GENETIC MECHANISMS THAT CONTRIBUTE TO DIFFERENCES IN BEEF TENDERNESS FOLLOWING ELECTRICAL STIMULATION

A Dissertation

by

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ABSTRACT

Tenderness is a key issue for consumers that is influenced by many environmental and genetic factors. Electrical stimulation (ES) is a postmortem treatment used to increase tenderness and reduce variation between carcasses within scientific treatment groups.

The purpose of this study was to examine genetic factors that influence tenderness in response to ES. Samples were obtained from a crossbred Nellore-Angus herd produced in McGregor TX. Muscle samples were obtained immediately after slaughter, and those selected for this study were based on divergent response to ES. Tenderness was determined by objective Warner-Bratzler shear force measurements following 14 d of aging. A commercial cattle microarray was used to identify large sets of genes with significantly different gene expression between tenderness groups. These data were used to find significantly enriched genetic signaling pathways. A subset of genes based on expression and pathway analysis was selected for verification by realtime quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). A subset of gene products was selected for western blot protein expression assays. In addition SNP haplotype blocks (hapblocks) were constructed that encompassed genes of interest to determine whether parent or breed of origin for these genes contributed to ES response.

The ECM and closely related focal adhesion pathways were significantly enriched from the microarray assay. From this pathway a total of 40 genes was assayed

by qRT-PCR. Of the assayed genes, many integrins were significantly upregulated in the group that responded well to electrical stimulation compared to the group that responded poorly. Through hapblock analysis, it was found that breed and parent of origin played a role in many production features including efficiency and growth rates. Breed of origin of integrin alpha-6 (*ITGA6*) could be linked to a 0.15 kg difference in ES residual tenderness values when inherited maternally ($P = 0.03$). The gene *FN1* had a breed of origin effect, with a difference in ES residual tenderness of 0.23 kg for different paternally-inherited hapblocks. Additionally, *ITGA6* protein expression was found to closely follow mRNA expression in a subset of animals chosen for western blot analysis.

These results suggest that components of the ECM may be a novel area of research for improving tenderness that will benefit both producers and consumers.

DEDICATION

I would like to dedicate this thesis to my amazing wife. Despite finishing a law degree, planning a wedding, taking the Texas bar exam, and working multiple jobs she always found the time to give me encouragement. Her assistance in translating my random notes into something coherent and keeping track of important deadlines was invaluable.

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NOMENCLATURE

ACTN2	Actinin, alpha 2
ACTN3	Actinin, alpha 3
ADG	Average daily gain, kg/day
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide
BYS	Birth year season, (S)pring or (F)all followed by year
CAM	Cell adhesion molecule
CAPN1	Calpain 1, (mu/I) small unit
CAPN2	Calpain 2, (m/II) small unit
CAPN3	Calpain 3, p94
CAST	Calpastatin, calpain inhibitor
COX3	Mitochondrially encoded cytochrome c oxidase III
COX7C	Cytochrome c oxidase subunit VIIc
DDMI	Daily dry matter intake, kg/day
DMI	Dry matter intake, kg
DoF	Days on feed
ECM	Extra-cellular matrix
ES	Electrically stimulated/Electrical stimulation
FLNA	Filamin A, alpha
FLNB	Filamin B, beta

FLNC	Filamin C, gamma
FN1	Fibronectin 1
GO	Gene ontology
G:F	Gain to feed ratio
HKII	Hexokinase 2
ITGA5	Integrin, alpha 5
ITGA6	Integrin, alpha 6
ITGA9	Integrin, alpha 9
ITGB1	Integrin, beta 1
ITGB4	Integrin, beta 4
ITGB5	Integrin, beta 5
ITGB6	Integrin, beta 6
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG Ontology
LDHB	Lactate dehydrogenase B
MYH1	Myosin heavy chain 1, skeletal muscle, adult
MYH2	Myosin heavy chain 2, skeletal muscle, adult
NES	Not electrically stimulated
qRT-PCR	Quantitative realtime reverse-transcriptase PCR
QTL	Quantitative trait loci
RPL5	Ribosomal protein L5
RPL19	Ribosomal protein L19

RPS20	Ribosomal protein S20
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SYS	Slaughter year season, (S)pring or (F)all followed by year
WBSF	Warner-Bratzler shear force

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Overview

Tenderness in beef has been identified as the main factor affecting consumer palatability ratings (Ramsey et al., 1963; Whipple et al., 1990b; Savell et al., 1991; Chambers and Bowers, 1993). In consumer sensory panel studies, tenderness accounts for more than 50% of the total value placed on meat by consumers (Miller et al., 2001). Consumers have expressed a willingness to pay a premium price for reliably labeled tender beef (Boleman et al., 1997; Lusk et al., 2001; Beriain et al., 2009). This preference makes tenderness an issue of concern to producers and consumers alike.

Applying an electrical current to the carcass postmortem is one commonly employed commercial method for improving end-product tenderness. Electrical stimulation has been shown to reduce, but not eliminate, variation in tenderness between carcasses. A better understanding of the genetic and biochemical factors affecting the improvement in tenderness (response) by electrical stimulation may enable breeders to improve the design of breeding programs and production strategies, providing benefits to both producers and consumers.

Measuring tenderness

Tenderness can be measured in a variety of ways. Within this dissertation, tenderness will be described in reference to Warner-Bratzler shear force (WBSF). This

method measures the total force necessary to shear a piece of cooked meat under controlled circumstances and reduces the subjectivity inherent to consumer panel ratings. WBSF is an objective measure of final tenderness that takes in to account all factors that affect this trait.

Factors affecting tenderness

Tenderness of beef is a complex trait that is influenced by many factors. These factors can be broadly grouped in to 3 main categories: postmortem metabolism, postmortem proteolysis, and muscle structure. These categories are not necessarily independent. For instance, the rate of postmortem metabolism can affect the rate of postmortem proteolysis.

Postmortem metabolism is a direct result of environmental and genetic factors affecting the living animal. The main component is pH decline caused by the glycolytic pathway converting energy stores, primarily glycogen, to lactic acid. This pH decline can have significant impacts on tenderness based both on rate of decline and ultimate pH (Harrell et al., 1978). A rapid pH decline can lead to increased toughness due to protein denaturation and increased water loss (Marsh et al., 1981; Huff-Lonergan and Lonergan, 2005). Pre-slaughter stress can increase overall toughness by promoting a rapid rate of postmortem glycolysis resulting in a rapid pH drop (Gruber et al., 2010). Higher temperatures lead to faster rates of pH decline (Bruce and Ball, 1990), supposedly due to greater glycolytic activity at higher temperatures.

Postmortem proteolysis is another factor that is heavily influenced by pre-slaughter conditions and individual animal characteristics. Proteins are not synthesized in the muscle postmortem to a significant degree (Konikova et al., 1972). Proteolysis is dependent on those proteases present in the muscle at the time of death and physical proximity to their specific substrates. Calcium-dependent proteases (known as calpains) and cathepsins have been implicated in this role (Calkins and Seideman, 1988) in addition to the proteasome (Briand et al., 1997).

Female animals and castrated males are generally more tender than bulls due to lower concentrations of inhibitors of muscle protein degradation (Hunsley et al., 1971). Indicine breeds are reported to be tougher and more variable than taurine breeds (Crouse et al., 1989). This is believed to be, in part, due to higher levels of the endogenous calpain inhibitor calpastatin. There is conflicting evidence on the role apoptotic pathways play in ultimate tenderness and response to aging (Plomgaard et al., 2005; Ouali et al., 2006; Cherel et al., 2012). The endogenous caspase enzymes have also been implicated, although their role too is controversial (Kemp et al., 2006; Huang et al., 2011; Kemp and Wheeler, 2011). Whereas the exact role of these pathways remains unclear, the overall function of proteolysis in tenderization process is well established (Koohmaraie, 1994; Wheeler and Koohmaraie, 1994).

The final major category, muscle structure, includes intramuscular fat deposits (marbling) and connective tissue. Total connective tissue content, collagen, is a factor in overall beef tenderness (Cross et al., 1973). However, solubility differences caused by variation in total crosslinks between collagen fibers may be more significant than total

connective tissue content (Light et al., 1985; Burson et al., 1986; Seideman, 1986; Mccormick, 1994). Cross linkage between muscle collagens increases with maturity of muscle fibers (Robins and Bailey, 1972; Shimokom.M et al., 1972); which may be why after attainment of maturity, beef cattle become increasingly tough with age (Zinn et al., 1970; Hunsley et al., 1971; Prost et al., 1975). Intramuscular fat deposits (marbling) can reduce shear force values by interspersing the muscle with a softer substrate and insulating against cooking induced toughness. Marbling is positively correlated with tenderness and palatability (Blumer, 1963; Campion et al., 1975; Berry, 1993; Wheeler et al., 1994).

It is possible for the function of any individual factor to be affected by an interaction with another factor. For instance, a low quality feed can contribute to increased toughness (Prost et al., 1975) by reducing proteolysis and collagen solubility. The rate of pH decline due to postmortem metabolism can alter the function of the calpain system (Maddock et al., 2005), thereby reducing the rate of postmortem proteolysis. Ultimate tenderness is a product of these 3 major categories, and complex interactions among them that are not entirely understood.

Breed differences

Breed composition has a significant impact on toughness and variability in tenderness. *Bos indicus* cattle are utilized in tropical and semi-tropical regions, including the southern United States, for their improved tolerance of heat, humidity, tropical diseases, and parasites as compared to *Bos taurus* cattle (Cartwright, 1980; Franke,

1980; Turner, 1980; Norris et al., 2003). These characteristics are beneficial in these regions to improve overall productivity (Carroll et al., 1955; Cole et al., 1963; Ramsey et al., 1963; Crockett et al., 1979). Indicine breeds seem to be more resistant than taurine breeds to stress associated with elevated temperatures and humidity, which affect feeding behavior less and appear less physiologically stressful for indicine breeds (Beatty et al., 2006). However, *Bos indicus* influence is often associated with increased meat toughness and variability (Carpenter et al., 1961; Ramsey et al., 1963; Dinius et al., 1976; Winer et al., 1981; Koch et al., 1982; McKeith et al., 1985; Crouse et al., 1988; Wheeler et al., 1990b).

Tenderness has been shown to be moderately to highly heritable in beef cattle (Shackelford et al., 1994; Wulf et al., 1996), presenting the possibility for successful selection for beneficial genetic factors influencing tenderness. A more recent study found that heritability for WBSF ranged from 0.27 to 0.47 (O'Connor et al., 1997). Although not a precise relationship, variability in tenderness increases with percentage *B. indicus* (Damon et al., 1960; Norman, 1982; Peacock et al., 1982). Indicine breeds contribute to greater WBSF values as well as greater variability in WBSF (Winer et al., 1981; Koch et al., 1982; Johnson et al., 1990; Wheeler et al., 1990b). Taurine breeds perform better in terms of eating quality on a high quality diet such as is found in feedlots as opposed to lower quality forages (Chaosap et al., 2011). Indicine breeds do not show the same decrease in toughness as taurine breeds following ES even after accounting for the activity of endogenous enzymes (calpains) at slaughter (Gursansky et al., 2010). As indicine breed composition adds to the overall toughness and variability of

a population of crossed animals it would be advantageous to study the role indicine influence plays in the underlying genetic factors influencing response to aging. It should be noted that fat thickness and different median age at slaughter could be confounding factors in directly comparing *B. indicus* to *B. taurus* animals (Huffman et al., 1990). A better understanding of the genetic components of these traits may make it possible to keep production traits without sacrificing meat quality.

