus software V4.0. The lengths were averaged and expressed in micrometers for statistical analysis.

Drip loss was measured to estimate the water holding capacity of the beef. Drip loss was performed after 72 hours post mortem. The samples were weighed, and this weight was considered the initial weight. The steak samples were then put in polystyrene over-wrapped trays with a Dri-Loc® pad (Cryovac Inc., Charlotte, North Carolina) at 2 °C for four days. After four days the samples were weighed again, and this was considered the final weight. Drip loss was calculated using the following equation:

Drip loss (mg/g) = ((initial weight (g) – final weight (g))/initial weight (g)) x 1000.

Warner-Bratzler shear force was measured to assess the toughness of the beef and cooking loss and cooking time were measured so that relationships between shear force, if any, could be established to see if shear force was related to moisture loss due to cooking or the time taken to reach a constant internal temperature during cooking. Cooking loss, cooking time, Warner-Bratzler shear force were measured on non-aged (3 days post mortem) and aged (13 days post mortem) steaks. Steaks 2.5 cm thick were weighed and grilled at 210°C (Garland Grill ED30B, Condon Barr Food Equipment Ltd., Edmonton, AB) to 71 °C in their geometric centre. Internal temperature

was measured throughout cooking using a spear point temperature probe (Type T copper-constantan,10 cm in length, AllTemp Sensors Inc., Edmonton, AB). The internal temperature for each steak was monitored using a Hewlett Packard HP34970A Data Logger (Hewlett Packard Co., Boise ID), and recorded. The cooking time was also recorded and expressed as seconds per gram cooked meat. Immediately after reaching 71 °C internal temperature, each steak was removed from the grill, placed in a polyethylene bag and cooled in an ice bath. Once cooled, steaks were stored at about 2 °C until the following day. The day after cooking each steak was weighed and the weight was recorded as the final weight, and cooking loss was calculated as ((raw weight – cooked weight)/raw weight) *1000 and expressed as mg of weight lost per g raw weight.

For shear force measurement, six cores per steak, each core measuring 1.9 cm diameter, were removed parallel to the direction of the muscle fibres. Using a Texture Analyser (Model TA. XT plus, Texture Technologies Corp, New York) equipped with a Warner-Bratzler shear head. Shearing was performed at 200 mm per minute crosshead speed, and peak shear force was measured and recorded in kg using software (Texture Exponent 32 Software, TextureTechnologies Corp., Hamilton, MA). Standard deviation of the cores (Core StdDev) was calculated as the standard deviation of the mean peak shear forces of the six cores recorded for each muscle.

2.2.5 Isolation of intramuscular connective tissue (IMCT)

Intramuscular connective tissue (IMCT) was isolated to prepare for measurement of collagen trivalent cross-links. Intramuscular perimysium connective tissue was isolated from the m. *semimembranosus* as described by Roy et al. (2015). Briefly, about 100 g cubed muscle were homogenized using a laboratory blender in 5 volumes of deionized water three times at low speed for 10 s and then high speed for another 10 s to separate perimysium from the myofibrillar proteins. The homogenate slurry was filtered through a metal sieve and then blotted dry using Whatman No. 4 filter paper (Fisher Scientific, Fisher Scientific, Mississauga, Ontario), then weighed and considered as wet perimysium. The filtrate of the perimysium was collected and reserved for isolation of the endomysium. The wet perimysium was then stored at – 20 °C away from light until it was thawed as pyridinoline has been shown to be degraded by ultraviolet light (Fujimoto et al. 1978), and fresh chloroform/methanol (2:1, v/v) was used to dehydrate and de-fat the perimysium.

Fat was removed to decrease its interference with isolation of the trivalent cross-links and the perimysium was dehydrated to establish a constant dry weight for the calculation of trivalent cross-link densities in the IMCT. The de-fatted perimysium was dried in a dark fume hood overnight at room temperature and was then weighed and considered dry perimysium. Dry perimysium was stored at – 20 °C away from light until EC and PYR collagen crosslinks were determined.

Intramuscular endomysium connective tissue (wet) was isolated from the filtrate of the perimysium by centrifugation at 500 × g for 10 minutes at 4 °C. The supernatant was discarded, and the pellet was re-suspended in 1000 mL 1.1 M KI in 0.1 M KH_2PO_4 overnight at 4°C with stirring to disperse and solubilize the myofibrillar proteins and separate them from the remaining connective tissue. The following day the pellet was collected using centrifugation at 10000× g for 15 minutes at 4 °C, and the supernatant containing myofibrillar proteins was discarded. The pellet was re-suspended in 1000 mL deionized water overnight at 4°C with stirring to precipitate the endomysium and the endomysium was collected using a small kitchen sieve. Wet endomysium was weighed and stored at – 20 °C. The total wet intramuscular connective tissue (total IMCT) was calculated using the following equation:

Total IMCT (wet basis) = Perimysium content (wet basis) + Endomysium content (wet basis).

2.2.6 Connective tissue characteristics measurement

2.2.6.1 Total collagen content measurement

Total collagen content was measured to determine the influence of collagen on meat toughness and to be able to calcluate the percentage heat solubility of collagen and the density of EC and PYR cross-links in collagen. Total collagen content was determined by the measurement of hydroxyproline content using the procedure derived from the method of Bergman and Loxley (1963). Approximately 0.1g of freeze-dried meat was hydrolyzed in 6 mL of 6 N HCl in a nitrogen atmosphere at 110 °C for 20 hours to liberate the hydroxyproline. After hydrolysis, 6 N HCl was removed by evaporation (Heidolph Collegiate rotary evaporator, Brinkmann, equipped with a DistiVac Ultra auto-purge vacuum system, Brinkmann, Mississauga, ON) at 40 °C. After evaporation, hydrolysates were reconstituted with 5 mL of distilled water and neutralized using NaOH as the Bergman and Loxley assay requires a neutral pH. Neutralization was confirmed using

pH paper. Neutralized hydrolysates were evaporated again and then reconstituted to a constant volume with 5 mL of distilled water.

For hydroxyproline content estimation, two aliquots of 1 mL each of the experimental hydrolysates were assayed along with 1 mL of deionized water as the blank. All tubes were dispensed into 20 mL tubes with 2 mL of isopropanol and 1 mL of oxidant solution that were mixed by vortex. The tubes were allowed to stand for 4 minutes to oxidize the hydroxyproline pyrrole to pyrrole-2-carboxylate at room temperature, and then 13 mL of p-dimethylaminobenzaldehyde (Ehrlich's reagent) were added to each tube which was capped and mixed by using vortex, to form a chromophore that absorbs light between 540 and 570 nm (Cissell et al. 2017). Tubes were then placed in a water bath at 60 °C for 25 minutes to facilitate the reaction, followed by cooling the tubes in an ice bath to halt the reaction.

After cooling, contents of the tubes were transferred to 50 mL volumetric flasks and filled to 50 mL with isopropanol. The absorbance of the chromophore was measured at 558 nm. The amount of hydroxyproline in the unknown samples was calculated by regression of absorbance against standards with known hydroxyproline concentrations of 2.5, 5, 10, 20 and 40 ppm trans-4-hydroxyproline (Sigma Chemical Co., St. Louis, USA) performed in duplicate. The assay was validated by comparing standard curves obtained in the assay to that described by Bergman and Loxley (1963). The total collagen was then determined by multiplying the hydroxyproline concentration by 7.14, as 14% of intramuscular collagen amino acids are hydroxyproline (Etherington and Sims, 1981).

2.2.6.2 Soluble collagen content measurement

Measurement of soluble collagen was conducted to estimate the heat stability and infer the extent of heat-stable cross-linking of collagen. Specifically, heat solubility of the collagen in the powdered muscle was determined to estimate its resistance to degradation during cooking using the procedure from Hill (1966). First, approximately 1 g of freeze-dried meat was weighed into a labelled centrifuge tube, and 12 mL of ¼ strength Ringers solution was added to each tube to simulate physiological fluid, and then the contents of the tubes were vortexed thoroughly. The tubes were incubated in a water bath for 63 minutes at 77 °C to simulate cooking meat to well done. After incubation was completed the tubes were centrifuged (Avanti J-E Centrifuge,

Beckman Coulter, Mississauga, ON) at 4000 × g for 20 minutes at 4 °C and the supernatant was retained. A further 8 mL of ¼ strength Ringers solution were added to each tube, and the previous step was repeated. The supernatants were combined and 1.8 mL was clarified by centrifugation for 25 seconds. From the clarified 1.8 mL, 1 mL was transferred into a clean labelled glass tube and prepared for acid hydrolysis by adding 1 mL of 12 N HCl and 3 ml of 6 N HCl to each tube. Hydrolysis was performed as described in Section 2.2.6.1 except the filtration step was omitted. Neutralization of the hydrolysates and determination of hydroxyproline concentration in the hydrolysates was performed also as described in Section 2.2.6.1. The collagen heat solubility was expressed as a percentage of the total collagen present in the powdered muscle and was calculated using the following equation:

$$\text{Collagen heat solubility (\%)} = (\text{soluble collagen}/\ \text{total collagen}) \times 100$$

2.2.6.3 Ehrlich chromogen crosslink measurement (EC crosslink)

Ehrlich chromogen cross link concentrations were measured to estimate the content of this trivalent cross-link in perimysium collagen and relate it to the Warner-Bratzler shear force value of the muscle. Ehrlich chromogen cross-link quantification was performed according to the method of Horgan et al (1990). Briefly, 0.1 g of dried perimysium was suspended in 50 mM Tris-HCl containing 1 mM calcium chloride overnight at 4 °C. The following day, trypsin was added at 0.5 mg/mL of supernatant to liberate the Ehrlich chromogen cross-links, and samples were incubated at 37 °C for 4 hours with mixing by vortex every 15 minutes. Immediately following the incubation, trypsinized solution was heated in a dry bath for 20 minutes at 65 °C to stop the reaction. The incubation solution was then cooled at room temperature and centrifuged at 28,000 x g for 30 minutes. Following centrifugation, the supernatant was filtered through a 0.45 µm nylon filter into a 5 mL Eppendorf tube. One mL of filtrate from each sample was added to 5% p-dimethylaminobenzaldehyde (p-DAB) in 4 M perchloric acid (containing 0.01% mercuric chloride) to form a chromophore with the cross-link and then underwent centrifugation at 14,000 x g for 2 minutes to clarify the solution. The absorbance of the supernatant was then measured immediately for each sample at 572 and 640 nm. The reading of absorbance from 640 nm was

subtracted from that performed at 572 nm as the latter absorbance reading was used as a baseline measurement.

EC cross-link concentration was expressed using a molar extinction coefficient of 25000 as previously validated by Kemp and Scott (1998) in nmol/g raw meat and mol/mol collagen based on the determination of total collagen by estimation of the hydroxyproline concentration of the tryptic digest.

2.3 Statistical analysis

The effect of breed and RFI group on proximate, meat quality and connective tissue measurements were performed using a mixed model within the statistical analysis software R (Version 3.5.1). Slaughter group within breed was included as a random effect and potential 2-way interactions were included. Interactions having no significance ($P > 0.05$) were removed from the final model. Cooking loss, cooking time, WBSF Core StdDev, soluble collagen, insoluble collagen and percentage collagen solubility were compared using a split plot model with breed and RFI as sources of variation in the main plot and two aging times (days 3 and 13 post mortem) as the source of variation in the sub-plot, thus a three-way interaction was included when significant ($P \leq 05$). Least square means of each treatment were compared for differences with significance at $P < 0.05$ using the Tukey's Honestly Significant Difference test (HSD). Pearson correlation analysis was performed to calculate correlation coefficients and determine linear relationships between 28 independent variables using R (Version 3.3.3) with the package Hmisc (Version 4.0-2).

2.4 Results

2.4.1 Meat quality and proximate analysis measured at day 3 post mortem

There was no effect of breed on intramuscular ultimate pH, muscle temperature at fabrication, intramuscular crude fat, protein or moisture content, sarcomere length or drip loss (Table 2.1) and no interactions with RFI. Similarly, there was no effect of RFI on muscle temperature at fabrication, intramuscular crude fat, protein or moisture, sarcomere length or drip

loss (Table 2.2). Steers with low RFI had an ultimate intramuscular pH 0.01 of a pH unit higher than those with high RFI (Table 2.2).

2.4.2 Intramuscular connective tissue (IMCT) and collagen characteristics measured at day 3 post mortem

There was no effect of breed or RFI on weights of wet and dry perimysium, wet endomysium or total wet IMCT as a percentage of initial raw steak weight (Tables 2.3 and 2.4). Likewise, there was no difference between the breeds or the RFI groups for total intramuscular collagen content or the concentration and density of Ehrlich's chromogen (EC) (Tables 2.3 and 2.4).

2.4.3 Effect of breed, RFI and post mortem aging on meat quality and collagen characteristics measured at both day 3 and 13 post mortem

The effects of breed, RFI group, 3 or 13 days post mortem aging and their interactions on least square means are shown in Table 2.5. Significant interactions were observed between breed x aging for cooking loss, breed x RFI for percentage collagen heat solubility, and RFI x aging for WBSF core standard deviation (StdDev), but none of the means within these interactions were significantly different ($P > 0.05$)(Table 2.5). There was an effect of breed ($P = 0.04$) for percentage collagen solubility, however, with muscle from Angus steer carcasses having a higher solubility than that of the Kinsella Angus crossbred (Table 2.5). There was a significant interaction between breed and post mortem aging that indicated that aged steaks (day 13 post mortem) from Charolais steer carcasses had a greater mean cooking time than steaks aged for 3 days (Figure 2.1) that was not different to that of other breeds. Also, there was a significant interaction ($P=0.003$) between breed and post mortem aging for WBSF where among all breeds, the non-aged steaks from Angus had a higher mean shear force than aged steaks (Figure 2.2). Similarly, there was a strongly significant interaction between breed and post mortem aging for actual and percentage soluble collagen ($P<.0001$) where collagen heat solubility increased with aging for steaks harvested from Angus steer carcasses (Figures 2.3 and 2.4).There was no effect of breed, RFI and post mortem aging on insoluble collagen. There was an effect of post mortem aging on WBSF and soluble collagen, with WBSF decreasing and soluble collagen increasing with post mortem aging time (Table 2.5).

2.4.4 Correlation analysis

The correlation coefficients for proximate, meat quality, intramuscular connective tissue and collagen characteristics are presented in Table 2.6. Sarcomere length was not correlated with any parameters while ultimate pH at fabrication had a slight negative correlation with temperature and was positively correlated with mean WBSF core StdDev for aged steak (13 dpm). The temperature at fabrication was negatively correlated with fat, drip loss and cooking time at 3 dpm while it was positively correlated with moisture and shear force of non-aged (3 dpm) steaks. Moisture was negatively correlated with fat and positively correlated with protein as percentage of muscle composition, while fat was negatively correlated with protein as a percentage of muscle composition. There was a positive correlation between drip loss and cook loss for non-aged steaks while there was a negative correlation between drip loss and cooking loss at 3 dpm and shear force at 13 dpm. There was a negative correlation between cooking loss for non-aged steaks and cooking loss for aged steaks and shear force for aged steaks, while there was a positive correlation with cooking time for non-aged steaks. Cooking loss from aged steaks was positively correlated with cooking time from non-aged steaks and shear force of aged steaks. There was a highly significant positive correlation between shear force of aged steaks and Core StdDev from aged steaks.

Dry perimysium weight was positively correlated with wet perimysium weight, total intramuscular connective tissue weight, total collagen, insoluble collagen at days 3 and 13, and intramuscular Ehrlich chromogen concentration and negatively correlated with percentage heat soluble collagen at days 3 and 13 post mortem (Table 2.6). Wet perimysium weight was positively correlated with total intramuscular connective tissue amount, total collagen, soluble collagen at day 3 dpm, insoluble collagen at days 3 and 13 post mortem, and intramuscular concentration of Ehrlich's chromogen (Table 2.6). Wet endomysium amount was positively correlated withi total wet connective tissue (Table 2.6). Total collagen was positively correlated with soluble collagen at day 3 post mortem and insoluble collagen at days 3 and 13 post mortem, and intramuscular concentration of Ehrlich's chromogen, and negatively correlated with percentage heat soluble collagen at days 3 and 13 post mortem (Table 2.6). Percentage heat soluble collagen was positively correlated to insoluble collagen at days 3 and 13 post mortem, percentage of soluble collagen on day 3 post mortem and intramuscular concentration of EC, and was negatively correlated to soluble collagen on day 13 post mortem and the percentage of soluble collagen on day 13 post mortem

(Table 2.6). Insoluble collagen at 3 dpm was positively correlated with insoluble collagen at 13 dpm and intramuscular concentration of EC, and negatively correlated with percentage heat soluble collagen at 3 and 13 dpm (Table 2.6). Insoluble collagen at 13 dpm was positively correlated with intramuscular EC concentration and negatively correlated with percentage soluble collagen at 3 and 13 dpm and density of EC in perimysium (Table 2.6). Percentage of soluble collagen at 3 dpm was negatively correlated with intramuscular concentration of EC,while percentage of soluble collagen at 13 dpm was negatively correlated with concentration of EC in muscle and perimysium collagen (Table 2.6).