Electrical stimulation

Electrical stimulation has been used successfully to increase tenderness and reduce variation in WBSF, particularly in those cuts that would otherwise be considered less tender (Savell et al., 1981; Roeber et al., 2000). Electrical stimulation works on carcasses from several livestock species and is effective in increasing tenderness in sheep and goat meat as well (Savell et al., 1977b). The process by which electrical stimulation of beef carcasses improves meat tenderness is not entirely understood.

Myofibril fractures caused by rapid muscle contraction are one proposed component of ES-induced tenderization (Savell et al., 1978; Takahashi et al., 1987). Another major factor in postmortem tenderization, pH decline, is believed to be a factor in response to ES (Hwang and Thompson, 2001). However, some studies have found no correlation between ES induced pH drop and reduced meat toughness (Hollung et al., 2007), whereas others have found a correlation between pH drop and reduced toughness only in certain muscle groups (Eilers et al., 1996). The main cause of pH decline, postmortem glycolytic rate, does not appear to play a direct role in ES response

(Ferguson et al., 2008), although glycolytic rate has been associated with overall tenderness (Martin et al., 1983). Reduced cold-shortening has been hypothesized to be one benefit of ES with regard to tenderness but was found to have a negligible role in final WBSF (Wheeler et al., 1990a). High voltage electrical stimulation has been shown to overcome some of the effects of breed on tenderness (Gursansky et al., 2010), enabling *B. indicus*-influenced carcasses to reach a level of tenderness more consistent with *B. taurus* carcasses under the same postmortem conditions. Electrical stimulation is not sufficient to entirely overcome breed effects and differences in shear force remain between carcasses from different breeds.

Not all carcasses respond equally to ES. Bull carcasses subjected to ES produced cuts with WBSF values comparable to those of steer carcasses that were not subjected to ES (Hopkinson et al., 1985), implying that ES can at least partially overcome the effect of being left intact. In general, steers respond better to ES than bulls (Klastrup et al., 1984). Additionally, indicine breeds do not show the same increase in tenderness as taurine breeds following ES. These results imply either a lesser susceptibility of muscle proteins to enzyme degradation following ES in indicine carcasses, or a yet unknown factor(s) influencing response to ES (Gursansky et al., 2010). Increased proteolysis due to ES, resulting in increased myofibrillar protein breakdown and thus increased tenderness, is a theory that is strongly supported by evidence (Ferguson et al., 2000; Bjarnadottir et al., 2011). Yet the role that increased proteolysis plays is still ambiguous as structural proteins such as titin, nebulin, or desmin have not been shown to degrade faster following ES than in similarly aged animals not subject to ES (Ho et al., 1997).

Some other factors believed to play a role in response to ES are changes in phosphorylation rates for glycolytic enzymes (Li et al., 2011) and differences in ATPase activity (Swatland, 1981).

Calpain system

Calpains and calpastatin are members of a well-studied proteolytic system believed to play a role in postmortem tenderization. Calpains are calcium activated cysteine-proteases found in muscle tissue (Ohno et al., 1984) and are present and active in muscle during the aging process (Morgan et al., 1993). Calpastatin is an endogenous inhibitor of calpain-mediated proteolysis (Averna et al., 2003). This proteolytic system is responsible for ordered protein turnover in living cells (Huang and Forsberg, 1998)and the general breakdown of muscle structure postmortem (Geesink et al., 2006).

Calpains, particularly μ-calpain, have been shown to be responsible for some of the myofibrillar protein degradation seen in aged muscle (Koohmaraie et al., 1988; Taylor et al., 1995a; Geesink et al., 2006). Calpains remain the best understood of all the proteases believed to function in muscle degradation (including cathepsins and ubiquitin mediated proteolysis). Commercially available genetic tests to determine postmortem tenderization and breeding value are primarily based on μ-calpain and calpastatin alleles.

Quantitative trait loci (QTL) for calpains and calpastatin have been used in tenderness based selection programs (Van Eenennaam et al., 2007), but have only been shown to account for a small amount of variance in WBSF in a population of *Bos taurus* crossbred cattle (Gruber et al., 2011). Differences in tenderness between Brahman and

Angus calpain variants can be recognized by consumers in some cuts that have not been subject to ES (Robinson et al., 2012) and several SNP in calpastatin were linked to significant differences in tenderness in aged meat not subjected to electrical stimulation (NES) (Schenkel et al., 2006; Chung and Davis, 2012). Preliminary data indicate that calpain may be less significant in postmortem tenderization of electrically-stimulated carcasses than previously believed. Analysis of QTL for tenderness in ES and NES sides of carcasses indicated that a QTL on chromosome 29 corresponding to μ-calpain failed to rise to the level of significance for electrically stimulated sides that was significant without ES (Gill, pers. comm.). Decline in pH as experienced early postmortem can reduce the ability of calpains to function (Maddock et al., 2005), and calpains become inactive at pH 5.8, or roughly 48 h postmortem (Camou et al., 2007). Calpastatin levels are useful in predicting overall tenderness in some animals at 24 h postmortem, but after this time period calpastatin levels are ineffective at predicting tenderness (Whipple et al., 1990a). Additionally, μ-calpain is mostly degraded before most postmortem tenderization occurs (Boehm et al., 1998). These data indicate that the role of the calpain system in determining response to ES is limited to the first 48 h postmortem.

The use of ES can partially reduce the effects of elevated calpastatin levels in animals treated with beta-agonist growth factors. However even with ES and aging the meat is still tougher than NES controls, indicating that some other factor is involved in animals with altered muscle turnover rates that affects ultimate tenderness (Hope-Jones et al., 2010). It has been shown that calpain differences can only account significantly

for early (24h) proteolysis, and after only play a negligible role (Uytterhaegen et al., 1992).

A study by O'Connor et al. (1997) found that calpastatin activity 24 h post-slaughter in *B. indicus-B. taurus* crossed cattle had both low heritability (0.15) and correlated poorly with tenderness (0.24). This finding would seem to make calpastatin a poor target for any directed breeding program to improve tenderness, although post-rigor calpastatin levels were found to be consistently higher in *B.indicus* carcasses (Whipple et al., 1990b). The implication seems to be that some factor in the indicine breeds unrelated to the calpain system is responsible for differential response in tenderness to postmortem ES. Other studies have indicated that it may not be economically desirable to select for low calpastatin levels to improve tenderness; a correlation was found between high calpastatin and improved feed efficiency (~13% higher calpastatin expression in high efficiency animals than low efficiency animals; $P < 0.05$) in cattle selected for divergent feed efficiency (McDonagh et al., 2001; Pintos and Corva, 2011). Given the role the calpain system plays in overall efficiency any breeding scheme based on altering calpain/calpastatin levels may run the risk of increasing production costs.

Cellular turnover

Cell turnover, either through the activation of internal proteolytic enzymes or regulated cell death (apoptosis), is a factor in muscle cell breakdown (Elmore, 2007). In addition to the calpain system,Laville et al. (2009) found that protein fragments associated with cellular apoptosis were more prevalent in tender than tough carcasses.

This indicates a possible role for pre-mortem muscle turnover in tenderization that utilizes a pathway other than the calpain system.

The proteasome is a proteolytic system found in muscle that is involved in planned muscle turnover, indicated by its activation following denervation of muscle tissue (Kimura et al., 2009). Incubating muscle extracts with the 20S subunit of the proteasome results in degradation of the Z-disks as well as the breakdown of nebulin, myosin, actin, and tropomyosin in an energy independent reaction (Taylor et al., 1995b; Robert et al., 1999). Inhibition of the proteasome prevents degradation of actin, troponin-T, myosin, and nebulin even in the presence of calpains (Houbak et al., 2008); this in conjunction with other studies of proteolysis postmortem indicates a sequential process wherein calpains act initially to allow for other proteolytic systems to function to further degrade muscle proteins with which calpains cannot react.

Meat from animals treated with muscle growth promotants that reduce cellular turnover tend to be tougher than meat from animals with greater rates of myofibrillar turnover (Bohorov et al., 1987). The type of growth promotant used does not seem to matter so long as it functions by suppressing cell turnover rates (Barham et al., 2003). Animals undergoing rates of increased muscle cell proliferation did not result in tougher meat; only those animals undergoing suppressed muscle cell turnover displayed higher WBSF values (Koohmaraie et al., 2002). A reduction in muscle cell degradation led to significantly tougher meat but increased muscle cell proliferation did not which supports the notion that the overall muscle degradation rate prior to slaughter is a key factor in

tenderization. Calpastatin and calpain levels can account for some but not all of the differences in toughness postmortem.

Pathway analysis of gene clusters responsible for differences in toughness between Chinese Qinchuan bulls and cows produced several groups of genes that correlate to differences in tenderness in beef cattle between genders (Zhang et al., 2011). Those pathways are predominantly associated with cell adhesion, collagen fibril organization and synthesis, immune response and cell-matrix adhesion. Gene expression studies have led to the identification of a variety of genes involved in apoptosis, heat shock, and response to oxidative stress that are differentially expressed based on postmortem tenderization rates (Bernard et al., 2007; Jia et al., 2007; Guillemin et al., 2011). Apoptotic response is not uniform among muscle groups (Burniston et al., 2005a; Burniston et al., 2005b; Kocturk et al., 2008; McMillan and Quadrilatero, 2011), offering a possible explanation for differences in response to tenderness between muscle groups following ES and aging.

Various muscle structural proteins have been shown to be affected by cathepsins (Mikami et al., 1987) and caspases (Communal et al., 2002; Kemp et al., 2006; Huang et al., 2011). The fact that calpains cannot explain a large portion of variance in WBSF combined with the demonstrable role of a variety of other proteolytic systems in muscle degradation indicates that postmortem tenderization is a complex system involving a number of variables and different systems.

Integrins

Integrins are a diverse class of structural proteins. In muscle, integrins play a key role in focal adhesion by serving as a structural link between the muscle fiber and the extracellular matrix. This link is central to muscle growth and survival and provides an essential regulatory element that promotes differentiation and prevents apoptosis (Velleman, 1999). The typical integrin structure in muscle involves a *beta* 1 subunit bound to an *alpha* subunit. The *alpha* isoform determines the function of the integrin unit and confers signaling specificity for a variety of other substrates and hormones. Integrin expression is associated with muscle differentiation and growth. The alpha-5 subunit binds fibronectin and is associated with muscle cell proliferation whereas the alpha-6 subunit binds laminin and is associated with myocyte differentiation (Taverna et al., 1998; Mayer, 2003; Wilschut et al., 2010). Fibronectin is responsible for myocyte migratory behavior and promotes cell fusion (Vaz et al., 2012). The role of specific integrins seems to be non-redundant. For instance, the loss of integrin alpha-5 leads to gradual cell death and inability to bind fibronectin despite normal expression levels for other integrin isoforms (Disatnik and Rando, 1999). Induced over-expression of integrins leads to a greater resistance to apoptosis in muscle cells without altering overall gene expression (Liu et al., 2008). Integrin signaling is associated with load-induced muscle hypertrophy and remodeling (Carson and Wei, 2000; Goldmann, 2012).

Integrin expression has been positively correlated with collagen production (Terracio et al., 1991). Collagen is one factor often cited in “background” toughness of meat, with a positive correlation between WBSF and collagen content in cooked meat

(Nakamura et al., 2010) and raw meat (Nishimura, 2010). In particular, collagen turnover rates are a factor in meat quality. Artificial induction of high turnover rates of collagen have been tied to an improvement in meat tenderness (Archile-Contreras et al., 2011). This effect is likely due to the increased cross linkages in mature collagen compared to newly synthesized collagen that results in reduced solubility upon cooking (Bailey, 1985).

A number of environmental and treatment factors can influence the formation of new collagen and collagen maturity. This leads to altered WBSF including altered nutrition leading to compensatory growth, testosterone, growth promotants, exercise and overall health (Mccormick, 1994). Because collagen content and type are traits subject to manipulation, some have speculated about the possibility of intentionally altering handling or breeding schemes or both to minimize postmortem background toughness due to collagen insolubility (Purslow et al., 2012). Increased growth rates immediately prior to slaughter are also associated with an increase in endogenous collagenases leading to increased collagen degradation postmortem (Sylvestre et al., 2002). Compensatory growth has also been linked to greater overall rates of muscle turnover (Kristensen et al., 2002). Laminin and other ECM proteins were found to be upregulated in meat selected for tenderness in bulls (Bernard et al., 2007). Connective tissue and muscle cell turnover are 2 factors commonly accepted to play a role in forming background toughness is beef. The integrins, as key regulators of connective tissue and muscle cell turnover rates, are an interesting but poorly understood area of research in meat quality.

Additional factors

Several other factors have been hypothesized to play a role in tenderness. Differences in fiber type are one possible factor; slow twitch fiber type generally have reduced WBSF values compared to fast twitch muscle fibers (Choi et al., 2006). It is not clear whether this is due to greater post mortem proteolysis, increased lipid content, or a factor yet to be determined. Muscle fiber size has been linked to WBSF but only early postmortem; after 6 d of aging muscle fiber size is not a significant factor in WBSF (Crouse et al., 1991). Fiber orientation may also be a factor with the location of a cut within a muscle group altering tenderness. Derington et al. (2011) found that location within a muscle group made a difference in tenderness due simply to differences in grain pattern throughout the muscle. This observation held true even though fiber type ratio and treatment were the same. High temperatures pre-rigor can inhibit proteolysis by causing protein denaturation and ultimately lead to tougher meat (Kim et al., 2012). Rapid protein denaturation also leads to increased drip loss with the accompanying reduction in overall beef quality. For reasons that are not entirely clear, bovine respiratory disease leads to increased toughness (Garcia et al., 2010).

Feed efficiency

Strategies used to improve production efficiency are another factor that may affect overall tenderness. Feed efficiency can be measured in a variety of ways (Carstens and Tedeschi, 2006). Essentially efficiency here applies to some measure of output to input. Gain to feed ratio (G:F) measures the amount of total gain by weight of the animal

compared to feed required to achieve that gain. Average daily gain (ADG) is a measure of total weight gain over a set period of time. Residual feed intake (RFI) models daily intake based on ADG and metabolic mid-weight (Archer et al., 1997).

As was discussed previously, growth promotants that are used to increase growth and efficiency, can adversely affect tenderness. According toMcDonagh et al. (2001), selection for improved efficiency by traditional breeding methods with no artificial growth promotants can have an impact on tenderness as well. They found that a single generation of selection for improved efficiency was enough to significantly increase the levels of calpastatin found in postmortem meat and reduce total myofibrillar fragmentation (a common measure for postmortem proteolysis).

Identification of novel factors that are separate from the calpain-calpastatin system is important for potential manipulation to affect or improve over all tenderness. Identification of genetic factors that improve efficiency but do not contribute to tougher meat will be of great value by reducing environmental and input costs without sacrificing consumer acceptance.

The purpose of this study was to investigate genes that influence tenderness. It is our hypothesis that genetic differences underlie differences in post-ES tenderness in beef.

CHAPTER II

GENETIC FACTORS AFFECTING BOVINE FEED EFFICIENCY

Introduction

Feed efficiency is a major concern for beef producers. Feed costs can constitute upwards of 70% of total livestock production costs (Becker, 2008). Grain prices are rising and they are predicted to continue increasing for the foreseeable future (USDA, 2010b). In the USA, most beef cattle are finished on grain to rapidly achieve sufficient weight and fat thickness prior to slaughter (USDA, 2010a). The predicted upward trend in grain prices means that production costs will necessarily continue to rise for the near future.

The environmental costs associated with beef production have become an increasingly important concern for the consumer. The beef industry has made substantial strides in reducing the environmental footprint of cattle production (Capper, 2011). However, with a growing population and increased consumer interest in environmental issues (Ilea, 2009), continued improvement in production efficiency with simultaneous reduction in emissions is desirable. A reduction in feed inputs is one possible method to reduce emissions caused by grain production and reduce the overall environmental impact of beef production.

The objective of this study was to identify genes responsible for improved feed efficiency in cattle. Our hypothesis is that after accounting for environmental variables there are genetic factors that influence feed efficiency in beef cattle.

Materials and methods

Herd structure

The cattle used in this study were produced in McGregor, Texas, from 2003 to 2007. The herd is a crossbred Nellore-Angus population, described by Sanders (2008). Specific animals used in this study were steers of the F_2 generation and consisted of 50% Angus and 50% Nellore background produced via embryo transfer. After weaning, the animals were grass fed for approximately 130 d. Cattle were fed a grain based diet in Calan gates beginning at an average of 11 to 13 mo of age, and individual feed intake was measured. Feeding was *ad libitum* and uneaten food was removed and measured every 7 d. Animals were weighed every 28 d.

All procedures were approved by the Texas A&M Institutional Animal Care and Use Committee (AUP #2008-234).

Sample selection

Animals were assigned to groups based on residual feed intake ($RFI_{(NRC)}$) as a measure of efficiency (Amen, 2009). Predicted daily feed intake was based on observed weight gain using standardized inputs from the NRC (2000) beef cattle model for animal type, age, sex, condition, and breed. This approach was used to enable comparisons among and within families raised in different contemporary groups. The difference between observed dry matter intake (DMI) and expected DMI is $RFI_{(NRC)}$. A positive $RFI_{(NRC)}$ indicates an animal consumed more than expected and is considered inefficient.

A negative $RFI_{(NRC)}$ indicates an animal consumed less than expected and is considered efficient.

For initial microarray study, 24 animals were selected for extremes of efficiency based on residuals of $RFI_{(NRC)}$ from a general linear model correcting for fixed effects of sire and family nested within sire, which were factors not previously accounted for with the NRC (2000) model. A total of 12 animals were classified as "efficient" negative $RFI_{(NRC)}$ residuals. A total of 12 animals were classified as "inefficient" with positive $RFI_{(NRC)}$ residuals. An additional 6 samples were added to each group for subsequent quantitative reverse-transcriptase (qRT-PCR) analysis bringing the total to 18 inefficient and 18 efficient. A statistically average group of 18 animals was also included for comparison purposes for a total of 54. Feed efficiency data for these groups are presented in Table 1.

Table 1. Simple means (+std err) $RFI_{(NRC)}$ residuals by efficiency groups

	Efficient	Average	Inefficient
n	18	18	18
$RFI_{(NRC)}$ residuals	-2.3 $\pm$ 0.1 [a]	0 $\pm$ 0.0 [b]	2.3 $\pm$ 0.1 [c]

[a,b,c] Means within a row with different superscripts differ significantly ($P < 0.01$).

Tissue collection

Approximately 1 g of muscle was collected shortly after death and prior to ES (less than 1 hr post-exsanguination) from the *Longissimus cervicis* in the neck region.

The sample was flash frozen in liquid nitrogen to prevent mRNA degradation. Samples were stored at -80°C prior to RNA extraction.

RNA extraction

RNA was extracted from approximately 100 to 200 mg of whole muscle tissue with TRI Reagent® (Molecular Research Center, Cincinnati, OH) and 1-bromo-3-chloropropane (Molecular Research Center). RNA was precipitated with isopropanol (Sigma Aldrich, St. Louis, MO), washed with 70% ethanol (Sigma Aldrich), and reconstituted in 50 µL nuclease-free water (Invitrogen, Carlsbad, CA). RNA was tested for quality on an Agilent 2100 series Bioanalyzer (Agilent Technologies, Palo Alto, Calif.) according to the manufacturer's protocol. Samples with an RNA integrity number (RIN) $\geq$ 8.0 and appropriate electropherogram image were treated with DNase (Invitrogen) and column-purified via RNeasy Mini kit (Qiagen, Valencia, CA). RNA extracts were stored at -80°C prior to microarray labeling.

Microarray

Twenty-four samples representing animals at the extreme tails of the efficiency distribution were labeled for microarray analysis using a two-color microarray-based gene expression analysis (Quick Amp Labeling Kit, Agilent Technologies) according to the manufacturer's protocol. RNA up to 8.3 µL was incubated at 40°C for 2 h, then 65°C for 15 min, then on ice for 5 min with the following reagents: 2.0 µL Spike A or Spike B mix, 1.2 µL T7 promoter, 4.0 µL 5X first strand buffer, 2.0 µL 0.1 M DTT, 1.0 µL

MMLV-RT, 0.5 µL RNaseOut for a total volume of 20.0 µL. Following this a master mix of 15.3 µL nuclease free water, 20.0 µL 4X transcription buffer, 6.0 µL 0.1 M DTT, 8.0 µL NTP mix, 6.4 µL 50% PEG, 0.5 µL RNaseOUT, 0.6 µL inorganic pyrophosphatase, 0.8 µL T7 RNA polymerase, and 2.4 µL cyanine 3-CTP or cyanin 5-CTP was added and incubated at 40°C for 2 h. Labeled cRNA was hybridized to bovine 4X44K stock arrays (Agilent array #015344). Fragmentation was performed by combining 825 ng cRNA, 11 µL 10X blocking agent, 2.2 µL 25X fragmentation buffer in a total volume of 55 µL per sample and heating 30 min at 60°C. To end fragmentation a total of 55 µL GEx Hybridization buffer was added for a total volume of 110 µL per sample. A total of 100 µL labeled sample was applied to each microarray and were hybridized in at 65°C for 17 h in a hybridization chamber. Arrays were washed and scanned immediately after this time.

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PCR

Synthesis of cDNA was accomplished with the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc.) with a starting quantity of 2 µg mRNA per 40 µL reaction. Oligo$(dT)_{20}$ primers (Integrated DNA Technologies, Coralville, IA) were used for reverse transcription. Samples were amplified in a total volume of 20 µL using the SYBR GreenER™ qPCR SuperMix (Invitrogen). Expression was normalized to *RPS20* as a reference gene (de Jonge et al., 2007). Realtime qRT-PCR was performed at 95°C for 10 min. followed by 40 cycles of 15 s at 95°C and 60 s at 60°C, in a 7900HT thermal cycler (Applied Biosystems, Inc.).