2.5 Discussion

The results indicated that there were no interactions between breed and RFI influencing meat quality and collagen characteristics. The implications of these results are that Angus, Charolais or Kinsella Composite steers exhibiting low RFI and therefore increased feed efficiency can be expected to have meat quality comparable to steers with high RFI. Although only three breed types were studied, the results could possibly be extrapolated to many crossbred *Bos taurus* steers as the Kinsella Composite was derived from seven different breeds (Nkrumah et al. 2007).

The results of the current study also showed there was no effect of breed on proximate composition, meat quality, connective tissue and collagen characteristics of the semimembranosus. The results are consistent with the findings of Guerrero et al. (2013) who reported there was a limited impact of breed on instrumental meat characteristics such as pH. Differences between breeds in terms of proximate composition, meat quality and collagen characteristics were likely not observed because the steers were finished to a similar subcutaneous fat level and thus similar physiological age at slaughter (Vieire et al.2006).

Similarly, residual feed intake had no effect on proximate, meat quality, connective tissue and collagen content measurements, excepting ultimate pH at the 72 hours. Muscle from low residual feed intake steers regardless of breed had a higher mean ultimate pH than muscle from steers with a high residual feed intake ($P = 0.04$). Although the difference in intramuscular pH was statistically significant, it was not biologically significant as the pH means for high and low RFI muscles differed by 0.01 pH units, and both means were well under the pH 5.8 of dark cutting muscle. The pH after cooling at fabrication should range from 5.6 to 5.8 to ensure meat with a long shelf life and acceptable beef quality (Zorzi et al. 2013; Fidelis et al. 2017). In the current study

the pH values ranged between 5.48 to 5.49 for high and low residual feed intake respectively, which was close to the recommended value range of Zorzi et al. (2013). These results are therefore consistent with results obtained by Baker et al. (2006) and Fidelis et al. (2017) who concluded that was no significant differences between low and high residual feed intake in terms of chemical composition or total collagen and shear force.

Collagen is the main protein of connective tissue, and plays an important role in meat quality particularly through its influence on meat toughness (Bailey, 1989; Rhee et al., 2004 ; Lepetit, 2008 ; Roy et al., 2015; Gonçales et al., 2018). In the current study all connective tissue characteristics were similar between residual feed intake groups. These results agree with those of Zorzi et al. (2013) who in their study compared low and high residual feed intake Nellore bulls and found there was no difference in total collagen between the two groups. Zorzi et al. (2013) reported that values for *longissimus thoracis* total collagen for high and low residual feed intake bulls were 1.32% and 1.22%, respectively. These values were close to those of Fidelis et al. (2017) who found 1.32% for low and 1.45% for high residual feed intake Nellore bulls.

The total intramuscular collagen content values observed in the current study ranged from 4.10 to 4.48 mg/g raw muscle, which were close to values reported by Campo et al. (1999) in their study of Spanish beef breeds where they observed values from 2.3 to 4.7 mg/g raw muscle. In comparison to the present work, these authors found a breed effect on collagen content. A study by Christensen et al. (2011) comparing fifteen European breeds including Aberdeen Angus and Charolais found there was no significant difference between Aberdeen Angus and Charolais in term of total intramuscular collagen content. Despite differences between the two studies in terms of results obtained, the value of mean total collagen content in the current study was 4.10 and 4.48 mg/g raw meat for the Aberdeen Angus and Charolais steer muscles, respectively, which was slightly higher than that of 3.76 and 3.68 mg/g raw muscle for Aberdeen Angus and Charolais, respectively, obtained by Christensen et al. (2011). Panea et al. (2018) studied differences between local Spanish and French beef breeds and they concluded that the breed production system has crucial impact which is responsible for the differences in total collagen content between the breeds.

Many authors agree that total collagen content is constant between animal at various ages however the quantity and quality of mature collagen cross-links are considered to be responsible for the meat toughness differences between animals and between different muscles

(Cranwell et al. 1996; Zorzi et al. 2013; Fidelis et al. 2017). Divalent collagen cross-links are those that link two collagen molecules and trivalent are those that link three collagen molecules (Eyre, 1987). As an animal ages, divalent cross-links become trivalent in order to support collagen strength and integrity (Snedeker and Gautieri 2014). But replacement of divalent with trivalent cross-links may influence the meat quality as trivalent cross-links are heat stable, thus decreasing the solubility of collagen and increasing meat toughness (Hill, 1966; Kuypers et al., 1994; McCormick, 1999; Purslow, 2018). The trivalent cross-links are comprised of the pyridinolines (PYR) and Ehrlich's chromogen (EC), both of which are consider stable at temperaures used to cook meat (Roy et al., 2015). Davey and Gilbert (1974) reported that the effects of collagen and its mature cross-links are significant on tenderness of cooked meat. In the current study, there was an absence of significant differences in collagen characteristics between residual feed intake groups. On the other hand, decreases in collagen cross link concentrations have been correlated with increased collagen heat solubility (Roy et al., 2015). This evidence was supported in the current study by significant negative correlations between both aged and non-aged collagen heat solubility and EC cross-link density ($r = -0.27$, $P<0.05$, for 3 days post mortem and $r = -0.37$, $P<0.01$, for 13 days post mortem aging). Nevertheless, there is limited literature examining how residual feed intake influences collagen cross-links, thus further studies are needed to investigate this aspect and should take different muscles into consideration.

Nowadays it is well known that post-mortem aging at refrigerated temperatures is essential for meat quality improvement (Vieira et al., 2006; Khan et al., 2016). During meat aging, muscle myofibrillar structures become weakened by proteolytic enzymes (Koohmaraie 1994; Maltin et al.2003; Brewer and Novakofski, 2008). Post mortem aging leads to improved meat quality mainly in meat tenderness, and that is the most important quality attributes for consumers (Miller et al., 2001), who are willing to pay extra for beef with known tenderness (Stolowski et al 2006). Post mortem aging had the largest influence on cooking time, Warner-Bratzler shear force and collagen solubility in this study through interactions with breed. Within the Charolais muscles, cooking time was shorter for 3 day than for 13 day aged muscle. Why this occurred was unclear, but lengthened cooking times can increase cooking loss and WBSF (Girard et al., 2012), and positive and negative correlations between cooking time and cooking loss and WBSF, respectively, supported this occuring in the day 13 samples. Despite this, with post mortem aging, WBSF decreased and collagen solubility increased the most in muscles from Angus steer

carcasses. The results also showed that post mortem aging significantly decreased shear force overall (P=0.007). The current results agreed with those of Stolowski et al. (2006) and Kołczak et al. (2008) who found that WBSF decreased during post mortem aging. Decreases in WBSF during post mortem aging are most likely due to the degradation of myofibrillar and cytoskeletal proteins of muscle fibres, proteoglycans and intramuscular connective tissue (Ouali, 1990; Koohmaraie, 1996; Nishimura et al. 1998). Beef from Angus with mixed muscle fibre types may have an increased predisposition toward substantial decreases in WBSF with ageing (Purchas and Barton, 1976), but the results of the current study are the first to show that collagen heat solubility may be increased most in Angus as well. If this is the case, then Angus cattle may not only have increased calpain activity post mortem through decreased calpastatin (Martins et al., 2017; Leal-Gutiérrez et al., 2018), they may also have increased matrix metalloproteinase capacity as well. Although collagen is considered to be resistant to proteolysis (Purslow 2018), Liu et al.(1995) noticed weakening of endomysium and perimysium in chicken muscle aged for 12 hours at 4 °C, and the increase in collagen heat solubility in the current study supports this observation.

Collagen is the most abundant protein in intramuscular connective tissues (Light Champion,1985) and provides strength to skeletal muscle (Nishimura, 2015) through formation of crosslinking between collagen molecules. Those that are considered heat stable (Roy et al. 2015) may contribute to the background toughness of meat (Bailey, 1989; Purslow et al. 2012; Du et al.2013). The increase in of heat soluble collagen with post mortem aging observed in this study agrees with the results of Palka (2003) who found that collagen solubility increased with 12 days post mortem aging at 4° C. Lucero-Borja et al. (2014) confirmed that extending the aging period up to 28 days significantly reduced the connective tissue score that was noticed by sensory panel evaluation. Furthermore a study by Stanton and Light (1987) to investigate the effect of conditioning on meat collagen showed that the amount of collagen was higher in conditioned muscles. On the other hand Jeremiah and Martin (1981) in their study found there was no relationship between collagen content or solubility with *longissimus dorsi* and *semitendinosus* muscles that were aged up to 20 days. These results confirmed those from the literature that indicated that collagen heat solubility can increase with post mortem aging. That this increase is not observed consistently may indicate that the effect of post mortem aging on collagen in beef may be muscle-specific.

2.6 Conclusion

There was no interaction between breed and residual feed intake or an effect of residual feed intake status on proximate, meat quality and connective tissue characteristics. As expected, the post mortem aging results confirmed that increasing aging time decreased the shear force and increased collagen heat solubility. This study examined beef steers that were categorized into high and low residual feed intake groups based upon their respective individual performances. Future studies will need to investigate the effect of prolonged selection for residual feed intake within a breed on meat quality and collagen characteristics. Based upon the results of this study, however, beef producers can preferentially select beef steers with low residual feed intake without anticipating a change in beef quality of the *semimembranosus* muscle.

2.7 Tables

Table 2. 1: Effect of breed on least square means (± standard error of the mean) for meat quality and proximate measurements on the m. *semimembranosus*

Measurement	**Breed**			**P value**[1]
	Kinsella Composite	**Angus**	**Charolais**	
n	24	23	24	
Ultimate pH	5.50 (0.04)	5.46 (0.03)	5.49 (0.04)	0.68
Intramuscular temperature (°C)	2.94 (0.46)	2.96 (0.46)	1.46 (0.46)	0.05
Crude fat (%)	3.09 (0.26)	3.80 (0.26)	4.19 (0.26)	0.18
Crude protein (%)	21.88 (0.22)	21.59 (0.21)	21.87 (0.22)	0.56
Moisture (%)	73.32 (0.19)	72.91 (0.20)	72.46 (0.19)	0.17
Sarcomere length (μm)	1.77 (0.04)	1.84 (0.04)	1.81 (0.04)	0.52
Drip loss (mg/g raw muscle)	45.49 (3.56)	51.13 (3.10)	57.33 (3.56)	0.25

[1] Probability of the F test, with significance at $P < 0.05$.

Table 2. 2 : Effects of RFI on least square means (± standard error of the mean) for meat quality and proximate measurements on the m. *semimembranosus*

Measurement	**Residual Feed Intake**		**P value**[1]
	High	**Low**	
n	35	36	
Ultimate pH	5.48 (0.02)	5.49 (0.02)	0.04
Intramuscular temperature (°C)	2.45 (0.43)	2.46 (0.43)	0.97
Crude fat (%)	3.95 (0.21)	3.44 (0.21)	0.10
Crude protein (%)	21.69 (0.19)	21.88 (0.19)	0.35
Moisture (%)	72.71 (0.16)	73.09 (0.16)	0.09
Sarcomere length (μm)	1.84 (0.03)	1.78 (0.03)	0.14
Drip loss (mg/g raw muscle)	51.28 (2.36)	51.35 (2.33)	0.97

[1] Probability of the F test, with significance at $P < 0.05$.

Table 2. 3: Effect of breed on least squares means (± standard error of the mean) for connective tissue characteristics on the m. *semimembranosus*

Measurement	**Breed**			**Pvalue[1]**
	Kinsella Composite	**Angus**	**Charolais**	
n	24	23	24	
Dry perimysium (%)	1.19 (0.13)	1.13 (0.13)	1.07 (0.13)	0.26
Wet perimysium (%)	4.77 (0.50)	4.26 (0.48)	4.01 (0.50)	0.19
Wet endomysium (%)	7.51 (0.69)	5.75 (0.77)	3.61 (0.69)	0.11
Total wet intramuscular connective tissue (%)	15.29 (1.13)	11.46 (1.03)	8.92 (1.13)	0.07
Total collagen (mg/g)	4.47(0.44)	4.10 (0.43)	4.48(0.44)	0.45
EC[2] (nmol/g raw meat)	7.99 (0.86)	7.70 (0.83)	8.13 (0.86)	0.72
EC[2] (mol/mol collagen)	0.55 (0.01)	0.56 (0.01)	0.55 (0.01)	0.64

[1] Probability of the F test, with significance at $P \leq 0.05$

[2] Ehrlich Chromogen cross-links.

Table 2. 4: Effect of RFI on least squares means (±standard errors of the means in parentheses) for connective tissue characteristics on the m. *semimembranosus*

Measurement	**Residual Feed Intake**		**P value[1]**
	High	**Low**	
n	35	36	
Dry perimysium (%)	1.14 (0.13)	1.12 (0.13)	0.63
Wet perimysium (%)	4.46 (0.48)	4.23 (0.47)	0.30
Wet endomysium (%)	5.25 (0.57)	5.10 (0.56)	0.36
Total wet intramuscular connective tissue (%)	11.65 (0.93)	12.13 (0.90)	0.54
Total collagen (mg/g)	4.28 (0.43)	4.42 (0.42)	0.49
EC[2] (nmol/g raw meat)	7.93 (0.83)	7.96 (0.82)	0.93
EC[2](mol/mol collagen)	0.56 (0.01)	0.54 (0.01)	0.13

[1]Probability of the F test, with significance at $P \leq 0.05$

[2] Ehrlich Chromogen cross-links

Table 2. 5: Effects of breed, RFI and post-mortem ageing (3 and 13 Days) on least squares mean (standard errors of the means in parentheses) for cooking loss, cook time, WBSF Core StdDev, soluble collagen, insoluble collagen and collagen solubility of the m. *semimembranosus*

Measurement	Breed				Residual Feed Intake			Post-mortem Ageing			Interaction		
	KC	AN	CH	P value[1]	High	Low	P value[1]	Day 3	Day 13	P value[1]	B[2]*R[3]	B[2]*A[4]	R[3]*A[4]
n	24	23	24		35	36							
Cook loss (mg/g)	274.10	285.10	275.96	0.63	274.29	282.48	0.28	279.79	276.98	0.74	NS[5]	0.04	NS
	(9.37)	(8.78)	(9.37)		(8.07)	(7.81)		(7.93)	(7.93)				
Cook time (sec/g)	3.86	4.11	4.57	0.26	4.00	4.36	0.15	3.97	4.39	0.08	NS	0.004	NS
	(0.21)	(0.22)	(0.21)		(0.17)	(0.17)		(0.17)	(0.17)				
WBSF (kg)	4.54	3.88	4.18	0.33	4.18	4.23	0.76	4.41[a]	3.10[b]	0.007	NS	0.003	NS
	(0.28)	(0.24)	(0.28)		(0.20)	(0.20		(0.20)	(0.20)				
Core StdDev[6]	0.95	0.68	0.88	0.10	0.85	0.83	0.71	0.87	0.81	0.26	NS	NS	0.04
	(0.05)	(0.05)	(0.05)		(0.04)	(0.04)		(0.04)	(0.04)				
Soluble collagen (mg/g)	0.63	0.85	0.70	0.16	0.71	0.74	0.44	0.59[a]	0.86[b]	<.0001	NS	<.0001	NS
	(0.05)	(0.05)	(0.05)		(0.04)	(0.04)		(0.04)	(0.04)				
Insoluble collagen	3.85	3.26	3.80	0.14	3.58	3.69	0.42	3.77	3.50	0.06	NS	NS	NS
(mg/g)	(0.40)	(0.39)	(0.40)		(0.39)	(0.38)		(0.38)	(0.38)				
Collagen heat solubility	13.21[a]	21.20[b]	14.78[ab]	0.04	15.90	16.89	0.33	13.08[a]	19.71[b]	<.0001	0.02	<.0001	NS
(%)	(0.87)	(0.87)	(0.87)		(0.70)	(0.69)		(0.69)	(0.69)				