From the microarray results *ACTN3* was selected for validation by qRT-PCR. Primers were designed for *ACTN3* using Oligo 6 Primer Analysis Software v6.71 (Molecular Biology Insights, Cascade, CO). Additional primers pairs used were *ACTN2*, *RPS20*, *COX7C*, *COX3*, *ADFP*, *ATP5B*, *HKII* and *LDHB*. Genes and primers used are described in Table 2. The *ACTN2*gene was selected for its complimentary role to *ACTN3*. The *RPS20, COX7C*, and *COX3*genes were selected as possible control genes based on the literature. And *ADFP*, *ATP5B*, *HKII* and *LDHB* were all selected as genes that played a role in muscle metabolism.

Primer pairs were evaluated by BLAST sequence similarity search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Primer pairs were selected that did not cross-amplify across species or between different mRNA transcripts. Additionally, primers were selected to lie across an exon junction to prevent genomic amplification.

Table 2. Complete list of primer pairs used for qRT-PCR assays and their function in muscle

Gene Symbol	Description	Sequence
ACTN2	actinin, alpha 2	GGTCTTTGACAACAAGCA
		TGATGGTTCTGGCGATA
ACTN3	actinin, alpha 3	CGGGAGACAAGAACTACATCA
		CGTAGAGGGCACTGGAGAA
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	CCCATCAAAACCAAGCAA
		TCAACACTCATCTCCACGAA
HKII	hexokinase 2	TCAACACTCATCTCCACGAA
		CACCACAGCAACCACATC
LDHB	lactate dehydrogenase B	CAGAAATGGGAACAGACAA
		GACTTCATAGGCACTCTCAAC
COX3	mitochondrially encoded cytochrome c oxidase III	CCACCACTTCGGCTTTGAAG
		GGAAAAGTCAGACTACGTCTACGAAA
COX7C	Cytochrome c oxidase subunit VIIa	TGCAGCCGCCATTTCTTC
		TAGCGCTGTTGGACGCTCTA
RPS20	ribosomal protein S20	ACCAGCCGCAACGTGAA
		CCTTCGCGCCTCTGATCA

COX3, *COX7C*, and *RPS20* primer sequences provided by (Kochan, 2009).

Muscle fiber type classification

Fiber type analysis as determined by gel electrophoresis was conducted in the laboratory of Dr. Min Du, at the Department of Animal Science, University of Wyoming, Laramie, WY. Longissimus muscle samples (0.1 g) were homogenized in 500 µl of buffer containing 250 mM sucrose, 100 mM KCl, 5 mM EDTA and 20 mM Tris-HCl pH 6.8. The homogenate was filtered through nylon cloth to remove debris and centrifuged at 10,000 x g for 10 min. The pellet obtained was re-suspended in a 500 µL of washing buffer containing 200 mM KCl, 5 mM EDTA, 0.5% Triton X-100 and 20

mM Tris-HCl pH 6.8. The suspension was centrifuged at 10,000 x g for 10 min. The pellet containing purified myofibrillar proteins was re-suspended in 200 μL water and 300 μL of standard 2 x sample loading buffer and then boiled for 5 min. After centrifugation at 12,000 x g for 5 min, the supernate was used for electrophoresis.

The stacking gels consisted of 4% acrylamide (acrylamide: bis = 50:1) and 5% (v/v) glycerol, 70 mM Tris-HCl pH 6.7, 0.4% (w/v) SDS, 4 mM EDTA, 0.1% (w/v) APS and 0.01% (v/v) TEMED. The separation gel contained 5% (w/v) glycerol, acrylamide: bis (50:1) at a concentration ranging from 5 to 20%, 200 mM Tris pH 8.8, 4 mM EDTA, 0.4% (w/v) SDS, 0.01% (v/v) TEMED and 0.1% (w/v) ammonium persulfate. The upper running buffer consisted of 0.1 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 150 mM glycine, and 10 mM mercaptoethanol, and the lower running buffer consisted of 50 mM Tris-HCl pH 8.8, 0.01% (w/v) SDS and 75 mM glycine. Gels were run at 8°C in a Hoefer SE 600 (Hoefer Scientific, San Francisco, CA) unit, at constant 200 V for 24 h (Bamman et al., 1999). After electrophoresis, gels were stained with Coomassie blue and scanned with a densitometer to determine the amount of each myosin isoform and percentage of Type I and Type IIx muscle fibers was reported (Underwood et al., 2007).

Haplotype analysis

All individuals (n = 776) in the first three generations of the McGregor Genomics population were genotyped with the Illumina BovSNP50 v1 assay. After filtering genotypes to remove animals with poor completion rate (< 0.9), SNP with low minor

allele frequency (< 0.05), poor completion rate (< 0.9), and those SNP that deviated from Hardy-Weinberg equilibrium ($P < 0.0001$), 39,890 genotypes per individual remained. To determine whether breed and parent of origin played a role in the efficiency phenotype, SNP genotypes spanning 1 Mb intervals flanking several genes of interest (Table 3) based on expression analyses were extracted using PLINK v1.07 (Purcell et al., 2007) and formatted for phase v2.1.1 software (Stephens et al., 2001). Haplotypes were established using 100 iterations of phase v2.1.1, with a thinning interval of 2 and a burn-in of 10. Resultant phased haplotypes were ordered by generation, and breed (Nellore or Angus) and parent of origin were manually tracked through the pedigree to assign the source of each haplotype block (hapblock) in the F_2 generation. The *CAPN1, CAPN2, CAPN3,* and *CAST* genes were selected for their role in muscle turnover. And the *FN1*, *MYH1*, and *MYH2*were selected for their role in muscle growth and maintenance.

Table 3. SNP hapblock location information

Gene	Chromosome	Location (UMD 3.1)		Number of SNP in 1 Mb region
CAPN1	29	44063463	44113492	24
CAPN2	16	27781671	27840011	24
CAPN3	10	37829007	37885645	24
CAST	7	98444979	98581253	18
ACTN2	28	9403203	9450920	16
ACTN3	29	45230630	45242406	16
MYH1/2	19	30110728	30165109	18
FN1	2	103881402	103950562	12

Statistical analysis

SPSS 16.0 software was used for all statistical analyses. To test for significance between efficiency groups, an independent samples t-test was used. Correlation analysis used a bivariate two-tailed Pearson's correlation. All qRT-PCR expression was normalized to the *RPS20* reference gene (de Jonge et al., 2007). Expression data are reported relative to the efficient group. Relative expression quantity for the efficient group is therefore equal to 1.0; the inefficient and average groups are represented as fold change relative to the efficient group. One sample, 8141, was removed from the efficient group due to the inability to determine expression reliably as a result of aberrant *RPS20* control values. It was removed from all further analyses. Unless otherwise noted averages reported are simple means.

Results

Microarray analysis yielded 58 genes that were expressed significantly different between efficiency groups and had a fold difference between groups of 1.5 fold or greater. The *ACTN3* gene was expressed 2.5 fold higher in the inefficient group than the efficient group but fell just short of significance ($P = 0.051$). However, due to its noted role in muscle efficiency in humans it was included for qRT-PCR verification. The full gene list can be found in the appendix.

Both sire and family nested within sire were found to be significant factors in the linear model ($P = 0.0356$ and $P < 0.001$, respectively) (Amen, 2007).

Following microarray analysis, several genes were selected for qRT-PCR analysis (Table 2). Expression of *ACTN3* was 1.6 fold greater ($P = 0.009$) in the average and inefficient groups than the efficient group (Table 4). Of the other genes assayed LDHB expression was significantly lower in the average group but not inefficient group. The remaining genes were not different between groups.

Table 4. Relative mRNA expression of selected genes by qRT-PCR analysis

	Classification	
Item	Average/Efficient	Inefficient/Efficient
N	18	18
ACTN3	1.6 ± 0.06[a]	1.6 ± 0.05[a]
ACTN2	1.2 ± 0.05	1.0 ± 0.05
COX3	1.7 ± 0.05[a]	1.4 ± 0.06[b]
COX7C	1.1 ± 0.03	0.9 ± 0.04
HKII	0.9 ± 0.04	0.8 ± 0.05
LDHB	0.6 ± 0.03[a]	0.9 ± 0.04[b]
PRDX3	0.9 ± 0.03	1.0 ± 0.04

Relative expression for the average and inefficient groups is calculated and presented as a fold-ratio compared to the efficient group. Expression was normalized to *RPS20*.
[a,b] Means with subscripts differ from efficient group $P < 0.05$. Means with different subscripts differ from each other $P < 0.05$.

Initially *COX3* and COX7 included as a potential reference gene for normalization. Due to varying expression levels between groups *COX3* was not used in that role. Instead, *RPS20* was used to normalize due to a high degree of expression stability between groups.

Post-transcriptional modifications and other regulatory mechanisms can make it difficult to directly correlate mRNA expression to protein expression (Greenbaum et al., 2002). To verify that the observed difference in *ACTN3* gene expression translated to actual differences in muscle protein expression a subset of 12 samples from each tail of the distribution (n = 24) was assayed for fiber type ratios based on gel separation of muscle fiber type specific isoforms. The ratio of fast to slow twitch muscle fiber (Type IIx/Type I) was 1.8 fold higher ($P = 0.027$) in the inefficient group compared to the efficient group (Figure 1).

ACTN2 expression did not vary significantly between any of the groups, nor did it correlate significantly with *ACTN3* expression or fiber type ratio. Hapblock analysis was conducted on *ACTN2*, *ACTN3*, *CAPN1*, *CAPN2*, *CAPN3*, *CAST*, *FN1*, and *MYH1*/2. Three of the hapblocks were associated with significant differences in efficiency, as measured by $RFI_{(NRC)}$ residuals, between the Angus and Nellore hapblocks in the larger population studied. The *CAPN2* and *ACTN3* Nellore hapblocks were associated with a superior efficiency when inherited maternally ($P = 0.03$). The *FN1* Angus hapblock was associated with a superior efficiency when inherited maternally ($P = 0.04$).

Neither the *CAST* nor the *CAPN1* haplotypes were associated with any improvement in efficiency. These data are presented in Table 5.

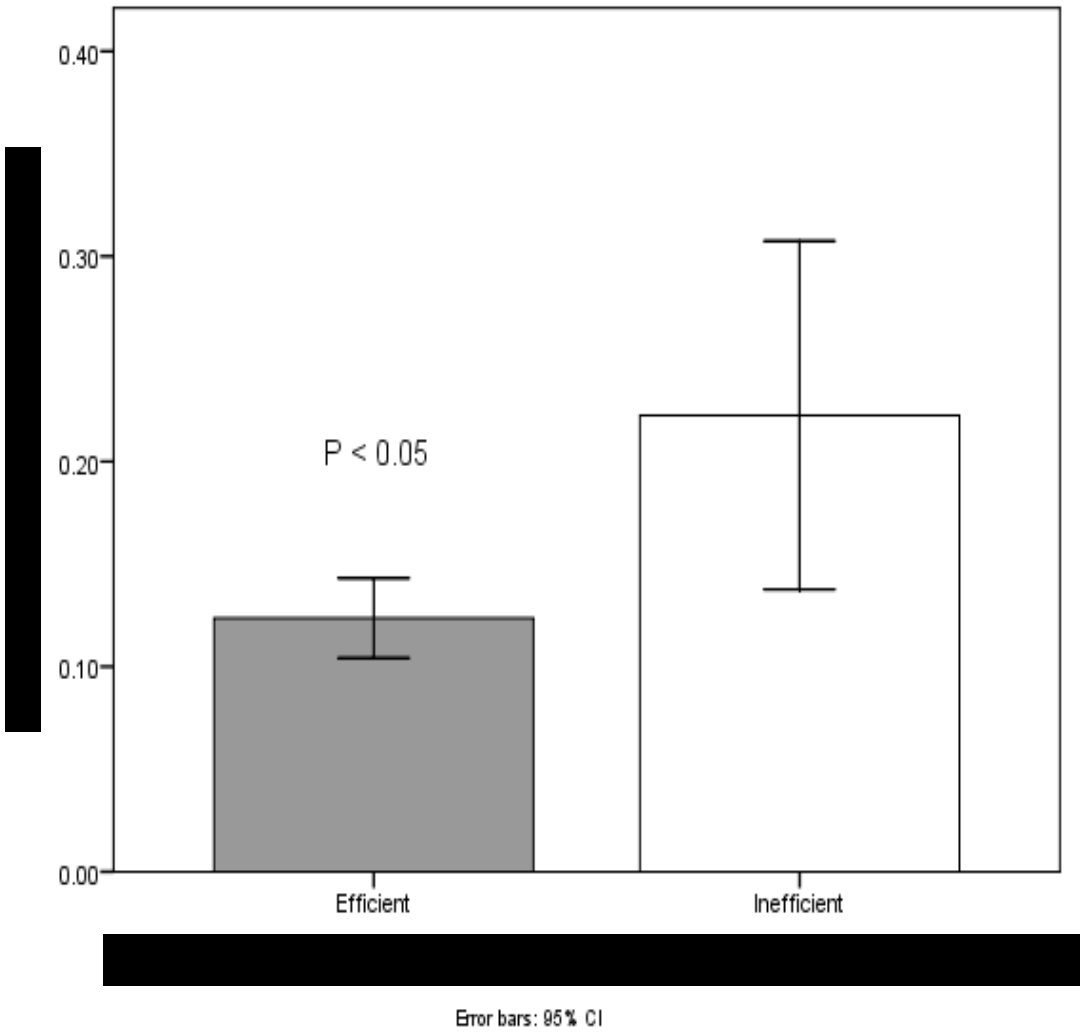

Figure 1. Proportion of fast to slow twitch (Type IIx/Type I) Fiber ratio was correlated with *ACTN3* expression (r = 0.622, $P < 0.001$).

Table 5. Efficiency statistics based on hapblock analysis

		Hapblock												Paternal							Maternal						
		NN			NA			AN			AA			NN/NA			AN/AA			*P*-value	NN/AN			NA/AA			*P*-value
ACTN3	n	41			49			40			44			90			84				81			93			
	eff.	-0.7	±	0.5	0.5	±	0.4	-0.3	±	0.4	0.5	±	0.4	0.0	±	0.3	0.1	±	0.3	0.74	-0.5	±	0.3	0.5	±	0.3	**0.03**
CAST	n	46			43			42			43			89			85				88			86			
	eff.	0.5	±	0.4	-0.8	±	0.4	0.2	±	0.3	0.2	±	0.5	-0.1	±	0.3	0.2	±	0.3	0.44	0.4	±	0.3	-0.3	±	0.3	0.13
CAPN1	n	23			39			37			75			62			112				60			114			
	eff.	-0.6	±	0.6	0.4	±	0.4	-0.3	±	0.4	0.2	±	0.4	0.0	±	0.3	0.0	±	0.3	0.98	-0.4	±	0.3	0.3	±	0.3	0.15
CAPN2	n	41			52			40			41			93			81				81			93			
	eff.	-0.7	±	0.5	0.5	±	0.4	-0.3	±	0.4	0.4	±	0.5	0.0	±	0.3	0.1	±	0.3	0.80	-0.5	±	0.3	0.5	±	0.3	**0.03**
FN1	n	59			52			32			31			111			63				91			83			
	eff.	0.4	±	0.3	-0.5	±	0.5	0.6	±	0.5	-0.4	±	0.5	0.0	±	0.3	0.1	±	0.3	0.81	0.5	±	0.3	-0.4	±	0.3	**0.04**

Season was one factor that could have had an impact on efficiency. A general linear model was used to produce least square means using $RFI_{(NRC)}$ residuals as the dependent variable with birth year season (BYS) as the fixed factor. There were 6 BYS groups in this study: F03 (n = 25), F04 (n = 32), F05 (n = 29), S03 (n = 22), S04 (n = 32), and S05 (n = 34), with a total of 86 animals weaned in the fall and 88 weaned in the spring. Steers weaned in the spring were more efficient than those weaned in the fall; linear contrast of fall $RFI_{(NRC)}$ residuals means minus spring $RFI_{(NRC)}$ residuals means was 1.09 $\pm$ 0.15 ($P < 0.0001$). There was one BYS, S05, which was more efficient than the other BYS groups ($P < 0.0001$). Within the subset selected for expression analysis the 15 of the 18 samples in the efficient group were from the S05 BYS (Table 6).

Table 6. Birth year season (BYS) distribution among efficiency groups

Group	n	F03	F04	F05	S03	S04	S05
Efficient	18	0	1	0	1	1	15
Average	18	3	4	4	1	4	2
Inefficient	17	5	4	6	1	1	0

Expression of *ACTN3* was significantly different between animals grouped by weaning season. *ACTN3* expression was higher in fall weaned animals than spring weaned animals by 2.1-fold ($P < 0.001$). No significant differences were noted in any

other genes assayed. Additionally there was a difference *(P* < 0.001) in fiber type by season of weaning. Those weaned in the fall (n =11) had a fast to slow twitch (Type IIx/Type I) fiber type ratio 1.6-fold greater than those weaned in the spring (n=13). These ratios were 0.23 and 0.14, respectively.

Discussion

By qRT-PCR analysis, it was confirmed that differences in *ACTN3* expression existed between efficiency groups (*P* = 0.009). Expression of *ACTN2* did not differ significantly between groups. Relative to the efficient group, the inefficient group overexpressed *ACTN3* by 1.6-fold. Fiber type ratio measured by Fast (Type IIx) / Slow (Type I) differed between groups (*P* = 0.027), with a 1.8 fold increase in the inefficient group relative to the efficient group. The inefficient group had a large standard error relative to the efficient group. A possible explanation for this would be that an animal could be inefficient due to a wide array of factors; fiber type ratios being just one variable among many that could possibly reduce overall efficiency.

In the total population (n = 174), from which a subset was used for gene expression assays, parent and breed of origin of the *ACTN3* hapblock had a significant impact on efficiency as measured by $RFI_{(NRC)}$ residuals. The Nellore hapblock was associated with greater efficiency when inherited maternally (-0.47 compared to 0.40, *P* = 0.05) regardless of the breed of origin of the paternal hapblock.

Season played a significant role in both feed efficiency, (with those animals weaned in the fall being more efficient than those weaned in the spring), *ACTN3*

expression (greater expression in animals weaned in the fall), and fiber type ratios (greater in those weaned in the fall).

Due to the very different methods of energy utilization between fast and slow twitch muscle fibers, it is possible that a shift in one direction can lead to overall differences in the utilization of energy during the lifespan of the animal. A reduction in *ACTN3* can lead to differences in glycogen phosphorylase activity in muscle as well as changes in calcium metabolism (MacArthur et al., 2007; Quinlan et al., 2009; Quinlan et al., 2010). Mice entirely deficient in *ACTN3* show an increase in expression of enzymes relating to the glycolytic pathway and a decrease in expression of enzymes of the aerobic cellular respiration pathway (MacArthur et al., 2008). Aerobic cellular respiration in total produces 38 molecules of ATP per molecule of glucose input compared to only 2 molecules of ATP produced by glycolysis per molecule of glucose, making an aerobic metabolism 19 times more efficient than glycolysis. Therefore any shift towards one over the other will have an effect on overall efficiency in energy metabolism.

Subsequent studies are necessary to further examine the interaction of genotype and environment and to verify if this trend holds up in other breeds and environments. Season has been shown to alter feeding behaviors and efficiency in cattle (Mujibi et al., 2010; Durunna et al., 2011). To our knowledge the impact of season on fiber type has not been studied in cattle, however in camels it was found that season has an effect on fiber type ratios, a season based shift in fast (type IIx) / slow (type I) ratios was observed with higher greater fast twitch muscle fiber expression in the fall months than the summer (Abdelhadi et al., 2012), which were similar to results in this study.

A relationship between *ACTN3* expression levels and metabolic rates has been reported in mice and humans (North and Beggs, 1996; North et al., 2003; Yang et al., 2003; Niemi and Majamaa, 2005; Macarthur et al., 2006; MacArthur et al., 2007; Moran et al., 2007; MacArthur et al., 2008; Papadimitriou et al., 2008; Roth et al., 2008; Ogura et al., 2009; Ahmetov et al., 2010; Fiuza-Luces et al., 2011). Cattle facing insufficient nutrition will lose fast twitch muscle fibers to preserve slow twitch muscle fibers (Lehnert et al., 2006), suggesting a preference for higher efficiency muscle under periods of nutritional stress. To our knowledge, however, a role for *ACTN3* expression in influencing bovine metabolic efficiency has not be previously reported. We believe *ACTN3* represents a novel gene target for selection to improve efficiency in cattle.

CHAPTER III

TENDERNESS FOLLOWING ELECTRICAL STIMULATION

Introduction

Tenderness in beef has been identified as the main factor affecting consumer palatability ratings (Ramsey et al., 1963; Whipple et al., 1990b; Morgan et al., 1991; Chambers and Bowers, 1993). In consumer sensory panel studies, tenderness accounts for more than 50% of the total value placed on beef by consumers (Miller et al., 2001). Consumers have expressed a willingness to pay a premium for reliably labeled tender beef (Boleman et al., 1997; Lusk et al., 2001). This preference makes tenderness a factor of interest for producers and consumers alike.

Applying an electrical current to the carcass postmortem is one commonly employed commercial method for improving end-product tenderness (Savell et al., 1977a). Electrical stimulation (ES) has been shown to reduce, but not eliminate variance between carcasses particularly for carcasses that would otherwise be less tender (Savell et al., 1981). Tenderness is moderately to highly heritable in cattle (O'Connor et al., 1997), indicating the possibility of using selection to improve this trait. A better understanding of the genetic and biochemical factors affecting the improvement in tenderness (response) following ES may enable breeders to improve the design of breeding programs, production, and marketing strategies. This would provide benefits to both producers and consumers. The objective of this study is to explore the genetic

factors that affect tenderization in beef following postmortem ES. It is our hypothesis that genetic differences underlie differences in post-ES tenderness in beef.

Materials and methods

Herd structure

All samples used in this project came from a herd established in McGregor, TX in 2003 as part of the McGregor Genomics project. The McGregor Genomics project was established to identify genetic factors that contribute to beef cattle productivity. This project enables QTL analyses for economically important traits in cattle including feed efficiency, carcass and meat traits, and behavior and female reproductive traits (Gill, 2004). The herd consists of 13 full-sibling F_2 embryos transfer families derived over a 5 year time period from 13 Nellore-Angus F_1 females and 4 Nellore-Angus F_1 males (Sanders, 2008). Progeny were created by a mixture of natural service and embryo transfer. Only steers in the F_2 population produced by embryo-transfer were used in this study.