KC = Kinsella Composite; AN = Angus; CH = Charolais.
[1]Probability of the F test, with significance at $P \leq 0.05$, [2] Breed , [3] RFI , [4] Post-mortem ageing , [5] NS, not significant
[6] Standard deviation of shear force [a, b] Least square means with different letters within a row within a source of variation differ ($P < 0.05$)

Table 2. 6 Pearson correlations coefficients between proximate, meat quality, intramuscular connective tissue and collagen characteristics.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sarcomere length (µm)[1]		-0.03	0.13	0.07	-0.05	-0.09	0.09	0.08	0.01	-0.01	-0.02	-0.01	0.05	0.1	-0.06
Ultimate pH[2]			0.28*	-0.03	-0.01	-0.1	0.01	-0.03	0.1	-0.04	0.06	0.07	0.28*	0.02	-0.02
Temperature[3]				0.30*	-0.26*	0.08	-0.40**	-0.10	-0.05	-0.27*	-0.21·	0.1	-0.03	0.41**	-0.12
Moisture (%) [4]					-0.95**	0.26*	-0.06	0.02	0.05	-0.15	-0.21·	0.14	0.02	0.14	-0.01
Fat (%) [5]						-0.39**	0.14	0.02	-0.1	0.15	0.14	-0.19	-0.08	-0.11	-0.06
Protein (%) [6]							-0.1	-0.22·	-0.01	-0.18	0.01	0.17	-0.02	-0.11	0.14
Drip loss(mg/g) [7]								0.25*	-0.23·	0.13	-0.04	0.05	-0.14	-0.17	-0.30*
Cook loss(mg/g) (3dpm)[8]									-0.26*	0.62**	-0.22·	-0.19	-0.07	0.18	-0.37**
Cook loss (mg/g) (13dpm)[9]										-0.05	0.79**	-0.08	0.05	0.17	0.37**
Cook time (Sec/g) (3dpm)[10]											0.01	-0.17	-0.09	0.13	-0.20·
Cook time (Sec/g) (13dpm)[11]												-0.02	0.11	0.07	0.42**
Core StdDev(3dpm)[12]													0.05	0.35**	0.1
Core StdDev(13dpm)[13]														0.07	0.42**
WBSF (Kg) (3dpm)[14]															0.13
WBSF (Kg) (13dpm)[15]															

	16	17	18	19	20	21	22	23	24	25	26	27	28
Dry perimysium (%)[16]		0.83**	0.00	0.31**	0.78**	0.28*	0.05	0.77**	0.68**	-0.35**	-0.33**	0.73**	-0.12
Wet perimysium (%)[17]			0.06	0.40**	0.64**	0.31**	0.03	0.62**	0.57**	-0.21·	-0.29*	0.62**	-0.08
Wet endomysium (%)[18]				0.87**	-0.03	0.11	0.00	-0.06	-0.03	0.13	-0.01	-0.04	-0.08
Total wet (IMCT) (%) [19]					0.16	0.15	-0.09	0.14	0.18	0.01	-0.18	0.10	-0.16
Total collagen (mg/g)[20]						0.42**	-0.06	0.98**	0.93**	-0.34**	-0.49**	0.91**	-0.21·
Soluble collagen (mg/g) (3dpm)[21]							-0.23*	0.25*	0.46**	0.69**	-0.37**	0.39**	-0.07
Soluble collagen (mg/g) (13dpm)[22]								-0.02	-0.42**	-0.18	0.87**	0.05	0.21·
Insoluble collagen (mg/g) (3dpm)[23]									0.90**	-0.49**	-0.45**	0.89**	-0.21·
Insoluble collagen (mg/g) (13dpm)[24]										-0.24*	-0.77**	0.81**	-0.27*
Solubility (%) (3dpm)[25]											0.02	-0.29*	0.11
Solubility (%) (13dpm)[26]												-0.37**	0.26*
EC (nmol/g raw meat)[27]													0.19
EC (mol/mol collagen) [28]													

*asterisks beneath each correlation indicate probability with *P < 0.05; **P < 0.01; ***P < 0.001

1 Shear force (3) (kg)	4 Moisture (%)	7 Drip loss(mg/g)	10 Cook time (3) mg/g)	13 Core StdDev (3dpm)	16 Dry perimysium (%)	19 Total wet IMCT (%)	22 Soluble collagen (mg/g) (13dpm)	25 Solubility (%) (3dpm)
2 Ultimate pH	5 Fat (%)	8 Cook loss (3) mg/g)	11 Cook time (13) mg/g)	14 WBSF (Kg) (3dpm)	17 Wet perimysium (%)	20 Total Collagen (mg/g)	23 Insoluble collagen (mg/g) (3dpm)	26 Solubility (%) (13dpm)
3 Temperature	6 Protein (%)	9 Cook loss (13) mg/g)	12 Core StdDev(3dpm)	15 WBSF (kg) (13dpm)	18 Wet Endomysium (%)	21 Soluble collag en (mg/g) (3dpm)25	24 Insoluble collagen (mg/g) (13dpm)	27 EC (nmol/g raw meat)
28 EC (mol/mol collagen)								

2.8 Figures

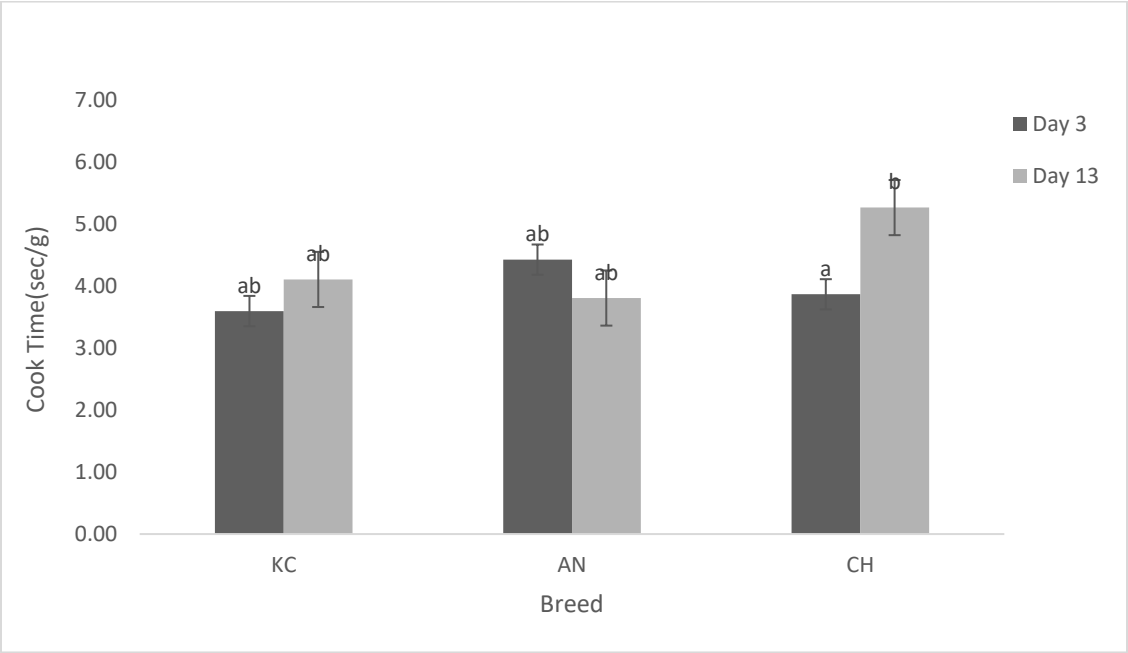

Figure 2- 1 Cook time (sec/g) of the m. semimembranosus as affected by an interaction between breed and post- mortem aging. Error bars are ± standard error of mean. Breed: AN = Angus; CH = Charolais; KC = Kinsella Composite. [a, b] columns with different letters are significantly different ($P < 0.05$)

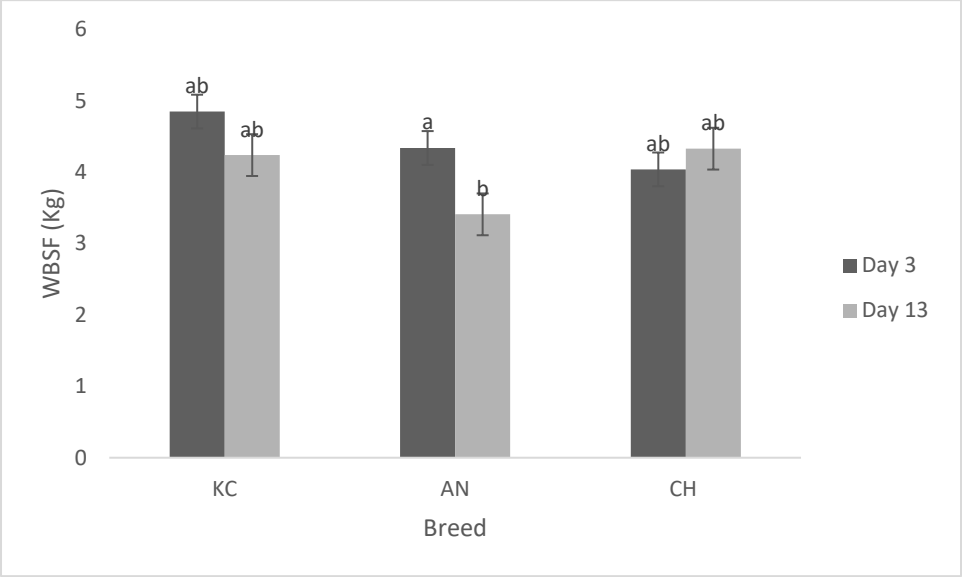

Figure 2- 2 WBSF (kg) of the m. *semimembranosus* as affected by an interaction between breed and post-mortem aging. Error bars are ± standard error of mean. Breed: AN = Angus; CH = Charolais; KC = Kinsella Composit. [a, b] columns with different letters are significantly different ($P < 0.05$)

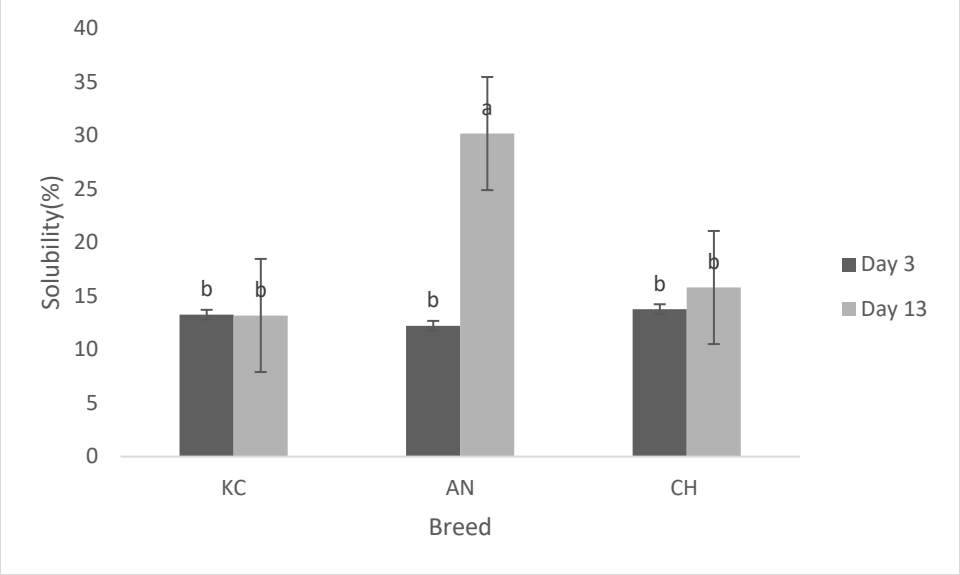

Figure 2- 3 Solubility (%) of the *m. semimembranosus* as affected by an interaction between breed and post- mortem aging. Error bars are ± standard error of mean. Breed: AN = Angus; CH = Charolais; KC = Kinsella Composite. a, b Columns with different letters are significantly different ($P < 0.05$)

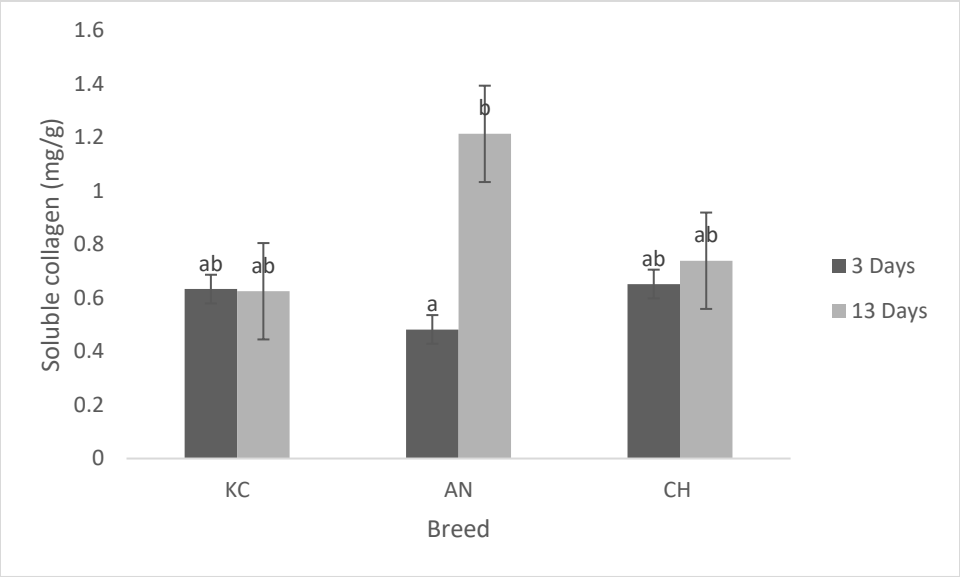

Figure 2- 4 Soluble collagen (mg/g) of the m. *semimembranosus* as affected by an interaction between breed and post-mortem aging. Error bars are ± standard error of mean. Breed: AN = Angus; CH = Charolais; KC = Kinsella Composite. a, b Columns with different letters are significantly different ($P < 0.05$)

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Chapter 3: Effect of breed and high and low collagen heat solubility on meat quality, connective tissue characteristics, and expression of genes related to collagen toughness of *m. semimembranosus* muscle

3.1 Introduction

The genetic make-up of an animal accounts for about 46% of the variation in meat tenderness (Smith et al. 2003). This suggests that improving beef tenderness through genetic manipulation and genetic selection is possible. Many studies confirmed that there was a breed influence on meat quality and collagen characteristics as well as in the expression of genes that are related to beef tenderness. Boccard et al. (1979) found that breed influenced biological differences and thus affected muscle characteristics. Hocquette et al. (2005) reviewed that beef may vary in its quality between different cattle breeds due to animal genotype differences. A study by Blanco et al. (2013) revealed that collagen characteristics were different between breeds due to variation in the physiological maturity of the breed. Similarly, Bruce and Roy (2019) confirmed that cattle breed is one of the biological factors that can affect beef quality variation by influencing intramuscular collagen content. Nowadays the genes related to myofibrillar proteins that influence beef toughness are known, and Page et al. (2002) concluded that there is a relationship between the calpain gene (*CAPN1*) and beef tenderness. As well, Nattrass et al. (2014) concluded that there is significant phenotypic variation in meat tenderness associated with *CAPN1* and calpastatin (*CAST*) genes and that these genes can explain about 44% of beef toughness. This result suggests that muscle structures other than proteins susceptible to the CAPN1 system may be responsible for the remaining 56% of the phenotypic variation.

The use of gene expression in the examination of the effects of cattle production practices on muscle biochemistry and relating them to meat quality has been limited (Byrne et al. 2005; Karisa et al. 2013; Liu et al.2018). As all biological characteristics are regulated by genes, identification of those genes is considered a key factor in understanding the phenotypic variation between animals (Hocquette et al. 2012). Gene expression study has been used to explain differences in meat quality as well as to predict and monitor meat quality through the development of biomarkers (Te Pas et al. 2017). Gene expression is one transcriptomic method that has been

applied in the study of differentially expressed genes within the same muscles of different breeds (Liu et al, 2018).

Gene expression study of collagen biosynthesis pathways and enzymes involved in the post-translational modification, as well as study of enzymes related to the degradation of collagen, may be able to explain the tenderness variation observed due to breed and between different muscles in the same animal. Expression of post-translational modification enzymes controlling quantity and quality of collagen crosslinking like lysyl oxidase (*LOX*), which are considered the key controlling steps in determining cross-link quantity, may affect the rate and extent of collagen crosslinking and subsequent collagen heat solubility. Additionally, lysyl hydroxylase (LH) appears to control the type of collagen cross-links (Yamauchi and Sricholpech 2012), and so its expression may determine the rate and extent of formation of the trivalent cross-links pyridinoline and Ehrlich's Chromogen. Matrix metalloproteinases (MMPs) are enzymes responsible for degrading intramuscular connective tissue, and their activities are regulated by the tissue inhibitor matrix metalloproteinases (TIMPs), which suppress the activity of the MMPs and therefore change in the expression of either family of proteins would impact collagen synthesis, degradation and heat solubility. Any alteration in gene expression that may exist would be anticipated to be found in phenotypic extremes; therefore the objectives of this study were to investigate the effect of breed and selection for high and low collagen heat solubility on proximate, meat quality and connective tissue characteristics as well as the expression of genes related to collagen associated toughness of 24 cattle selected from three breeds. A major muscle from the inside round muscle, the m. *semimembranosus,* which is considered to be of moderately high collagen content, was used to investigate the hypothesis that the expression of genes related to collagen synthesis and degradation are correlated with phenotypic meat quality and intramuscular collagen traits.

3.2 Materials and methods

3.2.1 Experimental design, animal management and sampling

Experimental design, animal management, muscle sampling and measurement of meat quality and connective tissue characteristics were as described in Section 2.2 of this thesis. For

gene expression analysis, samples were collected from all 72 cattle from the m. *semimembranosus* muscle within 30 min postmortem after removal of the hide. Approximately 10 g of tissue were removed using sterile procedures to prevent contamination with foreign DNA, and were frozen immediately in liquid nitrogen and preserved on dry ice until transported to the Meat Science laboratory. Samples were then stored at – 80 °C until gene expression analysis was performed. From the day 3 post mortem aging collagen heat solubility results, 12 steers were selected as having low [10.66 $\pm$ 2.99 (standard deviation, SD) %] and 12 steers with high [20.61 $\pm$ 7.51 (SD)%] intramuscular collagen heat solubility. Within the 24 steers selected, eight steers from each represented the extremes in collagen solubility breed (4 low and 4 high), and quantification of PYR cross-links and gene expression analysis were conducted on m. *semimembranosus* from each of the 24 steers.

3.2.2 Pyridinoline cross-link quantification

Pyridinoline cross-link concentration was measured to understand the contribution of this trivalent collagen cross-link to Warner-Bratzler shear force toughness. Intramuscular perimysium was isolated as described in Section 2.2 of this thesis. Following the procedure of Roy et al. (2015), pyridinoline cross-links were liberated by hydrolysed of about 0.20 g of intramuscular dry perimysium with 6 mL hydrochloric acid (6 M). The acid hydrolysates were then evaporated to dryness and reconstituted with 2.0 mL of 10% acetic acid to ensure that PYR did not adhere to laboratory glass. Pyridinoline was purified from re-constituted hydrolysates first by gel filtration (Bio-Gel P2, Bio-Rad, Canada) and then by cation-exchange chromatography (cellulose phosphate, Sigma-Aldrich, Canada), with fractions containing PYR identified by their natural fluorescence (excitation at 295 nm, emission at 395 nm). Pyridinoline cross-links were quantified using reversed phase high-performance liquid chromatography using fluorescence detection (excitation=295 nm, emission=395 nm). The method was validated using a pyridinoline standard (Quidel Corporation, San Diego, CA, USA), which was also used for calibration of the assay and quantification of PYR from experimental samples with unknown concentrations of PYR.

3.2.3 RNA extraction

RNA was extracted to measure gene expression of gene related to collagen toughness. Tissue samples stored at -80 °C were removed from storage and kept frozen on dry ice just prior

to RNA extraction. For RNA extraction, frozen tissue samples were ground in liquid nitrogen using a mortar and pestle free of RNA, and 0.1 g of ground tissue was stored in tube containing bead (Precellys, The Science of Lysing, France) at -80 °C until RNA extraction. RNA was extracted from the ground tissue using the TRizol reagent method where 1 mL of TRizol reagent (Ambion, Life Technology, USA) was added to the ground tissue to isolate high quality total RNA from DNA and protein, and then sample was homogenized by using a PRECEllys 24 Homogenizer (OMNI, International Homogenizer Company, Kennesaw. GA). RNA was precipitated using isopropanol (Fisher Scientific, Ottawa, Ontario) and 1.2 M sodium acetate in 0.8M NaCl. The precipitated RNA pellet was washed with 75% ethanol to remove residues of previous reagents. and the RNA was solubilized in 50 uL of molecular-grade water.

3.2.4 RNA Evaluation

RNA was evaluated to determine the purity and quality of the extracted RNA so that its suitability for use in gene expression analysis was known. The amount, purity and quality of the extracted RNA was evaluated based on absorbance at 260 and 280 nm using an ND-1000 spectrophotometer (Nanodrop Technologies, USA). RNA integrity number (RIN) was measured using an Agilent Tapestation (Agilent Tapestation, UK) where samples with a value higher than 7 were used for gene expression analysis.

3.2.5 Reverse transcription (cDNA synthesis)

Total RNA (1 µL) was reverse transcribed to synthesize cDNA using iScript RT Supermix (RNase H+ Moloney murine leukemia virus (MMLV) reverse transcriptase, RNase inhibitor, dNTPs, oligo (dT), random primers, buffer, MgCl2 and stabilizers) (Bio-Rad, USA). The final total reaction volume was 20 µL with the reaction mixture included 4 µL iScript RT Supermix, 2.5 µL total RNA and 13.5 µL nuclease free water. The NO-RT control reaction was prepared using iScript NO-RT control supermix (Bio-Rad, USA) to ensure cDNA samples were not contaminated with genomic DNA. All reaction tubes were incubated in a thermal cycler (AB Applied Biosystems, USA) following the manufacturer's protocol: priming 5 min at 25°C, reverse transcription 20 min at 46°C and RT inactivation 1 min at 95°C. After that, the cDNA was stored at -20 °C until gene expression analysis.

3.2.6 Primer design

The coding sequences for targeted genes (Tables 3.1) involved in collagen synthesis and degradation as well as the coding sequences for the candidate housekeeping genes *18 S rRNA* and *GAPDH* were obtained from the National Centre for Biotechnology Information database (NCBI database.www.ncbi.nlm.nih.gov/). The gene sequences were copied into Primer Express software (AB Applied Biosystems, USA) and then three pairs of forward and reverse primers of each gene were selected from designed primers. The default setting for the Primer Express software used a 20-24 base primer length. The %GC was 30-80, amplicon size was set to 50-300 base pairs, and melting temperature (MT) for primers was 58-60°C. The primers used for real-time quantitative PCR are listed in Table 3.2. Prior to polymerase chain reaction(PCR), the primers for the candidate genes were tested at different annealing temperatures (55, 58 and 60 ℃) so efficacy during the PCR technique was known.

3.2.7 Polymerase chain reaction for primer validation

cDNA was used as a template in the PCR method to check designed primers. To determine the PCR programme, the annealing temperature was calculated as 4 (number of G and C bases) + 2 (number of A and T bases). To amplify target genes using designed primers, a master mix was made for each gene in reaction tubes in this order: 2 µL of 10XPCR buffer into the master mix tube to maintain pH followed by 0.5 µL 10mM dNTP (dATP, dGTP , dCTP and dTTP which is adenine, guanine, cytosine and thymine, respectively), 1µL of $MgCl_2$, 1 µL for each forward and reverse primer, then 13µL of molecular water were added and finally 0.5 µL of Taq polymerase (5.0 units/ µL). The master mixture was mixed by vortex, spun using a mini plate spinner (MPS 1000), and then the master mix was added to the PCR template in this order: 19 µL master mix plus 1 µL cDNA with1 µL nuclease free water used instead of cDNA in the negative control. The program used in the thermocycler (Thermofisher, Applied Biosystems, USA) was preheating at 95 °C for 5 mins, denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 30 seconds, with the final extension having 7 minutes at 72 °C, with cooling at 40 °C. The number of cycles programmed was 35.

Agarose gel electrophoresis was used to confirm the PCR product. For agarose 1.5% gel electrophoresis, 2.7 g of agarose powder (Invitrogen, USA) were added to 180 mL of 1 XTBE

buffer (Thermofisher, Invitrogen, USA). The solution was boiled and dissolved completely in a microwave for 3 minutes, cooled to between 50 to 60 °C and then 1.2 µL of SyBR safe dye (Thermofisher, USA) were added. The mixed gel solution was poured into an electrophoresis tray, and the comb inserted to create wells and left to solidify. To monitor the migration of the samples on the gel, 4 µL of 6X loading dye (3 mL of glycerol (30%), 25 mg of bromophenol blue (0.25%), 25 mg of xylene cyanol (0.25%) and 10 mL dH20)) were added to and mixed in each PCR cell by vortex and then quick spin. Samples were injected into wells of the dlectrophoresis chamber, and a 100 bp ladder (Invitrogen, USA) was also loaded in the first and last wells to serve as molecular grade markers. Electrophoresis was run for 45 minutes at 150 volts. After the gel electrophoresis was completed, the gel was visualized in a 2D gel electrophoresis scanner (Typhon, General Electric FLA 9500).

3.2.8 Quantitative PCR (qPCR)

Primers for *18S rRNA* and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were tested as housekeeping genes on randomly selected RNA samples before gene expression analysis. *18S rRNA* was selected as the housekeeping gene for normalization because it was amplified at the same level with minimum variations in all the test samples and was not affected by the experimental treatment. Quantitative real-time PCR (qPCR) analysis was performed to analyze expression of target genes (Table 3.1) with StepOnePlusTM Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR green chemistry. For each reaction, the total volume of solution contained 5 µL Fast SYBR® Green Master Mix (Applied Biosystems),1 µL of each primer, 1 µL of nuclease-free water and 2 µL of cDNA template in a MicroAmp fast optical 348 well plate (Applied Biosystems, Singapore). qPCR was performed using the following program: 95°C for 20 seconds for initial denaturation and then 40 cycles of 95°C for 30 seconds followed by annealing/extension for 30 seconds at 60°C. The melting curve stage was set at 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds.

3.3 Statistical analysis

Effects of breed and collagen heat solubility ranking on meat quality and connective tissue characteristics were determined using statistical analysis software R (Version 3.5.1). In a two-way analysis of variance (ANOVA), breed and solubility levels were analyzed as fixed effects with a potential 2-way interaction. Interactions having ($P > 0.05$) were removed from the model, and the data were reanalyzed using reduced models. For post-mortem ageing effect, the split-plot procedure was used with breed and solubility levels as main plot effects and muscle aging time (day 3 and day 13) as the effect in the split plot. Least squares means of the treatments were compared for differences with significance at $P < 0.05$ using the Tukey's Honestly Significant Difference test (HSD). To analyze gene expression results, the expression was obtained based on the relative expression to the 18 S rRNA gene that was used as the housekeeping gene using the following formula: the ΔC_T mean was calculated as $\Delta C_{T\ means} = C_T$ (target gene) - C_T (reference gene), with a low ΔC_T indicating high gene expression. A two-way ANOVA was used to evaluate effects of breed and solubility level using the statistical analysis software R (Version 3.5.1). Differences between the three breeds and two solubility levels were considered significant when $P < 0.05$. When the F-test was significant, the HSD test was used to determine differences between effect means.

3.4 Results

The effects of breed treatment on proximate content, sarcomere length and drip loss of the 24 cattle selected are presented in Table 3.3. No significant difference in the means of the ultimate pH between the three breeds was observed. Kinsella Composite breed had the highest ($P \leq 0.05$.) muscle temperature at fabrication compared to that of Angus and Charolais muscles. There was no significant difference due to breed for percentage crude protein; however muscles from Charolais steer carcasses had the highest mean percentage of crude fat ($P \leq 0.05$). On the other hand, Angus muscles had the highest percentage of moisture and the lowest crude fat percentage. For sarcomere length and drip loss, there were no significant differences among the three breeds.

Results from the effect of selection for collagen heat solubility on the proximate content, sarcomere length and drip loss are presented in Table 3.4. The proximate content was not affected by high and low collagen heat solubility. Also, there was no significant difference in mean sarcomere length between muscles with high and low collagen heat solubility. For drip loss,

muscles with high collagen solubility had a greater mean drip loss (P= 03) than those with low collagen solubility.

There were no significant differences due to breed for percentage dry and wet perimysium ($P > 0.05$), whereas Kinsella Composite muscles had a higher percentage wet endomysium and total wet intramuscular connective tissue than muscles from Angus and Charolais ($P \leq 0.05$) (Table 3.4). EC concentration in raw meat (nmol/g raw meat), and within collagen (mol/mol collagen) were not affected by breed (Table 3.4). Also, there was no significant difference between breeds for PYR cross-link concentration in raw muscle (nmol/g raw meat) or collagen (mol/mol collagen) (Table 3.5).

Table 3.6 showed the effect of high and low collagen heat solubility on connective tissue characteristics. For the dry and wet perimysium content, there was no significant effect of collagen solubility ($P > 0.05$). Also, there was effect of collagen solubility on the mean endomysium content on a wet basis. For total collagen content, the high collagen solubility group had greater mean total collagen content than low collagen solubility muscles. As expected, collagen solubility at 3 dpm was significantly different as animals were segregated on the basis of this measurement, but there was no significant difference at 13 dpm between muscles segregated for collagen solubility at 3 dpm. For the density of EC cross-links in raw meat (nmol/g raw meat) high collagen solubility muscles had a higher mean EC muscle concentration than low collagen solubility muscles whereas there was no significant difference for EC cross-links density in collagen (mol/mol collagen). For the concentration of PYR cross-links in raw meat, there was no significant difference between high and low collagen heat solubility, however the concentration of PYR cross-links in collagen (mol/mol collagen) was higher (P= 0.008) in muscles with low collagen solubility at 3 dpm.

Measurements that were measured at 3 and 13 dpm are shown in Table 3.7. There were no differences in the means for cooking loss and WBSF core StdDev due to breed, collagen heat solubility, or post mortem aging period. For insoluble collagen, there were no significant differences due to breed and post- mortem aging, although the high collagen solubility muscles had higher mean insoluble collagen compared to the low collagen solubility muscle group. Interactions between breed and post mortem aging indicated that muscles from Angus carcasses had significantly greater collagen solubility as both a percentage and concentration of muscle at 13 dpm than at 3 dpm (Figures 3.1 and 3.2), whereas the muscles from the other breeds showed no change with aging.

Results of gene expression analysis are presented in Table 3.8. Breed did not affect the expression of *COL3A1, COL5A1, COL5A2, GSK3B, IGF-1, LOX, NFATC, PI3K, SMAD4* or MMP2 (Table 3.8). Expression of *CTGF* gene was significantly higher (P= 0.01) in the Charolais than in the Kinsella Composite and was similar to that of the Angus, while expression of *FOXO1* was lower in Charolais than in Kinsella Composite muscles (P=0.02). Expression of *P4HA1* was higher in Angus muscle than in Charolais muscle (P=0.04) but not different from Kinsella Composite muscles. Negative values were obtained for the ΔCt values for *SMAD2* and so these data were deemed inconclusive despite being statistically significant (P= < 0.0001).

Gene expression results were unaffected by categorization of muscles based upon high and low collagen heat solubility (Table 3.9). There were no significant differences due to selection for high and low collagen heat solubility in the expression levels of *COL3A1, COL5A1, COL5A2, CTGF, FOXO1, GSK3B, IGF1, LOX, NFATC, P4HA1, PI3K, SMAD2, SMAD4* and *MMP2* genes. There was a significant interaction between breed and collagen solubility for *CTGF* (Figure 3.3). Expression of *CTGF* was higher in low than in high collagen solubility muscles of Angus, while there was no difference in *CTGF* expression in the other breed types due to collagen solubility.