Steers were fed as yearlings and harvested at approximately 18 mo of age at the Texas A&M University Rosenthal Meat Science and Technology Center in College Station, TX. All carcasses were split laterally along the spine during processing. The right side was subjected to electrical stimulation, and as a control the left side was not electrically stimulated. Muscle samples were taken from between the 12^{th} and 13^{th} ribs, as this is the location for USDA grade assessment. Warner-Bratzler shear force and

sensory panel evaluation were performed in the Kleberg Center (Metteauer, 2009). All procedures involving animals were approved by the Texas A&M Institutional Animal Care and Use Committee (AUP #2008-234).

Sample selection

The samples used in this study were obtained from F_2 Nellore-Angus steers produced by embryo transfer. Females were kept for breeding and thus were excluded from any meat quality studies. This would have been done regardless as gender is already an established variable that affects tenderness (Baird, 2007). All animals used in this study were produced by embryo transfer (ET). Shortly postmortem (within 60 min after captive bolt stunning), muscle samples from the *Sternomandibularis* muscle were collected and flash frozen in liquid nitrogen to preserve RNA integrity. Samples were transferred to a -80°C freezer for long term storage. All muscle samples used in this experiment were collected prior to ES. It is unknown what effect ES has on mRNA and protein expression in the short term postmortem. This means that every animal has two recorded shear force values, one for the half that was not subjected to electrical stimulation (NES) and the other for the half that was subjected to electrical stimulation (ES). For every animal there is only one muscle sample collected prior to ES.

To further reduce the total number of variables, a statistical model was created to address factors known to affect tenderness. A mixed-model in SPSS v16.0 was utilized to produce this model. Fixed class effects were: calving group (year and season) and slaughter date. Random class effects were: sire and family nested within sire. Total age

at time of slaughter, measured in days, was factored in as a linear covariate. This same process was performed separately for both ES and NES WBSF values. Residual differences were used to assign tenderness groups. Those with positive residuals (actual WBSF greater than model predicted) were designated as "high" and those with negative residuals (actual WBSF lower than model predicted) were deemed "low."

Twelve extremes of tenderness from each tail of the distribution were selected using ES residuals, those that were tougher than predicted ("high") and those that were more tender than predicted ("low"). This produced a total of 24 samples for the ES residual group. Additionally, 12 extremes were selected using the NES residual values at either end. A total of 24 unique samples were selected from the NES group. In this way, a total of 48 samples were selected for microarray analysis.

It should be noted again that samples were all collected prior to electrical stimulation for all animals. The ES grouping is therefore based on WBSF measurements made following ES. The muscle sample used for gene and protein expression assays, however, was not subject to ES. This was necessary to reduce variability due to currently unknown effects ES would have on muscle gene and protein expression. One sample, 7203, was absent from the muscle sample stocks. It was removed from further analysis.

Tissue collection

Approximately 1 g muscle was collected shortly postmortem prior to ES (less than 1 h post-exsanguination) from the *Sternomandibularis*. The sample was flash frozen in liquid nitrogen to prevent mRNA degradation. Samples were stored at -80°C.

RNA extraction

RNA was extracted from approximately 100 to 200 mg of whole muscle tissue with TRI Reagent® (Molecular Research Center, Cincinnati, OH) and 1-bromo-3-chloropropane (Molecular Research Center). RNA was precipitated with isopropanol (Sigma Aldrich, St. Louis, MO), washed with 70% ethanol (Sigma Aldrich), and reconstituted in 50 µL nuclease-free water (Invitrogen, Carlsbad, CA). RNA was tested for quality on an Agilent 2100 series Bioanalyzer (Agilent Technologies, Palo Alto, Calif.) according to the manufacturer's protocol. Samples with an RNA integrity number (RIN) $\geq$ 8.0 were treated with DNase (Invitrogen) and column-purified via RNeasy Mini kit (Qiagen, Valencia, CA). Samples were stored at -80C until use.

Microarray

An initial microarray study was used to identify candidate genes for follow up study. Samples were selected based on ES residuals for 24 (12 high and 12 low) animals and on NES residuals for 24 animals (12 high and 12 low) for a total of 48 samples.

A single color 44K bovine array (*B. taurus* (Bovine) Oligo Microarray v2 Agilent 4X44K GPL11649) was used according to manufacturer's instructions. The

single color array was chosen as natural variation in gene expression would have made pairing samples on a 2 color array impractical. Spike-in procedure was performed in the same manner as described in Chapter II with the exception that only Spike A mix was used here. Following this a master mix of 15.3 µL nuclease free water, 20.0 µL 4X transcription buffer, 6.0 µL 0.1 M DTT, 8.0 µL NTP mix, 6.4 µL 50% PEG, 0.5 µL RNaseOUT, 0.6 µL inorganic pyrophosphatase, 0.8 µL T7 RNA polymerase, and 2.4 µL cyanine 3-CTP or cyanine 5-CTP was added and incubated at 40°C for 2 h. Labeled cRNA was hybridized to bovine 4X44K stock arrays (Agilent array #015344). Fragmentation was performed by combining 825 ng cRNA, 11 µL 10X blocking agent, 2.2 µL 25X fragmentation buffer in a total volume of 55 µL per sample and heating 30 min at 60°C. To end fragmentation a total of 55 µL GEx Hybridization buffer was added for a total volume of 110 µL per sample. A total of 100 µL labeled sample was applied to each microarray and were hybridized in at 65°C for 17 h in a hybridization chamber. Arrays were washed and scanned immediately after this time. Normalization and quality control were performed using embedded functions in the Genespring GX v11.0.2 software.

Quantile normalization was used to reduce variability between arrays. Array data were subjected to baseline normalization to the median expression level and had a minimum threshold of 1.0 expression all using standard quality control options built in to the Genespring software. Quality control on arrays was performed via correlation plots. If expression between an array and all other arrays within a population dropped below a correlation of 0.9 it was deemed unreliable. Five arrays were found to be

outliers using this method (7151, 7227, 7606, 7732, and 8148). These samples were removed from further consideration in this project. In order to maintain a sufficient population size for statistical analysis groups were recalculated at this point to ensure that the ES group had a total of 12 extremes at each end.

Importantly, reassignment of animals was performed before any statistical analyses were performed. Adjusting labels to account for removed samples did not result in any significant change to population metrics (Table 7).

Table 7. Original and revised population statistics for samples used in microarray analysis

	Group (ES)	N	WBSF (ES)[a]	Predicted WBSF (ES)[b]	Residuals (ES)[c]
Revised	High	12	4.14	2.88	1.1
	Low	12	2.11	2.79	-0.79
Initial	High	12	4.14	2.83	1.06
	Low	12	2.11	2.82	-0.71

[a] WBSF (ES) = observed Warner-Bratzler shear force on carcass half subjected to electrical stimulation (ES).
[b] Predicted WBSF (ES) = predicted Warner-Bratzler shear force on carcass half subjected to ES based on model predictions.
[c] Residuals (ES) = difference between observed ES and predicted ES shear force values.

All further analysis is based on these 42 samples (5 removed as outliers based on microarray results and 1 where the sample was missing from the stocks).

Probes were filtered based on flags. Flags for "population outlier", "saturated", "not positive or significant", "not uniform", and "not above background" were assigned automatically by Agilent Extraction Software v9.5. A further explanation of the calculations used to assign these flags is available in the Agilent Extraction Software v9.5 manual, downloaded from the following uniform resource locator (URL): http://www.chem.agilent.com/Library/usermanuals/Public/ReferenceGuide_050416.pdf.

For this experiment all flags except "not uniform" and "not above background" were assigned the label "marginal". Probes flagged as "not uniform" or "not above background" were deemed failed or aberrant and were labeled "absent" and were not used further. A probe without flags was given the default label "present". As a cut off, 8 out of 24 probes for any given point must have a value of "present" or "marginal" to be considered for statistical analysis.

The Mann-Whitney unpaired test was used for both ES and NON in separate analyses. A False Discovery Rate (FDR) was not used as these were these samples failed the assumption of independence. A non-parametric test was used to avoid relying on the *a priori* assumption of a normal distribution of gene expression within this population selected for extremes. A cut off of $P \leq 0.05$ and fold change 1.4 fold or higher between groups was employed as the thresholds to generate lists of probes for subsequent pathways analysis.

A total of 43,713 unique probes were on each array. Of these 32,900 passed the quality control for acceptable levels of present or marginal probes. Filtering by significance and fold change reduced this to 1,046 unique probes. Of these, 867 had

usable identifiers (gene annotations that could be recognized by the DAVID software). This is presented in Table 8.

Table 8. Numbers of unique probes at various stages of the microarray analysis

	Total Number of Probes	% of Total
Total Probes on array	43,713	100.00
Passed quality control[a]	32,900	75.26
1.4 Fold expression difference[b]	1,046	2.45
Usable identifiers	867	1.98

[a] Quality control based on flags
[b] $P \leq 0.05$.

Pathway analysis

A pathway analysis was performed using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/). A total of 1,071 genes were differentially expressed from the microarray at a significant level. Of those, 867 were associated with known genes. DAVID software was able to reliably identify 752 of those gene symbols in the bovine specific pathway set. A minimum count of 2 and cutoff of $P \leq 0.1$ was used to determine if a KEGG pathway was significantly enriched.

Gene Ontology analysis was also performed within the DAVID software using the same data set that was used for pathway analysis. The GO-fat category was used with the default ease setting of 0.1 and minimum count of 2.

PCR

To verify expression data obtained from microarray analysis quantitative realtime reverse-transcriptase PCR (qRT-PCR) was used. Quantitative realtime RT-PCR can be used to validate expression results obtained from large scale gene expression assays such as microarrays. Messenger RNA extracted for the microarray assay was reverse transcribed using oligo(dT) primers Integrated DNA Technologies (Coralville, IA) and the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc.) with a starting quantity of 2 µg mRNA per 40 µL reaction.

The population used in this study was the same as before with the same 5 samples that were found to be unreliable in the microarray set removed (7151, 7227, 7606, 7732, and 8148) for a total of 42 samples remaining. Because one goal of this step was to verify and add to the results of the microarray study, any samples not being used previously were considered undesirable for follow-up studies.

A total of 0.6 µg of RNA were used per reaction well for cDNA generation. Each sample was amplified in duplicate in batches of 20 µL and recombined following amplification; the protocol used was in accordance with manufacturer specifications with no deviations. A 20 µL reaction was prepared as follows: 9.2 µL of RNA quality water, 2.0 µL 10X RT buffer stock, 2.0 µL oligo(dT) primers, 0.8 µL dNTP, 1.0 µL RT enzyme, and 5.0 µL diluted RNA sample. These were then amplified using an ABI2720 thermal cycler following the manufacturers protocol; 25°C for 10 min, 37°C for 2 h, and 70°C for 2 min.

Following amplification samples were diluted 1:5 in 25 ng/µL yeast tRNA following Riggs lab protocol. Working stocks were stored at 4°C and master aliquots were stored long-term at -20°C.