Pearson correlations between meat quality, connective tissue and collagen characteristics are presented in Table 3.10. Results showed that there was a significant positive correlation between pyridinoline cross-link concentration (mol/mol collagen) and sarcomere length ($r = 0.50$, $P < 0.05$). Dry perimysium was highly positively correlated with wet perimysium ($r = 0.75$, $P < 0.001$), total collagen ($r = 0.76$, $P < 0.001$), 3 and 13 day insoluble collagen ($r = 0.75$, 0.66, respectively, $P < 0.001$), EC concentration in muscle (nmol/g raw meat) ($r = 0.78$, $P < 0.001$) and pyridinoline cross-link concentration in meat ($r = 0.58$, $P < 0.01$). Total collagen content was highly positive correlated with soluble collagen at day 3 post mortem ($r = 0.66$, $P < 0.001$), soluble collagen at day 3 and 13 post mortem aging ($r = 0.98$ and 0.91, respectively, $P < 0.001$) as well as with EC cross-link concentration ($r = 0.91$, $P < 0.001$) and pyridinoline cross-link concentration in muscle ($r = 0.69$, $P < 0.001$); however it was negatively correlated with collagen heat solubility at day 3 post mortem aging ($r = -0.50$, $P < 0.05$) and pyridinoline cross-link concentration in collagen (mol/mol collagen)($r = -0.48$, $P < 0.05$). As expected, collagen heat solubility at day 13 post mortem aging was positive correlated with soluble collagen at day 13 post mortem aging ($r = 0.92$, $P < 0.001$) and soluble collagen at 3 days post mortem aging ($r = 0.46$, $P < 0.05$) as well as positively correlated with EC cross-link in collagen (mol/mol collagen) ($r = 0.49$, $P < 0.05$)

while it was negatively correlated with insoluble collagen at day 3 ($r = -0.46$, $P < 0.05$) and day 13 ($r = 0.81$, $P < 0.001$). Insoluble collagen at day 3 post mortem aging was highly positive correlated with insoluble collagen at day 13 post mortem aging ($r = 0.87$, $P < 0.001$), EC and pyridinoline cross-links concentrations (nmol/g raw meat) ($r = 0.88$ and 0.71 , respectively $P < 0.001$), however was negatively correlated with pyridinoline cross-link concentration in collagen (mol/mol collagen) ($r = -0.43$, $P < 0.05$). EC cross link measured in muscle was highly positively correlated with pyridinoline cross-link concentration in muscle ($r = 0.66$, $P < 0.001$) but was negatively correlated with pyridinoline cross-link concentration in collagen (mol/mol collagen) ($r = -0.41$, $P < 0.05$).

Pearson correlation coefficients between genes are presented in Table 3.11. Results indicated that *COL3A1* ΔCt was positively correlated with that of *COL5A1* ($r = -0.44$, $P < 0.05$), *COL5A2* ($r = 0.96$, $P < 0.001$) and *FOXO1* ($r = 0.61$, $P < 0.01$). *COL5A1* ΔCt was moderately positively correlated with that of *COL5A2* ($r = -0.49$, $P < 0.05$), *GF-1* ($r = 0.48$, $P < 0.05$) and *LOX* ($r = 0.54$, $P < 0.01$). *COL5A2* ΔCt was positively correlated with that of *FOXO1* ($r = 0.53$, $P < 0.01$) and *P4HA1* ($r = 0.56$, $P < 0.01$). *FOXO1* ΔCt was positively moderate correlated with that of *P4HA1* ($r = 0.50$, $P < 0.05$) while *P4HA1* ΔCt was highly positive correlated with that of *SMAD4* ($r = 0.64$, $P < 0.001$), and *SMAD2* ΔCt was moderately correlated with that of *MMP2* ($r = 0.49$, $P < 0.05$). The ΔCt values of *CTGF, GSK3B, IFG1, LOX, NFATC, PI3K* and *SMAD4* were not correlated with those of any other genes.

Pearson correlation coefficients between genes and proximate analysis and meat quality measurements are presented in Table 3.12. Correlation results indicated that the ΔCt values of *IGF-1, LOX, NFATC, PI3K* and *SMAD4* were not correlated with proximate analysis and meat quality measurements. *COL3A1, COL5A2, FOXO1* ΔCt values were highly negatively correlated with sarcomere length ($r = -0.74$, -0.76, and -0.65, respectively, $P < 0.001$), however *COL3A1* and *FOXO1*ΔCt values were moderately negatively correlated with temperature at fabrication ($r = -0.47$ and -0.57, respectively, $P < 0.05$). *CTGF* and *SMAD2* ΔCt values were positively correlated with temperature at fabrication ($r = 0.67$ and 0.57, respectively, $P < 0.05$). *GSK3B* ΔCt was negatively correlated with percentage of intramuscular crude fat ($r = -0.43$, $P < 0.05$) and highly negatively correlated with sarcomere length ($r = -0.57$, $P < 0.01$). *P4HA1* ΔCt was negatively correlated with sarcomere length ($r = -0.44$, $P < 0.05$).

Pearson correlations coefficients results between genes and cooking loss, cooking time and WBSF at 3 and 13 dpm are presented in Table 3.13. While *COL3A1* ΔCt was negatively correlated with shear force at 3 dpm, *SMAD2* ΔCt was positively correlated with shear force value at 3 dpm ($r = -0.45$ and 0.45, $P < 0.05$). *FOXO1* ΔCt was highly negatively correlated with shear force at 3 dpm ($r = -0.60$, $P < 0.01$) while *IGF-1* ΔCt was highly positively correlated with shear force at 3 dpm ($r = 0.54$, $P < 0.01$). *CTGF* ΔCt was negatively and positively correlated ($P < 0.05$) with cooking time and shear force at 3 dpm ($r = -0.46$ and 0.42) respectively.

Pearson correlation results between genes and connective tissue characterization are presented in Table 3.14. *COL5A2* ΔCt was positively correlated ($P < 0.05$) with soluble collagen concentration in muscle at 3 dpm ($r = 0.44$, $P < 0.05$). *FOXO1* ΔCt was highly negatively correlated ($P < 0.01$) with wet endomysium connective tissue ($r = -0.57$) and total wet IMCT ($r = -0.61$). *LOX* ΔCt was positively correlated ($r = 0.45$, $P < 0.05$) with dry perimysium connective tissue. *SMAD2* ΔCt was highly positively correlated with wet endomysium connective tissue and total wet IMCT ($r = 0.53$ and 0.57, respectively, $P < 0.01$). *MMP2* ΔCt was positively correlated ($P < 0.05$) with soluble collagen and percentage collagen heat solubility at 3 dpm ($r = 0.47$ and 0.48).

Pearson correlation results between genes and collagen cross-links measurements are presented in Table 3.15. *COL3A1* and *COL5A2* ΔCt values were negatively correlated ($r = -0.42$ and -0.49, respectively, $P < 0.05$) with pyridinoline cross-link concentration in collagen (mol/mol collagen) but *COL5A1* ΔCt was positively correlated ($r = 0.45$, $P < 0.05$) with the same measure. *GSK3B* ΔCt was positively correlated with Ehrlich Chromogen cross-link density in collagen (mol/mol collagen) ($r = 0.41$, $P < 0.05$). *LOX* ΔCt was slightly positively correlated with Ehrlich Chromogen cross-links concentration in muscle (nmol/g raw meat) but was highly positively correlated ($P < 0.01$) with pyridinoline cross-link concentration in muscle (nmol/g raw meat). *SMAD4* ΔCt was positively correlated with pyridinoline cross-link concentration in muscle (nmol/g raw meat) as well.

3.5 Discussion

Results related to breed effects on meat quality and collagen characteristics were similar to those observed in Section 2 of this thesis and so will not be discussed further. Rather, the results related to collagen will be the focus of this discussion as this study provided the unique opportunity

to investigate the relationships between collagen and meat quality characteristics and the expression of genes related to intramuscular collagen synthesis and degradation. Muscles were sorted into phenotypic extremes of high and low collagen solubility to examine collagen cross-link and gene expression differences, if any, associated with those extremes.

Mean PYR and EC intramuscular concentrations for bovine m. *semimembranosus* muscles in this thesis were higher than those observed by Roy et al. (2015) for bovine m. *gluteus medius*, but similar to those obtained by the same authors for the bovine m. *semitendinosus*. The values for PYR and EC molar concentrations in this thesis were higher however than those observed by Roy et al. (2015) and those observed by Ngapo et al. (2002), who presented PYR and EC concentrations values for the *semitendinosus* and *gluteobiceps* combined. The molar values obtained by Roy et al. (2015) for the *semitendinosus* and the *gluteus medius* were similar to those obtained by Ngapo et al. (2002), indicating that the difference between the values obtained in this thesis for the *semimembranosus* when using the same method as Roy et al. (2015) were due to difference in muscle type rather than methodological anomaly. As a result, the differences in intramuscular concentrations of PYR and EC observed when comparing Roy et al. (2015), Ngapo et al. (2002) and this thesis were a function of total collagen content, and the means for total collagen content across the studies support this hypothesis. These results indicate that the densities of PYR and EC in the *semimembranosus* may be higher than those of the *gluteus medius* and *semitendinosus*, implying that the collagen in this muscle is under greater strain that that of the *gluteus medius* and *semitendinosus*, but this was not investigated any further in this thesis.

Muscles with high collagen heat solubility at 3 dpm had a higher concentration of EC and decreased intra-collagen density of PYR than those with low collagen heat solubility on the same day post mortem. These results are supported by the Pearson correlation coefficients in this thesis, which indicated that absolute amount of soluble collagen at day 3 post mortem increased as PYR concentration in collagen decreased, and that the amount of insoluble collagen at day 3 post mortem increased as EC and PYR concentrations in muscle increased. These results do not agree with those of Roy et al. (2015) who did not find significant Pearson correlations between soluble collagen and PYR and EC cross-link concentrations in muscle or collagen. These results do agree with those of Ando et al. (2006), however, who found that PYR concentration in fish muscle were greatest in the insoluble and least in the acid soluble collagen portion of the muscle. Differences in crosslinking may have been most observable in this study because specific extreme phenotypes

were selected and studied in this thesis, and this may indicate in conjunction with the results of Roy et al. (2015) that statistical differences in crosslink concentrations, although statistically significant in Roy et al. (2015), may not have been large enough to cause differences in heat solubility. The results of this thesis, however, indicate that statistical differences in collagen heat solubility are indeed accompanied by statistical differences in collagen trivalent crosslinks.

These results potentially imply that Ehrlich's Chromogen is not heat-stable, given the positive correlation between its density and the percentage heat soluble collagen at day 13 post mortem. Supporting it being heat stable are the positive correlations between the concentrations of EC in muscle and insoluble collagen at days 3 and 13 post mortem. The results obtained by Horgan et al. (1991) found that EC cross link concentration increased then decreased with animal age in intramuscular connective tissue and these authors suggested that the EC cross-link may be converted to another collagen cross-link through an unknown chemical reaction. Additionally, the results obtained by Roy et al. (2015) found that the density of EC cross-links was decreased in slow-growth steers that were supplemented with ractopamine. The inconsistent results between these studies clearly indicate that further research is needed to fully understanding the relationship between EC cross links and soluble and insoluble collagen.

The results of this study confirmed that collagen solubility increases with post mortem aging and this challenges the common perception that connective tissue is resistant to post mortem proteolysis (Purslow, 2018). The current results do not agree with those of Herring et al. (1967) who found that the collagen solubility did not change with up to 10 days post-mortem aging. A study by Nishimura et al. (1998) also confirmed that up to 14 d post mortem aging did not change the connective tissue content of bovine semitendinosus muscle. The results obtained in the present thesis may be due to the small size of population examined at just eight steers per breed, and additional research is clearly needed.

Again, as found in Section 2 of this thesis, the Angus breed exhibited the only increase in collagen solubility with post mortem aging. This increase was unrelated to *MMP2* expression, and this is not unexpected as *MMP2* is a Type IV collagenase and gelatinase (Birkedal-Hansen et al. 1993; Ruiz-Gómez et al., 2019). MMPs are a family of enzymes that have the potential to degrade various connective tissue extracellular matrix components (Carmeli et al. 2004; Chen and Li, 2009; Christensen and Purslow, 2016). Sylvestre et al. (2002) found that the activity of *MMP2* was increased in the muscle of rapidly growing lambs, and this increased activity was associated

with an increase in the amount of free intramuscular hydroxyproline. In contradiction to the results of Sylvestre et al. (2002), the results of this thesis indicated that increased expression of *MMP2* was associated with decreased percentage of collagen heat solubility, and it was unrelated to the percentage of collagen solubility at 13 dpm. Approximately 25 types of MMPs have been found and each degrades specific tissues (Qi et al., 2016; Christensen and Purslow, 2016). Qi et al. (2016) investigated the correlation between the expressions of *MMP-1*, *-2*, and *-8* and the meat quality traits of *longissimus dorsi* muscle and found that *MMP2* was expressed more in marbling fat rather than in muscle, and this may explain the current results. Also, the expression of intramuscular *TIMPs* was not considered in this thesis, and these results may also be explained by the m. *semimembranosus* muscle of Angus not only having increased expression or activity of other *MMPs* but decreased expression or concentration of *TIMPs*. This hypothesis was not, however, pursued further in this thesis.

Decreased expression of *COL3A1* and *FOXO1* and increased expression of *CTGF*, *IGF-1* and *SMAD2* were correlated with decreased shear force at 3 dpm. *COL3A1* codes for the alpha 1 chain of Type III collagen in skeletal muscle (Kovanen, 2002) and comprises about 40% of the fibrous collagen in beef muscle (Purslow, 2018). Its increased expression was related to increased density of PYR in collagen in this thesis, suggesting that Type III collagen may preferentially form PYR cross-links. There was a weak negative correlation between the abundance of this gene and PYR cross-links concentration ($r=-0.42$, $P<0.05$). Bao et al. (2007) found that Laiwu Black pig muscles with high amounts of total and insoluble intramuscular collagen tended to have a higher amount of *COL3A1* mRNA. Burson and Hunt (1986) however found no connection between beef toughness and the proportions of intramuscular collagen Types I and III in the m. *longissimus thoracis et lumborum* and without an estimation of the expression of *COL1A1* and *COL1A2* in this thesis, whether expression *COL3A1* was disproportionate in the m. *semimembranosus* used in this thesis is unclear. Further research is needed to understand the relationship between Type III collagen and beef toughness.

Forkhead Box O1 (*FOXO1*) is a protein-coding gene that regulates the expression of the ubiquitin protein ligase in muscle through IGF-1 signalling pathways (Velloso, 2008). Down-regulation of *FOXO1* has been linked to increased collagen re-modelling and reduced collagen deposition (Mori et al., 2014), which would be conducive to reduced contribution of collagen to cooked muscle toughness. Connective tissue growth factor (CTGF) is also known as insulin-like

growth factor binding protein 8 and it belongs to the CCN family of matricellular proteins (Liu et al. 2011). Its main function is to act as a central mediator of tissue remodelling and fibrosis, and its upregulation has been linked to new collagen synthesis and scar tissue formation (Cheong et al. 2019). Blackstock et al. (2014) concluded that *IGF-1* increased collagen synthesis in smooth muscle through post-transcriptional and translation thus inducing *LARP6* expression (la ribonucleoprotein domain family member 6), which is responsible for regulation of collagen type 1 mRNA translation. Additionally, Reiser et al., (1996) revealed that *IGF-1* had effect on the enzymes related to post-translational modifications of collagen, therefore elevating collagen synthesis. These expression levels together suggest that cattle with low WBSF values at day 3 post mortem may have had increased collagen synthesis.

Increased expression of *SMAD2* (*SMAD* family number 2) was also correlated with reduced shear force at 3 dpm. *SMAD2* is a protein-coding gene that belongs to the family of mothers against decapentaplegic homologs (SMAD). *SMAD2* and two other members from the SMAD family (*SMAD3* and 4) mediate multiple signaling pathways. This protein mediates the signaling of the transforming growth factor (TGF)-beta by first binding to the *TGF-β* receptor (TβR) II which activates TβRI. Then *SMAD2* and *SMAD3* are phosphorylated and bind with *SMAD4* to form a SMAD complex which moves into the cell nucleus. In the cell nucleus the SMAD complex binds specifics components of target genes, such as those activating procollagen synthesis and enzymes catalyzing collagen cross-linking (Du et al. 2013). This pathway is very important because it is related to collagen cross-linking formation, which can lead to meat toughness. Although there were no significant correlations between *SMAD2* and the concentrations of EC and PYR, there was a significant positive correlation between *SMAD4* and intramuscular PYR concentration, indicating that as *SMAD4* expression decreased, the concentration of intramuscular PYR increased. *SMAD4* plays a central role in the TGF-beta-mediated fibrotic process (Ahmed et al. 2017; Lin et al. 2017).

The Kinsella composite had the lowest level of *CTGF* and highest level of *FOXO1* expression followed by Angus and then Charolais which had the highest level of *CTGF* and lowest level of *FOXO1* expression. These results suggest that differences in expression of genes related to collagen synthesis and degradation may have genetic origins, and may potentially be targets for selection. These results may also simply reflect differences in physiological age between the breed types at slaughter, as the Kinsella composite had the highest amount of IMCT and wet

endomysium. The Kinsella composite steer is an early maturing line, and reduced *CTGF* and increased *FOXO1* expression would be expected in muscle that is no longer experiencing exponential growth.