Primers were chosen based on results from the microarray and pathway analysis as well as from the literature. Primer design followed lab protocol using Oligo Primer Analysis Software v6.71 by Molecular Biology Insights (Cascade, CO). Primers were selected where at least one primer within a pair covered an exon junction as a precaution against amplifying genomic DNA. Additionally, they were selected to have a melting temperature (Tm) close to 60°C, an approximate length of 20 base pairs, roughly equal balance of nucleotides not skewed towards G/Cs or A/Ts, and a dimerization of ΔG no less than -4.0 at any point particularly at the 3' end. Potential primers were then analyzed using Basic Local Alignment Search Tool (BLAST) provided freely by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A search of the *Bos taurus* database (taxid: 9913) for highly similar sequences (megablast) was employed to determine if the primer sequence would amplify non-specifically. Primers were ordered from Integrated DNA Technologies (Coralville, IA) and diluted in RNA-grade water to a working concentration of 10µM. Genes that were candidates for primer design consisted of those selected from the pathway analysis, those believed to be significant from the literature although not found to be significant in our assays, and potential control genes selected from the literature. A total of 40 primer pairs were ultimately used (Table 9).

Table 9. Primers used for qRT-PCR

Primer	Forward	Reverse
18S	TGCCGGAGTCTCGTTCGT	GGTGCATGGCCGTTCTTAGT
ACTA1	CGACGGTCAGGTCATCA	TGCTGTTGTAGGTGGTCTCAT
ACTN1	AAGATGGAGGAGATTGGAAGGAT	GGCTTGTAGTTGACGATGCTCT
ACTN2	GGTCTTTGACAACAAGCA	TGATGGTTCTGGCGATA
ACTN3	CGGGAGACAAGAACTACATCA	CGTAGAGGGCACTGGAGAA
ACTN4	AGCAGAGCAAACAACAGTCCAA	CCTCCAGCGTCCCATTCAT
B2M	AGTAAGCCGCAGTGGAGGT	CGCAAAACACCCTGAAGACT
C4A	GCCGCCTTCAGTTGGAA	GGTCTCCCTTGAGGTCGTAAGTG
CAPN1	GACCATAGGCTTCGCTGTCT	AGGTTGATGAACTGCTCGGA
CAPN2	CGACTGGAGACACTGTTCAGGA	CTTCAGGCAGATTGGTTATCACTT
CAST	GCTGTCGTCTCTGAAGTGGTT	GGCATCGTCAAGTTCTTTGTTGT
COL1A2	GGGCAACAGCAGATTCACTTACA	TCAAGGATAGGCAGGCGAGAT
COL6A1	TGAAAATGTGCTCTTGCTGTGAGT	AATGACCTTGACGATGAAGTCCTT
COX7C	TGCAGCCGCCATTTCTTC	TAGCGCTGTTGGACGCTCTA
CTSF	GCTGGAGACGGAGGATGA	CACTGAGTCGTTGATGTAGACCTT
FLNA	CTGACCAAGACTGCCACCAT	ATGTTGTTGCCTGCTTTGCT
FLNB	CGTCTCCACCAAGTTCGCT	TCACAGATGCTGCCAACAGT
FLNC	AAGCAGGCACCAATATGATGAT	CCACTTGACGATGAGGATGTAGT
IPO7	CTTCCCTAATAATGTTGAACCAGTTAC	GAACACACATCTTTCTGTCGTGAA
ITGA5	GCAAGAATCTCAACAACTCGCAA	GCCAGTCGCTCATCGGAAATA
ITGA6	AAGACAGACAGATGATGGCAGA	GGCACTTGATGTTCACACAGTT
ITGA9	AGCCTGTGAACTGCCTCAA	ACATCAGCCGTCAGAACATAAT
ITGB1	CTTCTATTGCTCACCTTGTCCA	AGATAATGTTCCTACCGCTGACTT
ITGB4	CACAACCTCCAGCAGACCAA	CCATCAGCACAGTGTCCACAA
ITGB5	CGGAGAGAGTTCGCCAAGTT	CTGTGCCATTGTAGGATTTGTTGA
ITGB6	CATCAATGAGAAAGACTGTCCAA	CAATAAGAAGAATAGCCAGCGAA
Loc518180	ATACGCACAGCCGCAGA	CACGATGTCACCCACCTCTAA
MYH1	TGAGGAAGCGGAGGAACAAT	TGGGACTCGGCAATGTCA
MYH2	CAATGACCTGACAACCCAGA	CCTTGACAACTGAGACACCAGA
NRAS	GCTAATCCAGAACCACTTTGTAGAT	GCCTTCGCCTGTCCTCAT
PRDX3	AAGTTGTGGCAGTGTCAGTGGA	CGTAGTCTCGGGAAATCTGTTTG
RPL19	ACCCCAATGAGACCAATGAA	GCAGTACCCTTTCGCTTACCTAT

Table 9. Continued

Primer	Forward	Reverse
RPL5	GACTGGAGATGAATACAATGTGGAA	CGTTTGGTACTGTGAGGGATAGA
RPS20	ACCAGCCGCAACGTGAA	ACCAGCCGCAACGTGAA
SDHA	GCAGAACCTGATGCTTTGTG	CGTAGGAGAGCGTGTGCTT
TLN1	AAATGCCAAGAACGGAAACTT	GTCAGACACGCCAACCAGATA
TLN2	GCTGGGACATAAGGTGACACA	CTGCGAGCGTCTTGGTCT
UFM1	AAATAGCCTTCAGGGAGAAAGTGTAA	AATGCTTCCTTAAATGTTCGGTCTTC
YWHAZ	GCATCCCACAGACTATTTCC	GCAAAGACAATGACAGACCA

IDs are based on official gene symbol as provided by NCBI

For all quantitative RT-PCR the *7900HT* Fast Real-Time PCR System (Applied Biosystems, California) was used in the 9600 Fast Emulation mode. This consisted of a 10 min 95°C melting phase, 40 cycles of 95°C for 15 s, 60°C for 1 min, followed by a 95°C dissociation phase. All reactions were performed in a 20 µL reaction consisting of 6.8 µL RNA-grade water, 10 µL SYBR Green Master Mix, 0.6 µL of each primer, and 2.0 µL of cDNA sample.

SDS (v.2.4) software from Applied Biosystems (Carlsbad, CA) was used to quantify qPCR results. A threshold value of 0.20 was used to determine C_t value. Relative expression was calculated using the method described by Livak and Schmittgen (2001). In summary, raw C_t values were normalized to the geometric mean of *RPL19*, *RPL5*, and *RPS20* based on internal expression stability between groups. The normalized value was subtracted from the raw C_t for each sample (ΔCT). From the ΔCT the average value of the high group was subtracted (ΔΔCT). The ΔΔCT was linearized

by taking the inverse negative log-$_{base\ 2}$ (RQ). The high group will thus always have a median expression value of 1.0. It should be noted that these are arbitrary units (AU) and that no direct comparison between different genes in total expression levels can be reliably made. All values are relative and applicable directly only as a within-group comparison of relative expression.

Haplotype analysis

Haplotype analysis was performed in the manner described in Chapter II. Genes selected for haplotype analysis were those that either showed significant differences in gene expression as assessed by qPCR, were found in pathways of interest, were shown to be of interest by a literature search, or some combination of the three. These genes can also be classified in to 3 main functional categories: the calpain system, muscle structural proteins, and components of the ECM.

A list of these genes along with chromosome location is given in Table 10. The total number of SNP present within the defined region is listed in the "SNP in hapblock" column.

Table 10. Location of genes used in the construction of hapblocks

Category	Gene	Chromosome	Coordinates		SNP in hapblock
Calpain System	*CAPN1*	29	44063463	44113492	24
	CAPN2	16	27781671	27840011	24
	CAPN3	10	37829007	37885645	24
	CAST	7	98444979	98581253	18
Muscle Structure	*ACTN2*	28	9403203	9450920	16
	ACTN3	29	45230630	45242406	16
	MYH1/2	19	30110728	30165109	18
ECM/ Focal Adhesion	*ITGA5*	5	25778012	25799053	14
	ITGA6	2	-24230649	-24118992	18
	ITGA9	22	10892055	11361218	23
	ITGB1	13	20248978	20290982	11
	ITGB4	19	-56511970	-56467165	20
	ITGB5	1	-69914350	-69787160	15
	ITGB6	2	36242459	36362623	16
	FN1	2	103881402	103950562	12

Coordinates are based on the UMD 3.1 bovine genome assembly.

Protein analysis

From a subset of 8 samples (8208, 8133, 7123, 7115, 8156, 7232, 8157, and 8050) protein was extracted in RIPA buffer from approximately 1 g sample stored at -80°C. The solution used for RIPA was: 73.5 mL H_2O, 5.0 mL 3M NaCl, 5.0 mL conc Tris HCl, 10.0 mL, 10% Triton X-100, 0.2 mL 0.5 M EDTA (pH 8.0), 1.0 mL protease inhibitor, 1 mL phosphatase inhibitor cocktail I and 1 mL phosphatase inhibitor cocktail II. Protein samples were quantified using Coomassie Plus Bradford Assay Kit supplied by Pierce Biotechnology (Rockford, IL) using serum albumin as a standard. Samples

were diluted to a standard concentration based on quantity as determined by quadratic best fit curve of absorbance at 595 nm.

A 5% stacking gel and 10% resolving gel were used for sufficient separation of bands. The stacking gel recipe was 2.7 mL H_2O, 0.67 mL 30% acrylamide mix, 0.5 mL 1.0M Tris (pH 6.8), 0.04 mL 10% SDS, 0.04 mL10% ammonium persulfate, and 0.004 mL TEMED. The resolving gel recipe was 4.0 mL H_2O, 3.3 mL 30% acrylamide mix, 2.5 mL 1.5M Tris (pH 8.8), 0.1 mL 10% SDS, 0.1 mL10% ammonium persulfate, and 0.004 mL TEMED. Gels were run in pairs using a Bio-Rad Mini-PROTEAN Tetra Cell setup according to manufacturer's instructions. A total of 25 µg of sample in 30 µL Laemmli buffer were added to each well. Gel separation and blotting were both performed at 100V for 1 h. Antibodies were diluted according to manufacturer's instructions in a solution of 7.5% dry milk solution in TBST according to lab protocol. Primary antibodies were allowed to bind overnight (12 or more h) with steady agitation at 4°C. Secondary antibodies were allowed to attach for 1 h at room temperature. All secondary antibodies contained horse radish peroxidase and were labeled using the ECL Western Blotting Detection kit provided by GE Healthcare (Waukesha, WI). Fluorescence was detected by the Gel Doc XR and ChemiDoc XRS gel documentation system and quantified by Quantity One 1-D Analysis software by Bio-Rad (Hercules, CA). The antibodies used are presented in Table 11.

Table 11. Antibodies used in western blot analysis

Antibody	kDa	Target	Company	Product number
ITGB1	138	N20	Santa Cruz Biotechnology	sc-6622
ITGA6	120	C18	Santa Cruz Biotechnology	sc-6596
ITGB6	97	N20	Santa Cruz Biotechnology	sc-6633
m-CAPN	80	80kDa	Thermo Scientific	MA3-942
u-CAPN	80	80kDa	Thermo Scientific	MA3-940
CAST	80	80kDa	Thermo Scientific	MA3-944
anti-goat secondary			Santa Cruz Biotechnology Inc.	sc-2304
anti-mouse secondary			Thermo Scientific	SA1-72018

Integrins are compatible with anti-goat antibody and the calpains and calpastatin were compatible with the anti-mouse antibody. All were compatible with use in bovine tissues according to manufacturer's specifications.

Statistical analysis

SPSS v16.0 was used for all statistical analysis with the exception of the microarray expression levels. Microarray analysis procedures were based on Genespring software as described previously in the methods section of Chapter II. Unless otherwise noted an independent samples t-test was used to determine significance. Measures of averages are given as mean values. Estimations of error are given as standard error of the mean (SEM).