Prolyl 4-hydroxylase subunit alpha 1 (*P4HA1)* codes for a key enzyme in collagen biosynthesis (Myllyharju, 2003; Myllyharju and Kivirikko, 2004) and was also differentially expressed between the breed types, with Angus having higher expression than Charolais. In collagen, 4-hydroxyproline residues are necessary for triple helical molecule formation and maintenance of collagen molecule stability (Myllyharju, 2003; Holster et al., 2007; Gilkes et al., 2013; Aro et al., 2015). *P4HA1* belongs to the family of prolyl 4-hydroxylases (P4H) that are composed of two alpha and two beta subunits, but the alpha subunit type 1 is the main form in most cell types and tissues (Myllyharju, 2003); therefore, the study of the transcriptome or expression for this gene in meat quality is very important because it plays a role in catalyzing the post-translational formation of 4-hydroxyproline in -Xaa-Pro-Gly- sequences in collagens. There was no significant variation between high and low collagen heat solubility groups but there was a significant correlation between this gene and *COL5A2* ΔCT suggesting that it was linked to the formation of Type V collagen at the time of slaughter.

The abundance of *LOX* mRNA was highly positively correlated with the concentration of PYR in muscle, indicating that as the expression of *LOX* decreased the concentration of PYR cross link increased ($r=0.60$, $P<0.01$). *LOX* is responsible for the rate and density of collagen cross-linking, and is the enzyme most active during collagen synthesis. Lysyl oxidase is an extracellular enzyme that plays a key role in the maturation collagen and elastin (Giampuzzi et al. 2000). Thus, it plays a crucial role in collagen cross-links through post-translational modification. Furthermore, it is essential for connective tissue formation and stability (Yamauchi and Sricholpech 2012). A study by Jeong et al. (2017) correlated extracellular matrix-related gene expression levels with different adipose tissues from Korean cattle and found that there was a significant positive correlation between expression of *LOX* and marbling score. In this light, Roy et al. (2018) found that there was a significantly positive correlation between PYR cross-link and marbling (intramuscular fat content) in horse meat. Similarly, Huang et al. (2010) found that the contribution of lysyl oxidase to collagen synthesis was higher in the fetal skeletal muscle of lambs gestated in obese ewes. The relationship observed in this thesis may indicate that there was active deposition of marbling in the muscles of the cattle in this study, as would be expected in finishing steers.

3.6 Conclusion

The results of this study confirmed that increased collagen solubility of the m. *semimembranosus* is associated with increased intramuscular EC and decreased PYR density in perimysium collagen. These results also suggested that the expression of genes related to collagen synthesis and degradation are different between cattle breed types, and thus have potential as candidate genes for selection.

3.7 Tables

Table 3. 1: Candidate genes related to collagen synthesis and degradation used in the gene expression study

Gene symbol	Gene name	Gene function
COL3A1	Collagen type III alpha 1 chain	Provides instructions for making alpha 1 chain of type III
COL5A1	Collagen type V alpha 1 chain	Provides instructions for making alpha 1 chain of type V collagen
COL5A2	Collagen type V alpha 2 chain	Provides instructions for making alpha 2 chain of type V collagen
CTGF	Connective tissue growth factor	Upregulation of new collagen synthesis
FOXO1	Forkhead box protein O1	Regulates the expression of the ubiquitin protein ligase in muscle
GSK3B	Glycogen synthase kinase 3 beta	Controls the progression of wound healing and fibrosis
IGF-1	Insulin-like growth factor-1	Increases collagen synthesis in smooth muscle
LOX	Lysyl oxidase	Responsible for the rate and density of collagen cross-linking
NFATC	Nuclear Factor of Activated T Cells 1	Controls cell proliferation and extra-cellular matrix remodeling
P4HA1	Propyl 4 hydroxylase	A key enzyme in collagen biosynthesis
PI-3K	Phosphoinositide 3-kinases	Plays a role in relaying the TGF-β2 signal to induce type I collagen synthesis through SMAD pathways
SMAD2	SMAD family member 2	Mediate multiple signaling pathways related to collagen cross-linking formation
SMAD4	SMAD family member 4	Plays a central role in the TGF-beta-mediated fibrotic process
MMP2	Matrix metalloproteinase 2	Degrades denatured collagen types IV, VII, and X
18S rRNA	18S ribosomal RNA	Building block for the ribosomal 40S subunit. Tested and used in this study as a housekeeping gene.
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. Tested in this study as a housekeeping gene.

Table 3. 2: The primers used for gene expression analysis in this study[1]

Targeted gene	Primers (5' - 3')	Accession Number	Product size (bp)
COL3A1	F[2]:ATTGGCCCTGTTTGCTTTTTATAA	BC123469.1	110
	R: TGGTCACTTGTACTGGCTGACAA		
COL5A1	F: CGCCTCCCACAGTGTAACG	XM_024999726.1	112
	R: GCCTCAATTCAGTTCTTGCAAA		
COL5A2	F: AGCTGGGATGGACTTACTCTACTTG	XM_024979774.1	110
	R:TAATAACAATACATTTTAGCCCTGCCTAT		
CTGF	F: TCCCAAAATCTCCAAGCCTATC	NM_174030.2	160
	R: GACCTCCCCATCAGGACACTT		
FOXO1	F: CATGTTTATCGAGCGCTTGGA	XM_025000053.1	112
	R: GAAGCTTTGGTTGGGCAAAA		
GSK3B	F:TACCAAATGGGCGAGACACA	NM_001101310.1	110
	R:TGCTTGAATCCGAGCATGAG		
IGF-1	F:TAGAGCCTGCGCAATGGAAT	NM_001077828.1	155
	R:ACTGCTGATTTTTCCCATTGCT		
LOX	F: TGATCACAGGGTGCTGCTAAGA	NM_173932.4	109
	R: GCTGGTGACAACTGTGCCATT		
NFATC	F:AACTCTCTGCTCTTCCCACTTACTTT	NM_001166615.2	110
	R:TCTGCCATTCCTTCTCAAAATTC		
P4HA1	F: GGACTGTTTTGAGTTGGGCAAA	NM_001075770.1	109
	R: CGGTAGAAACCTCGCCTTCA		
PI3K	F:AAGGAAAAACAGGAAACACAGAAAA	M61745.1	109
	R:CAGTGCAAAGAGCGTGATGAA		
SMAD2	F: GGAACTGCCCCCTCTGGAT	NM_001046218.1	109
	R: ATCCAGGAGGTGGCGTTTCT		
SMAD4	F: CCCCAGTTCTACCTCCTGTGTT	NM_001076209.1	220
	R: GGAGAACCTGCATCCATGCT		
MMP2	F: TGATGGCGCCCATTTATACC	NM_174745.2	110
	R: GCCGGTGCCAGTATCAATGT		
18S rRNA	F:GGATCCATTGGAGGGCAAGT	NR_036642.1	110
	R:CCGCTCCCAAGATCCAACT		
GAPDH	F:CACCAGGGCTGCTTTTAATTCT	NM_001034034.2	110
	R:TGCCGTGGGTGGAATCATA		

[1]The annealing temperature for all targeted genes and reference gene was 60 °C and species for all genes sequences was *Bos taurus*.

[2]F: forward, R: reverse

Table 3. 3: Effects of breed on least squares mean (± standard error of mean) for proximate composition and meat quality characteristics

Measurement	**Breed**			**P value[1]**
	Kinsella Composite	**Angus**	**Charolais**	
n	8	8	8	
Ultimate pH	5.49 (0.02)	5.50 (0.02)	5.50 (0.02)	0.92
Temperature (˚C)	3.29[a] (0.16)	2.69[b] (0.16)	1.91[c] (0.16)	< 0.0001
Crude fat (%)	3.40[b] (0.41)	2.85[b] (0.41)	4.93[a] (0.41)	0.0050
Crude protein (%)	21.46 (0.25)	21.63 (0.25)	21.64 (0.25)	0.85
Moisture (%)	73.14[ab] (0.35)	73.61[a] (0.35)	72.07[b] (0.35)	0.02
Sarcomere length, (μm)	1.78 (0.06)	1.85 (0.06)	1.75 (0.06)	0.55
Drip loss(mg/g)	45.92 (2.74)	49.39 (2.74)	51.68 (2.74)	0.35

[1]Probability of the F test, with significance at $P \leq 0.05$.
[a, b, c] Least square means with different letters within a row differ ($P < 0.05$) due to breed.

Table 3. 4: Effects of selection for collagen heat solubility on least squares mean (± standard error of mean in parenthesis) for proximate and meat quality

Measurement	**Selection for collagen heat solubility**		**P value[1]**
n	**High**	**Low**	
	12	12	
Ultimate pH	5.51 (0.01)	5.49 (0.01)	0.34
Temperature (˚C)	2.70 (0.13)	2.56 (0.13)	0.44
Crude fat (%)	3.87 (0.33)	3.59 (0.33)	0.56
Crude protein (%)	21.40 (0.33)	21.75 (0.33)	0.25
Moisture (%)	72.93 (0.29)	72.95 (0.29)	0.96
Sarcomere length (μm)	1.74 (0.05)	1.85 (0.05)	0.18
Drip loss(mg/g)	52.83[a] (2.24)	45.16[b] (2.24)	0.03

[1]Probability of the F test, with significance at $P \leq 0.05$

[a, b] Least square means with different letters within a row differ ($P < 0.05$) due to selection for collagen heat solubility

Table 3. 5: Effects of breed on least squares mean (± standard error of mean in parenthesis) for connective tissue characteristics

Measurement	**Breed**			**P value[1]**
	Kinsella Composite	**Angus**	**Charolais**	
n	8	8	8	
Dry perimysium (%)	1.35 (0.06)	1.15 (0.06)	1.27(0.06)	0.11
Wet perimysium (%)	4.100 (0.40)	4.45 (0.40)	4.95 (0.40)	0.57
Wet endomysium (%)	9.02[a] (1.15)	5.91[ab] (1.15)	3.49[b] (1.15)	0.01
Total wet intramuscular connective tissue (%)	15.59[a] (1.17)	11.45[ab] (1.17)	9.53[b] (1.17)	0.005
Total collagen (mg/g raw meat)	4.84 (0.28)	3.100 (0.28)	4.90 (0.28)	0.06
Collagen heat solubility (3dpm[2]) (%)	14.54 (1.19)	13.16 (1.19)	13.51 (1.19)	0.70
Collagen heat solubility (13dpm[2]) (%)	13.66[b] (3.08)	33.99[a] (3.08)	17.55[b] (3.08)	0.0003
EC[3] (nmol/g raw meat)	8.72 (0.54)	7.48 (0.54)	8.53 (0.54)	0.24
EC[3](mol/mol collagen)	0.54 (0.02)	0.56 (0.02)	0.53 (0.02)	0.37
PYR[4](nmol/g raw meat)	4.08 (0.26)	3.32 (0.26)	3.92 (0.26)	0.12
PYR[4](mol/mol collagen)	0.26 (0.02)	0.25 (0.02)	0.26 (0.02)	0.96

[1] Probability of the F test, with significance at $P \leq 0.05$
[a, b] least square means with different letters within a row differ ($P < 0.05$) due to Breed
[2] Post mortem aging
[3] Ehrlich Chromogen cross-links
[4] Pyridinoline cross-links

Table 3. 6: Effects of selection for collagen heat solubility on least squares mean (± standard error of mean in parenthesis) for connective tissue characteristics

Measurement	Selection for collagen heat solubility		P value[1]
	High	Low	
n	12	12	
Dry perimysium (%)	1.30 (0.05)	1.21 (0.05)	0.23
Wet perimysium (%)	4.95 (0.33)	4.64 (0.33)	0.51
Wet endomysium (%)	6.83 (0.94)	5.45 (0.94)	0.31
Total wet intramuscular connective tissue (%)	13.07 (0.96)	11.32 (0.96)	0.21
Total collagen content (mg/g raw meat)	4.97[a] (0.23)	4.18[b] (0.23)	0.02
Collagen heat solubility (3dpm[2]) (%)	15.78[a] (0.97)	11.69[b] (0.97)	0.01
Collagen heat solubility (13dpm[2]) (%)	19.86 (2.51)	23.60 (2.51)	0.31
EC[3] (nmol/g raw meat)	8.91[a] (0.44)	7.58[b] (0.44)	0.04
EC[3](mol/mol collagen)	0.54 (0.01)	0.54 (0.01)	0.81
PYR[4](nmol/g raw meat)	3.74 (0.21)	3.80 (0.21)	0.83
PYR[4](mol/mol collagen)	0.23[a] (0.01)	0.28[b](0.01)	0.008

[1] Probability of the F test, with significance at $P \leq 0.05$
a, b, least square means with different letters within a row differ ($P < 0.05$) due to selection for collagen heat solubility.
[2] Post mortem aging
[3] Ehrlich Chromogen cross-links
[4] Pyridinoline cross-links

Table 3. 7:Effects of breed, selection for collagen heat solubility and post-mortem aging on least squares mean (± standard error of mean in parenthesis) for cook loss, cook time, WBSF, coreStdDev, soluble collagen, insoluble collagen and collagen heat solubility

Measurement	Breed			P value[1]	Selection for collagen heat solubility		P value[1]	Post-mortem Ageing		P value[1]	Interaction
	KC	AN	CH		High	Low		Day 3	Day 13		Breed × Aging
n	8	8	8		12	12		12	12		
Cook loss (mg/g)	270.69 (11.89)	295.62 (11.89)	272.83 (11.89)	0.43	275.95 (9.71)	283.47 (9.71)	0.59	288.15 (9.71)	271.28 (9.71)	0.23	NS
Cook time (sec/g)	3.70 (0.48)	4.44 (0.46)	4.45 (0.48)	0.44	3.99 (0.42)	4.40 (0.41)	0.34	4.33 (0.41)	4.06 (0.41)	0.53	0.02
WBSF (kg)	4.72 (0.33)	3.85 (0.30)	4.04 (0.33)	0.27	4.23 (0.27)	4.18 (0.26)	0.94	4.40 (0.26)	4.01 (0.26)	0.12	0.03
Core StdDev	0.99 (0.08)	0.66 (0.08)	0.85 (0.08)	0.21	0.80 (0.06)	0.87 (0.06)	0.45	0.87 (0.06)	0.80 (0.06)	0.44	NS
Soluble collagen (mg/g raw meat)	0.68 (0.07)	0.95 (0.07)	0.74 (0.07)	0.20	0.87 (0.06)	0.71 (0.06)	0.07	0.63[a] (0.06)	0.94[b] (0.06)	0.001	0.0003
In soluble collagen (mg/ g raw meat)	4.16 (0.19)	3.05 (0.19)	4.163 (0.19)	0.08	4.11[b] (0.16)	3.48[a] (0.16)	0.01	3.94 (0.16)	3.64 (0.16)	0.17	NS
Collagen heat solubility (%)	14.10 (1.71)	23.57 (1.71)	15.53 (1.71)	0.101	17.82 (1.40)	17.65 (1.40)	0.93	13.74[a] (1.40)	21.73[b] (1.40)	0.0003	0.0002

KC= Kinsella composite AN= Angus CH = Charolais

[1] Probability of the F test, with significance at $P \leq 0.05$

[a, b], least square means with different letters within a row differ ($P < 0.05$) due to selection for collagen heat solubility and post mortem aging.

Table 3. 8: Effects of breed on least squares mean (± standard error of mean in parenthesis) for gene expression (expressed as ΔCt) associated collagen toughness

Genes	Breed			P value[1]
	Kinsella Composite	Angus	Charolais	
n	8	8	8	
COL3A1	-0.56 (0.65)	-0.91 (0.65)	0.48 (0.65)	0.30
COL5A1	12.25 (2.38)	10.75 (2.38)	13.14 (2.38)	0.79
COL5A2	3.72 (0.58)	2.94 (0.58)	4.23 (0.58)	0.30
CTGF	17.50[a] (1.87)	11.25[ab] (1.87)	8.75[b] (1.87)	0.01
FOXO1	4.14[b] (0.51)	5.05[ab] (0.51)	6.36[a] (0.51)	0.02
GSK3B	6.93 (0.54)	7.77 (0.54)	6.78 (0.54)	0.39
IGF-1	8.31 (0.67)	6.34 (0.67)	7.38 (0.67)	0.15
LOX	14.38 (2.48)	10.63 (2.48)	10.86 (2.48)	0.50
NFATC	14.38 (2.57)	9.88 (2.57)	13.25 (2.57)	0.45
P4HA1	8.47[ab] (0.54)	7.63[b] (0.54)	9.70[a] (0.54)	0.04
PI3K	8.00 (1.69)	11.50 (1.69)	12.38 (1.69)	0.18
SMAD2	1.52[a] (0.69)	-2.85[b] (0.69)	-3.68[b] (0.69)	< 0.0001
SMAD4	8.26 (1.72)	6.34 (1.72)	6.23 (1.72)	0.65
MMP2	7.34 (0.80)	6.32 (0.80)	6.68 (0.80)	0.66

[1]Probability of the F test, with significance at $P \leq 0.05$.

[a, b] Least square means with different letters within a row differ ($P < 0.05$) due to breed.

Table 3. 9: Effects of selection for high and low collagen heat solubility on least squares mean (± standard error of mean in parenthesis) for gene expression (expressed as ΔCt) associated collagen toughness

Genes	Selection for collagen heat solubility		P value[1]
	High	Low	
n	12	12	
COL3A1	-0.04 (0.53)	-0.62 (0.53)	0.45
COL5A1	9.55 (2.05)	14.25 (1.95)	0.12
COL5A2	3.98 (0.47)	3.27 (0.47)	0.29
CTGF	12.92 (1.53)	12.08 (1.53)	0.71
FOXO1	5.37 (0.42)	4.99 (0.42)	0.52
GSK3B	7.52 (0.44)	6.80 (0.44)	0.27
IGF-1	7.57 (0.57)	7.13 (0.55)	0.58
LOX	11.55 (2.12)	12.42 (2.12)	0.74
NFATC	12.33 (2.10)	12.67 (2.10)	0.91
P4HA1	8.78 (0.44)	8.42 (0.44)	0.57
PI3K	10.00 (1.39)	11.25 (1.39)	0.53
SMAD2	-1.39 (0.57)	-1.94 (0.57)	0.50
SMAD4	7.20 (1.40)	6.68 (1.40)	0.79
MMP2	7.50 (0.65)	6.06 (0.65)	0.13

[1]Probability of the F test, with significance at $P \leq 0.05$.
[a, b] Least square means with different letters within a row differ ($P < 0.05$) due to selection for collagen heat solubility.

Table 3. 10: Pearson correlation coefficients between meat quality, intramuscular connective tissue and collagen characteristics

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	0.04	0.063	0.11	0.24	−0.04	0.33	−0.20	−0.23	−0.18	0.32	0.28	−0.27	−0.28	0.27	−0.37.	0.16	−0.20	−0.29	−0.31	−0.055	0.16	0.50*
2		0.32	−0.49*	0.039	−0.36.	−0.08	−0.50	−0.26	−0.31	−0.0097	−0.094	0.001	0.25	−0.05	0.15	−0.05	−0.041	0.019	−0.23	−0.45*	−0.24	−0.16
3			−0.42*	0.44*	−0.37.	−0.15	−0.56**	−0.097	0.0047	−0.0078	−0.0085	−0.093	−0.0067	0.33	−0.04	0.37.	−0.097	−0.24	−0.15	−0.093	−0.35.	−0.34
4				−0.08	0.65***	0.27	0.43*	0.16	-0.066	0.02	0.015	0.096	−0.34	0.025	−0.20	0.092	0.17	0.042	0.14	0.069	0.29	−0.01
5					−0.08	−0.07	−0.15	−0.36.	−0.36.	−0.017	−0.13	−0.37.	−0.27	0.34	−0.38.	0.22	−0.32	−0.41*	−0.33	0.12	−0.28	0.08
6						0.06	0.57**	0.23	0.053	−0.29	−0.26	0.33	−0.39.	0.059	−0.12	0.058	0.42*	0.26	0.38.	0.13	0.39.	0.01
7							0.09	0.28	−0.14	0.55**	0.51*	0.20	−0.21	0.04	0.09	0.14	0.26	0.11	0.26	0.15	0.31	0.05
8								0.21	0.087	−0.07	0.01	0.12	−0.11	−0.26	−0.03	−0.23	0.15	0.20	0.20	0.16	0.24	0.06
9									0.75***	0.023	0.22	0.76***	0.062	-0.34	0.47*	−0.033	0.75***	0.66***	0.78***	0.04	0.58**	0.31
10										−0.10	0.16	0.53**	0.23	-0.26	0.49*	0.056	0.47*	0.47*	0.59**	0.12	0.36.	-0.24
11											0.95***	0.18	0.25	0.029	0.086	0.02	-0.23	-0.14	-0.11	0.13	-0.18	-0.04
12												0.06	0.31	-0.07	0.20	-0.06	-0.12	-0.02	0.004	0.10	0.09	-0.09

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
13													0.16	−0.50*	0.66***	-0.15	0.98***	0.91***	0.91***	−0.21	0.69 ***	−0.48*
14														−0.24	0.84 ***	0.20	0.06	0.22	0.15	-0.07	-0.10	-0.28
15															0.46*	0.92***	−0.46*	−0.81***	−0.31	0.49*	−0.39.	0.20
16																0.24	0.48*	0.65 ***	0.60**	−0.15	0.31	−0.46*
17																	−0.11	-0.55**	0.021	0.45*	−0.17	0.035
18																		0.87 ***	0.88 ***	−0.20	0.71 ***	−0.43*
19																			0.76 ***	−0.36.	0.65 ***	−0.39.
20																				0.22	0.66 ***	−0.41*
21																					-0.04	0.21
22																						0.24

* asterisks beneath each correlation indicate probability with *P < 0.05; **P < 0.01; ***P < 0.001

1 Sarcomere length (µm)	2 Drip loss(mg/g)	3 Cook loss(mg/g) (3dpm)	4 Cook loss (mg/g) (13dpm)	5 Cook time (Sec/g) (3dpm)	6 Cook time (Sec/g) (13dpm)	7 WBSF (Kg) (3dpm)	8 WBSF (Kg) (13dpm)	9 Dry perimysium (%)9	10 Wet perimysium (%)10
11 Wet endomysium (%)	12 Total wet (IMCT) (%)	13 Total collagen (mg/g)	14 Solubility (%) (3dpm)	15 Solubility (%) (13dpm)	16 Soluble collagen (mg/g) (3dpm	17 Soluble collagen (mg/g) (13dpm	18 Insoluble collagen (mg/g) (3dpm)	19 Insoluble collagen (mg/g) (13dpm)	20EC (nmol/g raw meat)
21 EC (mol/mol collagen)	22PYR(nmol/g raw meat)	23 PYR(mol/mol collagen)							

Table 3. 11:Pearson correlation coefficients between candidate genes associated with collagen toughness

Genes	COL3A[1]	COL5A[1]	COL5A[2]	CTGF	FOXO[1]	GSK3B	IGF-1	LOX	NFATC	P4HA1	PI3K	SMAD[2]	SMAD[4]	MMP[2]
COL3A1		-0.44*	0.96***	-0.18	0.61**	0.35$^{\cdot}$	-0.27	-0.29	-0.06	0.49	-0.02	0.23	-0.21	-0.09
COL5A1			-0.49*	0.16	-0.04	-0.24	0.48*	0.54**	0.001	-0.17	0.13	-0.37$^{\cdot}$	0.32	-0.03
COL5A2				-0.06	0.53**	0.31	-0.22	-0.32	-0.05	0.56**	-0.12	0.39.	-0.28	0.08
CTGF					0.05	0.18	0.19	0.17	-0.07	-0.01	-0.07	0.35.	0.29	0.33
FOXO1						0.39$^{\cdot}$	-0.20	-0.23	-0.25	0.50*	0.18	-0.39.	-0.26	-0.09
GSK3B							-0.21	-0.29	-0.15	0.01	0.33	-0.02	0.17	0.00
IGF-1								0.20	-0.01	0.09	-0.25	-0.01	0.10	0.13
LOX									0.38$^{\cdot}$	-0.06	0.02	0.05	0.14	0.12
NFATC										0.21	-0.17	0.20	-0.23	0.03
P4HA1											-0.13	0.14	0.64***	0.30
PI3K												-0.39$^{\cdot}$	0.20	-0.34
SMAD2													-0.16	0.49*
SMAD4														-0.26

* asterisks beneath each correlation indicate probability with *P < 0.05; **P < 0.01; ***P < 0.001

Table 3. 12: Pearson correlation coefficients between candidate genes associated with collagen toughness, proximate analysis and meat quality.

Genes	Ultimate pH	Temperature (˚C)	Moisture (%)	Crude fat (%)	Crude protein (%)	Sarcomere length, (µm)	Drip loss(mg/g)
COL3A1	0.22	- 0.47 *	-0.13	0.10	- 0.08	-0.74***	0.09
COL5A1	- 0.11	-0.05	-0.10	0.14	0.16	0.40·	-0.06
COL5A2	0.21	-0.35·	-0.15	0.12	-0.13	-0.76 ***	0.09
CTGF	0.22	0.67**	0.32	-0.33	0.32	-0.10	-0.14
FOXO1	0.27	- 0.57**	- 0.21	0.18	0.27	-0.65 ***	0.29
GSK3B	-0.16	-0.04	0.33	- 0.43*	0.31	-0.57 **	-0.14
IGF-1	0.00	0.18	- 0.03	0.06	0.12	0.33	0.18
LOX	0.29	0.26	- 0.04	0.05	- 0.07	0.20	-0.23
NFATC	0.32	0.02	0.03	- 0.01	- 0.31	0.01	-0.06
P4HA1	0.17	- 0.27	- 0.33	0.32	0.21	-0.44 *	0.33
PI3K	-0.05	- 0.19	- 0.03	0.01	0.25	-0.06	-0.06
SMAD2	0.05	0.57**	0.19	- 0.23	- 0.23	-0.25	-0.23
SMAD4	-0.23	0.21	0.15	-0.16	-0.03	0.21	-0.32
MMP2	0.01	0.40·	-0.22	0.16	0.03	-0.10	-0.12

* asterisks beneath each correlation indicate probability with *P < 0.05; **P < 0.01; ***P < 0.001

Table 3. 13: Pearson correlation coefficients between candidate genes associated with collagen toughness and cook loss (3 and 13 days), cook time (3 and 13 days) and shear force (3 and 13 days)

Genes	Cook loss 3 days	Cook loss 13 days	Cook time 3 days	Cook time 13 days	Shear force 3 days	Shear force 13 days
COL3A1	-0.10	0.07	-0.18	0.10	-0.45*	0.06
COL5A1	-0.10	-0.02	0.06	0.12	0.20	0.05
COL5A2	-0.09	0.03	-0.28	0.03	-0.37·	0.04
CTGF	-0.29	0.05	-0.46*	-0.24	0.42*	0.05
FOXO1	-0.04	-0.23	-0.06	0.01	-0.60**	0.04
GSK3B	0.07	-0.30	0.07	-0.23	-0.40·	-0.06
IGF-1	0.04	-0.02	0.24	0.03	0.54 **	-0.04
LOX	-0.30	0.34	-0.09	0.40·	0.38·	0.17
NFATC	0.20	0.01	0.08	0.17	0.07	0.02
P4HA1	-0.01	-0.31	-0.13	0.07	-0.23	0.03
PI3K	-0.18	0.09	0.14	0.31	-0.37·	0.07
SMAD2	-0.17	0.12	-0.30	-0.20	0.45 *	-0.04
SMAD4	-0.24	0.28	-0.19	0.16	0.24	0.20
MMP2	0.07	-0.25	0.00	-0.33	0.28	-0.06

*asterisks beneath each correlation indicate probability with *P < 0.05; **P < 0.01; ***P < 0.001

Table 3. 14: Pearson correlations coefficients between candidate genes associated with collagen toughness and connective tissue characterization

Genes	Dry perimysium	Wet perimysium	Wet endomysium	Total. wet IMCT	Total collagen	Soluble collagen 3 days	Soluble collagen 13 days	In soluble collagen 3 days	In soluble collagen 13 days	collagen heat solubility (%) 3 days	collagen heat solubility (%) 13 days
COL3A1	0.04	0.18	-0.27	-0.25	0.21	0.33	-0.19	0.15	0.26	0.26	-0.24
COL5A1	0.24	0.08	-0.10	-0.07	0.06	-0.21	0.12	0.13	0.00	-0.30	0.09
COL5A2	0.09	0.23	-0.16	-0.12	0.25	0.44 *	-0.21	0.17	0.30	0.39·	-0.27
CTGF	0.18	-0.05	0.35 ·	0.31	0.13	0.17	-0.06	0.10	0.13	0.19	-0.13
FOXO1	-0.13	-0.05	-0.57 **	-0.61 **	0.10	0.16	-0.06	0.07	0.11	0.15	-0.10
GSK3B	-0.05	0.09	-0.18	-0.18	-0.10	0.10	0.00	-0.15	-0.09	0.20	0.02
IGF-1	-0.15	-0.31	0.40 ·	0.33	-0.14	-0.18	0.25	-0.11	-0.22	-0.07	0.26
LOX	0.45*	0.09	0.07	0.10	0.38·	0.20	0.00	0.39	0.32	-0.01	-0.13
NFATC	0.06	-0.10	0.27	0.25	0.05	0.13	-0.20	0.02	0.13	0.06	-0.21
P4HA1	-0.16	-0.14	-0.10	-0.15	0.13	0.37	-0.20	0.05	0.20	0.42	-0.19
PI3K	-0.05	-0.12	-0.54	-0.57	-0.02	-0.22	0.08	0.04	-0.05	-0.27	0.12
SMAD2	0.32	0.20	0.53**	0.57**	0.15	0.33	-0.18	0.08	0.20	0.30	-0.21
SMAD4	0.30	0.20	0.08	0.11	0.21	-0.05	0.15	0.26	0.12	-0.22	0.04
MMP2	0.27	0.22	0.39·	0.40·	0.19	0.47 *	0.07	0.09	0.13	0.48*	0.01

*asterisks beneath each correlation indicate probability with *P < 0.05; **P < 0.01; ***P < 0.001

Table 3. 15: Pearson correlations coefficients between candidate genes associated with collagen toughness and collagen cross links measurements

Genes	EC[1] (nmol/g raw meat)	EC[1](mol/mol collagen)	PYR[2](nmol/g raw meat)	PYR[2](mol/mol collagen)
COL3A1	0.19	-0.07	-0.14	-0.42*
COL5A1	0.01	0.07	0.37·	0.45*
COL5A2	0.24	-0.07	-0.17	-0.49*
CTGF	0.18	0.10	0.17	-0.05
FOXO1	0.01	-0.25	-0.26	-0.32
GSK3B	0.09	0.41*	-0.33	-0.28
IGF-1	-0.12	0.06	-0.08	0.12
LOX	0.35·	-0.05	0.60**	0.23
NFATC	0.03	-0.04	0.15	0.13
P4HA1	0.08	-0.10	-0.15	-0.15
PI3K	0.04	0.22	0.16	0.25
SMAD2	0.26	0.23	0.08	-0.11
SMAD4	0.33	0.27	0.42*	0.06
MMP2	0.28	0.21	-0.07	-0.23

*asterisks beneath each correlation indicate probability with *P < 0.05; **P < 0.01; ***P < 0.001.

[2] Ehrlich Chromogen cross-links

[3] Pyridinoline cross-links

3.8 Figures

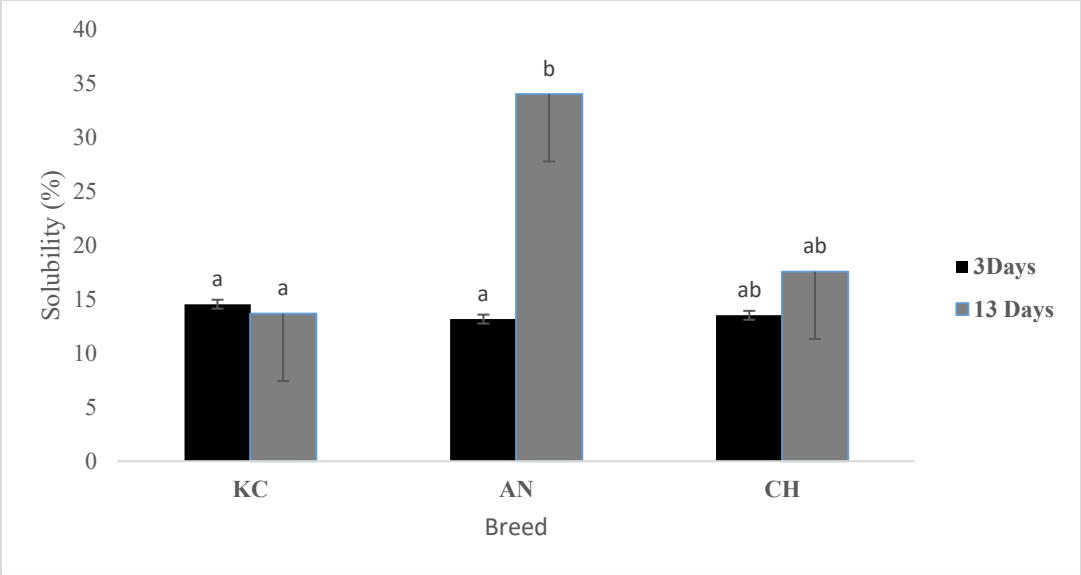

Figure 3- 1 Mean solubility (%) of the m. semimembranosus as effected by an interaction between breed and 3- and 13-days post-mortem aging. Error bars are ± standard error of mean. Breed: AN = Angus; CH = Charolais; KC = Kinsella Composite. a, b columns with different letters are significantly different ($P < 0.05$). ΔCT: the relative amplification of the targeted gene to the house keeping gene.

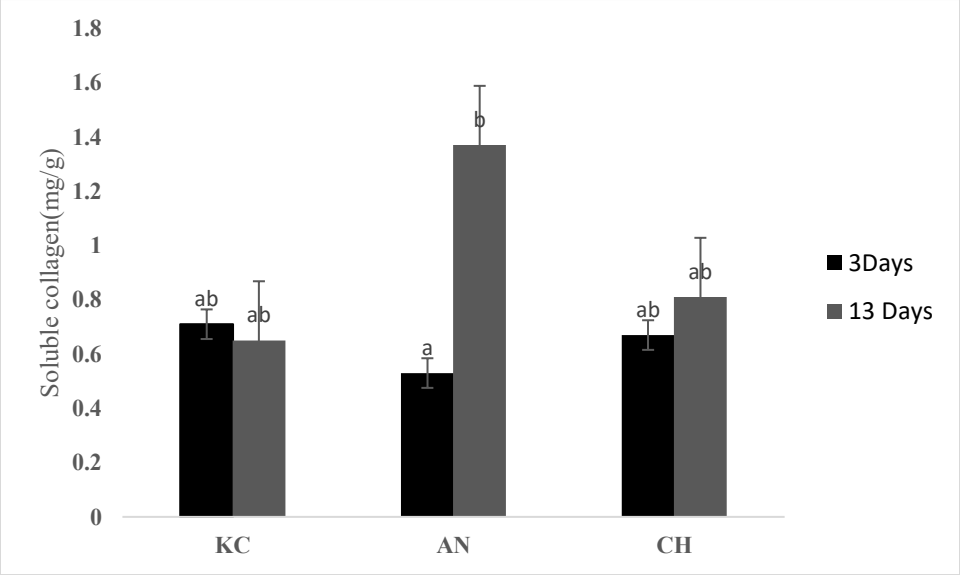

Figure 3- 2 Mean soluble collagen (mg/g) of the m. Semimembranosus as effected by an interaction between breed and 3- and 13-days post-mortem aging. Error bars are ± standard error of mean. Breed: AN = Angus; CH = Charolais; KC = Kinsella Composite. a, b columns with different letters are significantly different ($P < 0.05$). ΔCT: the relative amplification of the targeted gene to the house keeping gene.

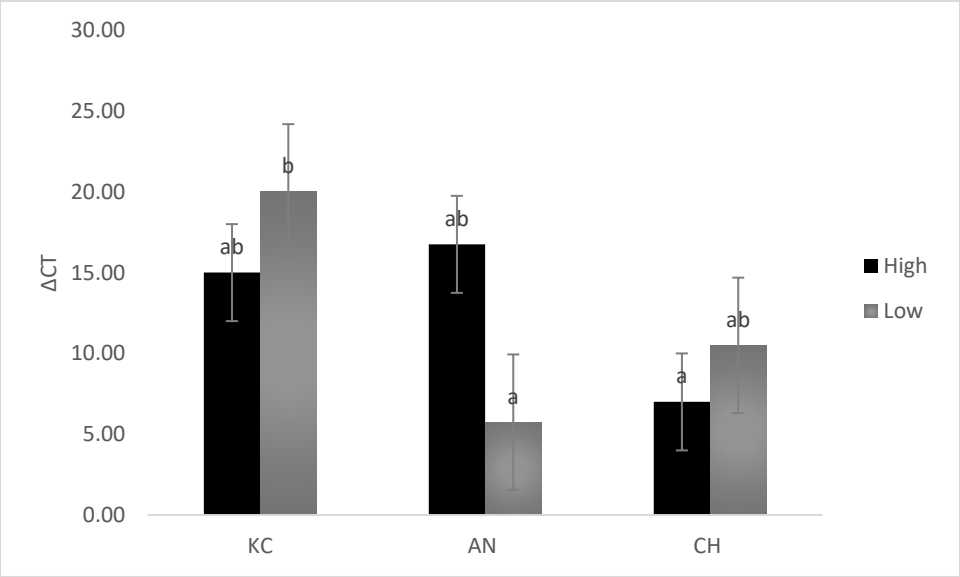

Figure 3- 3 Mean ΔCt of CTGF gene of the *m. semimembranosus* as effected by an interaction between breed and selection for high and low collagen heat solubility. Error bars are ± standard error of mean. Breed: AN = Angus; CH = Charolais; KC = Kinsella Composite. [a, b] columns with different letters are significantly different ($P < 0.05$). ΔCT: the relative amplification of the targeted gene to the house keeping gene.

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Chapter 4: General Summary

4.1 General summary of results

The null hypothesis for the first study (Chapter Two) was that there were no differences in the meat quality and collagen characteristics of the m. *semimembranosus* (inside round muscle) between Angus, Charolais and Kinsella composite crossbred steers or between steers in those breeds selected for low (negative) and high (positive) residual feed intake. The results revealed that there were no differences either between the three breeds or the two residual feed intake groups in meat quality or collagen characteristics. As a result, the null hypothesis for this study was accepted. The results of the first study confirmed that we can selection for highly feed efficient cattle by selecting for low residual feed intake without an impact on the quality of the beef. Thus, selecting for low RFI may be a profitable strategy for cattle producers by reducing their cattle production feed costs.

That post-mortem aging can enhance meat quality is well known and it is especially effective at decreasing meat toughness. This study investigated the effects of three breeds, low and high residual feed intake status, and post mortem aging of beef from 3 to 13 days post mortem on meat quality. Also, for the first time, the interactions between breed, RFI and aging were studied, as breed may affect the efficacy of selecting for RFI. In the first study, the results showed that post-mortem aging significantly decreased the Warner Bratzler shear force of the *semimembranosus* from the carcasses of the three breeds; however, it will be additionally beneficial if the toughness was also evaluated either by consumer or trained panelists. Warner-Bratzler shear force has been shown to correlate with the sensory tenderness/toughness of only one muscle, the m. *longissimus thoracis et lumborum* (Shackelford et al. 1997), and so it may not be suitable for characterizing or extrapolating tenderness to high connective tissue muscles such as the m. *semimembranosus*. Furthermore, the first study revealed that post-mortem aging significantly increased the collagen heat solubility, which is considered one of the factors that plays a critical role in decreasing meat toughness (Seideman, 1986; Weston et al., 2002; Moon, 2006).

These results suggested that breed influenced post mortem aging, as the Angus steaks had the greatest reduction in toughness during aging. The results also revealed a significant increase in collagen heat solubility for the Angus steaks, suggesting that changes in collagen

structure possibly due to post mortem enzymatic activity may have contributed to the overall decrease in WBSF in this breed with aging. Further work is needed to substantiate if this is a repeatable phenomenon in the Angus breed, as this could be a distinct meat quality advantage for this breed.

The main objective of the second study (Chapter Three) was to investigate if the expression of specific genes related to connective tissue. Study of gene expression in muscle tissue is considered one of the best methods to identify genes that are related to meat quality (Plastow and Bruce, 2014). Plastow et al. (2005) concluded that collection of tissue and the analysis of its isolated mRNA allows researchers to explore new genes that are potentially responsible for meat quality variation. Cassar-Malek et al. (2008) reported that information of meat quality attributes is difficult to obtain before animals are slaughtered thus it has also been difficult to address the inconsistent toughness and marbling of beef through genetics or epigenetics. As a result, gene expression research associated with beef quality has focussed on the identification of molecular processes involved in meat quality traits so that the beef industry could select live animals with the desired improved quality characteristics. Likewise, Cassar-Malek and Picard (2016) reported that the expression of mRNA early post mortem aging can be correlated with meat quality and these correlations used to identify biomarkers important to improving overall beef quality. Meat scientists nowadays are putting their effort into investigating the influence of genetics on meat quality traits regulation by identifying the molecular mechanisms and biochemical processes that are involved in meat quality (Guo and Dalrymple, 2017). Many studies have applied a transcriptomics approach to investigate the expression of genes associated with increased intramuscular fat (Clark et al. 2011) as well as myofibrillar degradation by calpain and calpastatin activities during post-mortem aging (Hocquette et al., 2005). But there has been limited research using gene expression in the identification of molecular mechanisms and biochemical processes related to intramuscular connective tissue and collagen characteristics especially collagen heat solubility and collagen cross-links in beef.

Therefore, to provoke differences in gene expression with regard to collagen heat solubility and collagen trivalent crosslink concentrations, muscles with low and high collagen heat solubility were selected based on the collagen heat solubility results on day 3 post mortem. The density of Ehrlich Chromogen (EC) (nmol EC/ g raw meat) was significantly higher in cattle selected for high collagen heat solubility while the density of pyridinoline (PYR) in collagen (mol

PYR/mol collagen) was lower in cattle selected for high collagen heat solubility. Although the selection for collagen heat solubility failed to be related to differences in the expression of the genes examined, cattle breed significantly influenced the variation in the expression of *CTGF*, *FOXO1*, *SMAD2* and *P4HA1*. An interaction between breed and level of collagen heat solubility for expression of *CTGF* suggests that increasing the replication with regard to extreme phenotypes of high and low collagen heat solubility muscles may provide a better understanding of how the expression of this gene influences collagen heat solubility. The current results showed significant positive and negative correlations between Ehrlich Chromogen and pyridinoline with genes related to collagen synthesis, suggesting that combining these measurements with collagen heat solubility may help with selection for tender m. *semimembranosus*. The correlation results of this study showed that sarcomere length and Warner-Bratzler shear force were significantly correlated with some genes related to collagen synthesis. This suggests that including these measurements in consideration with collagen heat solubility may provide a more complete description of the variation in beef toughness arising from both myofibrillar and connective tissue characteristics, and they may then be useful for selection of tender inside rounds.

Notably, this thesis research is some of the first work to use phenotypic extremes of collagen heat solubility to investigate the influence of gene expression on collagen characteristics. It is also among the first studies to examine the effects of breed, residual feed intake and their interaction on intramuscular collagen characteristics of beef muscle considered to have moderate levels of collagen.

4.2 Future research

The results of the first study (Chapter Two) showed that cattle breed and RFI selection for a feed efficient animal did not influence connective tissue characteristics of the *semimembranosus* muscle, and thus further studies should investigate the influence of breed and RFI selection on collagen characteristics of other muscles. Other muscles may have different levels of matrix metalloproteinase activity and physiological maturity, and may therefore be affected differently than the m. *semimembranosus*.

In the second study (Chapter Three), because a limited number of cattle were selected for the low and high collagen heat solubility groups (only 4 cattle in each group), statistical power

was not high and this may have affected the significance of the statistical analysis results. Further studies with sufficient animals are warranted to gain greater understanding of how selection for high collagen heat solubility can reduce the toughness of high connective tissue muscle such as the *semimembranosus*.

The collagen cross-link results in the second study (Chapter Three) showed that the density of Ehrlich Chromogen (EC) (nmol EC/g raw meat) was increased in the muscles of cattle selected for high collagen heat solubility, suggesting that the EC cross-link may not be heat-stable, thus further research is needed to understand the relationship between collagen heat solubility and the EC cross-link. The processes that control the formation of collagen cross-links and determine whether EC or PYR is formed are not yet known, but understanding this would potentially enable control of cross-link formation in bovine muscle and ultimately the contribution of collagen to beef toughness. Two pathways can result in the formation of PYR cross links, with the most common routes beginning with the hydroxylation of lysine residues in either the C-telopeptide region of an α 1 chain or in the N-telopeptide region of the α 1 and α 2 chains, which lead to hydroxylated forms of the PYR cross-link (Yamauchi and Sricholpech, 2012). Hydroxylation of lysine is enzymatically catalyzed by lysyl hydroxylase (LH) (Yamauchi and Sricholpech, 2012). There are three individual isoenzymes in the lysyl hydroxylase family, specifically LH1, LH2 and LH3 (Takaluoma et al. 2007), which are encoded by procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (*PLOD1*), *PLOD2,* and *PLOD3,* respectively (van der Slot et al., 2003). The LH2 has two isoforms, LH2a and LH2b (Mercer et al.2003). Both LH2a and LH2b contain dioxygenase which is responsible for catalyzing hydroxylation but only LH2b contributes to the formation of PYR by selective hydroxylase activity on the telopeptide lysines of collagen (Gjaltema et al. 2016). LH1 and LH3 catalyze the hydroxylation of lysine that is present in the helical domain of collagen while LH2 hydroxylates the lysine residues in the telopeptides of collagen (Gjaltema et al. 2016). Van Der Slot et al. (2004) concluded that LH2b is the main isoform responsible for PYR cross-link formation. Similarly, Piersma, and Bank (2019) in their review noted that only LH2b was responsible for hydroxylation of lysines in collagen telopeptides, and overexpression of this enzyme led to increased PYR cross-link concentration. Likewise, Gjaltema and Bank (2016) indicated that a high activity level of LH2b was correlated with increased PYR cross-link production. Mercer et al. (2003) meanwhile found that high expression of LH1 and LH2a was accompanied by increased production of PYR cross-links and concluded that LH2a controlled the

activity of telopeptide lysyl hydroxylase. Thus, further research to examine how LH2a and LH2b mRNA levels relative to the levels of other LH isozymes will provide better understanding as to how these genes can be used to control meat toughness in high connective tissue muscles such as the *semimembranosus*.

This thesis offered some insight into the formation of PYR as there was a significant positive correlation between *SMAD4* and intramuscular PYR concentration, indicating that as *SMAD4* expression decreased, the concentration of intramuscular PYR increased. *SMAD4* plays a central role in the TGF-beta-mediated fibrotic process (Ahmed et al. 2017; Lin et al. 2017) and may do so in the process of PYR cross-links formation by enhancing the activity of *PLOD2* (Gjaltema et al. 2015). A study by Remst et al. (2013) revealed that TGF-β is responsible for the up-regulation of the *PLOD2* gene encoding LH2b, and concluded that increased LH2b expression was correlated with the presence of TGF-β in fibrosis. Furthermore, Remst et al. (2014) also confirmed that TGF-β played an important role in stimulating the expression of *PLOD2* and the formation of LH2 in human fibroblasts. Moreover, Rosell-García et al. (2019) found that collagen deposition was increased in extracellular matrix by TGF-β1–mediated regulation of *PLOD2* expression. Thus, taking *TGF-β1* and *PLOD2* genes into account and correlating them with intramuscular collagen characteristics may provide geneticists with the basis for selection of cattle that produce more tender high collagen muscles like the m. *semimembranosus*.

The gene expression results presented in the second study (Chapter Three) established numerous relationships between gene expression and collagen biochemistry not observed before. *COL3A1* expression was related to increased density of PYR in collagen, suggesting that Type III collagen may preferentially form PYR cross-links, and this hypothesis was supported by a negative correlation between the ΔCT of this gene and PYR cross-link concentration ($r=-0.42$, $P<0.05$). Also, the abundance of *LOX* mRNA was highly positively correlated with the concentration of PYR in muscle, indicating that as the expression of *LOX* decreased the concentration of PYR cross link increased ($r=0.60$, $P<0.01$), suggesting that the rate of collagen synthesis was very much linked to PYR concentration in muscle. Future research needs to consider animal growth rate, muscle sizes and the proportions of Type I, III and IV collagens to test if Type III collagen preferentially establishes PYR cross-links and if collagen types and their cross-links are related to animal growth rate.

The relationships between gene expression and intramuscular collagen characteristics observed in this thesis need to be validated with additional research; however, they do at this time provide insight into the possible mechanisms for the formation of trivalent collagen cross-links and the importance of their contribution to beef toughness. The results observed in this thesis will provide collagen researchers with many hypotheses and much to think about regarding mechanisms of collagen cross-link formation in the coming years.

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