Results

Bovine microarray analysis was conducted on skeletal muscle RNA samples from 48 steers. Samples represented 4 tenderness groups in which loin steaks from the carcasses were evaluated for Warner-Bratzler shear force. For both the ES and NES carcass halves, model predicted WBSF values for both, residuals, and initial classifications for the ES or NES group is presented in Table 12. It is worth noting that some samples could be classified in an extreme group in both the ES and NES. Following the removal of unusable outliers the groups were reassigned. These data are presented in Table 13. All subsequent references to ES residual tenderness groups will come from Table 13.

The random effects of sire ($P = 0.03$) and family nested within sire ($P = 0.02$) as well as the fixed effects of calving group ($P < 0.01$) and slaughter date ($P < 0.01$) were significant components of the model, developed and described in the methods section, and were retained.

Table 12. Tenderness and classification information on 48 animals that were used in this study

ID	WBSF, kg (ES)[a]	Predicted WBSF, kg (ES)[b]	Residuals (ES)[c]	Group (ES)	WBSF, kg (NES)[d]	Predicted WBSF, kg (NES)[e]	Residuals (NES)[f]	Group (NES)
7009	4.59	2.82	1.72	High	3	3.36	-0.36	
7126	3.48	2.4	1.16	High	3.44	3.58	-0.14	
7232	4.61	2.64	1.95	High	3.88	3.81	0.07	
7715	4.06	2.99	1.04	High	4.75	3.98	0.77	
7730	4.23	3.09	1.11	High	3.17	3.98	-0.81	
7732	3.86	2.85	0.92	High	4.68	3.85	0.83	
8010	3.78	2.64	1.08	High	4	3.67	0.33	
8050	4.9	3.67	1.18	High	5.38	4.5	0.88	
8113	3.75	2.66	0.97	High	3.54	3.68	-0.14	
8146	3.97	2.79	1.04	High	3.34	3.75	-0.41	
8157	4.67	3.53	0.94	High	5.36	6.13	-0.77	
8420	4.32	3.49	0.93	High	5.61	4.64	0.97	
7005	2.18	2.82	-0.68	Low	3.56	3.36	0.2	
7115	1.72	2.83	-0.91	Low	3.62	3.07	0.55	
7123	1.64	2.4	-0.68	Low	2.39	3.58	-1.19	
7142	2.52	3.53	-0.97	Low	6.81	6.13	0.68	
7203	2.42	2.96	-0.58	Low	3.39	4.34	-0.95	
7606	1.96	2.82	-0.74	Low	2.3	3.36	-1.06	
7728	2.23	2.79	-0.58	Low	5.12	3.75	1.37	
7731	1.9	2.79	-0.91	Low	3.18	3.75	-0.57	
8004	2.04	2.64	-0.65	Low	2.11	3.67	-1.56	
8115	2.28	2.83	-0.6	Low	3.07	3.07	0	
8133	1.61	2.4	-0.95	Low	3.54	3.58	-0.04	
8419	2.41	3.49	-0.98	Low	4.7	4.64	0.06	
7021	3.68	2.79	0.9		6.15	3.75	2.4	High
7127	2.43	2.39	0.16		5.5	3.39	2.11	High

Table 12. Continued

ID	WBSF, kg (ES)[a]	Predicted WBSF, kg (ES)[b]	Residuals (ES)[c]	Group (ES)	WBSF, kg (NES)[d]	Predicted WBSF, kg (NES)[e]	Residuals (NES)[f]	Group (NES)
7151	2.77	2.95	-0.11		7.17	4.92	2.25	High
7215	2.77	2.45	0.36		7.06	3.98	3.08	High
7227	2.75	2.64	0.09		5.74	3.81	1.93	High
7303	2.57	2.96	-0.36		6.37	4.34	2.03	High
7742	2.72	2.66	0		5.22	3.32	1.9	High
8019	2.88	2.99	-0.14		6.89	3.98	2.91	High
8035	2.5	2.39	0.1		5.14	3.39	1.75	High
8125	2.47	2.21	0.17		4.43	2.71	1.72	High
8148	2.76	2.64	-0.07		5.86	3.81	2.05	High
8159	2.81	2.59	0.16		7.78	6.1	1.68	High
7101	4	2.96	1.07		2.72	4.34	-1.62	Low
7112	2.61	2.45	0.28		2.66	3.98	-1.32	Low
7518	2.4	2.99	-0.39		2.41	3.98	-1.57	Low
7736	2.37	2.59	-0.16		4.43	6.1	-1.67	Low
7738	3.23	2.78	0.43		2.67	3.98	-1.31	Low
8053	3.59	3.67	-0.13		3.13	4.5	-1.37	Low
8156	4.4	2.95	1.27		3.47	4.92	-1.45	Low
8208	1.47	2.64	-1.1		2.58	3.81	-1.23	Low
8303	2.36	2.45	0.01		2.54	3.98	-1.44	Low
8306	2.7	2.99	-0.2		2.16	3.98	-1.82	Low
8314	2.27	2.68	-0.34		2.2	3.65	-1.45	Low
8328	2.33	2.64	-0.26		1.97	3.81	-1.84	Low

[a] WBSF (ES) = observed Warner-Bratzler shear force on carcass half subjected to electrical stimulation (ES).
[b] Predicted WBSF (ES) = predicted Warner-Bratzler shear force on carcass half subjected to ES based on model predictions.
[c] Residuals (ES) = difference between observed ES and predicted ES shear force values.
[d] WBSF (NES) = observed Warner-Bratzler shear force on carcass half not subjected to electrical stimulation (NES).
[e] Predicted WBSF (NES) = predicted Warner-Bratzler shear force on NES carcass half not subjected to ES based on model predictions.
[f] Residuals (NES) = difference between observed NES and predicted NES shear force values.

A total of 48 single-color microarray hybridizations were conducted on catalog bovine arrays (Agilent). Six hybridizations did not pass quality control screening and were removed. These 6 samples were removed from all subsequent analysis. The amended list is presented in Table 13.

Table 13. Tenderness and classification information on revised list of 42 animals used for the tenderness study following removal of statistical outliers as determined by microarray analysis

WBSF, kg (ES)[a]	Predicted WBSF, kg (ES)[b]	Residuals (ES)[c]	Group (ES)	WBSF, kg (NES)[d]	Predicted WBSF, kg (NES)[e]	Residuals (NES)[f]	Group (NES)
4	2.96	1.07	High	2.72	4.34	-1.65	Low
4.4	2.95	1.27	High	3.47	4.92	-1.34	Low
4.59	2.82	1.72	High	3	3.36	-0.36	
3.48	2.4	1.16	High	3.44	3.58	-0.17	
4.61	2.64	1.95	High	3.88	3.81	0.06	
4.06	2.99	1.04	High	4.75	3.98	0.67	
4.23	3.09	1.11	High	3.17	3.98	-0.87	
3.78	2.64	1.08	High	4	3.67	0.33	
4.9	3.67	1.18	High	5.38	4.5	0.82	
3.75	2.66	0.97	High	3.54	3.68	-0.03	
3.97	2.79	1.04	High	3.34	3.75	-0.31	
4.67	3.53	0.94	High	5.36	6.13	-0.68	
2.04	2.64	-0.65	Low	2.11	3.67	-1.56	Low
2.27	2.68	-0.34	Low	2.2	3.65	-1.39	Low
2.18	2.82	-0.68	Low	3.56	3.36	0.2	
1.72	2.83	-0.91	Low	3.62	3.07	0.4	
1.64	2.4	-0.68	Low	2.39	3.58	-1.22	
2.52	3.53	-0.97	Low	6.81	6.13	0.59	
2.23	2.79	-0.58	Low	5.12	3.75	1.32	
1.9	2.79	-0.91	Low	3.18	3.75	-0.63	
2.28	2.83	-0.6	Low	3.07	3.07	0.03	
1.61	2.4	-0.95	Low	3.54	3.58	0.11	
1.47	2.64	-1.1	Low	2.58	3.81	-1.24	

Table 13. Continued

ID	WBSF, kg (ES)[a]	Predicted WBSF, kg (ES)[b]	Residuals (ES)[c]	Group (ES)	WBSF, kg (NES)[d]	Predicted WBSF, kg (NES)[e]	Residuals (NES)[f]	Group (NES)
8419	2.41	3.49	-0.98	Low	4.7	4.64	0	
7021	3.68	2.79	0.9		6.15	3.75	2.34	High
7127	2.43	2.39	0.16		5.5	3.39	2.11	High
7151	2.77	2.95	-0.11		7.17	4.92	2.18	High
7215	2.77	2.45	0.36		7.06	3.98	3.07	High
7227	2.75	2.64	0.09		5.74	3.81	1.92	High
7303	2.57	2.96	-0.36		6.37	4.34	2.06	High
7742	2.72	2.66	0		5.22	3.32	1.79	High
8019	2.88	2.99	-0.14		6.89	3.98	2.8	High
8035	2.5	2.39	0.1		5.14	3.39	1.75	High
8125	2.47	2.21	0.17		4.43	2.71	1.76	High
8148	2.76	2.64	-0.07		5.86	3.81	2.15	High
8159	2.81	2.59	0.16		7.78	6.1	1.75	High
7112	2.61	2.45	0.28		2.66	3.98	-1.39	Low
7518	2.4	2.99	-0.39		2.41	3.98	-1.5	Low
7736	2.37	2.59	-0.16		4.43	6.1	-1.75	Low
7738	3.23	2.78	0.43		2.67	3.98	-1.32	Low
8053	3.59	3.67	-0.13		3.13	4.5	-1.43	Low
8303	2.36	2.45	0.01		2.54	3.98	-1.32	Low
8306	2.7	2.99	-0.2		2.16	3.98	-1.74	Low
8328	2.33	2.64	-0.26		1.97	3.81	-1.72	Low
7203	2.42	2.96	-0.58		3.39	4.34	-0.91	
7606	1.96	2.82	-0.74		2.3	3.36	-1.06	
7732	3.86	2.85	0.92		4.68	3.85	0.81	
8420	4.32	3.49	0.93		5.61	4.64	0.92	

[a] WBSF (ES) = observed Warner-Bratzler shear force on carcass half subjected to electrical stimulation (ES).
[b] Predicted WBSF (ES) = predicted Warner-Bratzler shear force on carcass half subjected to ES based on model predictions.
[c] Residuals (ES) = difference between observed ES and predicted ES shear force values.
[d] WBSF (NES) = observed Warner-Bratzler shear force on carcass half not subjected to electrical stimulation (NES).
[e] Predicted WBSF (NES) = predicted Warner-Bratzler shear force on NES carcass half not subjected to ES based on model predictions.
[f] Residuals (NES) = difference between observed NES and predicted NES shear force values.

A non-parametric Mann-Whitney test with no FDR was used to determine significance between ES and NES tenderness groups. A total of 32,900 genes passed quality control for both ES and NES tenderness groups. Within the ES tenderness group 1,937 probes that were significantly different between groups. From those results 1,046 probes had a 1.4 fold or greater difference in expression levels between ES tenderness groups.

A pathway analysis of microarray results was performed using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/). A total of 1,046 genes were differentially expressed (1.4 fold or greater) from the microarray at a significant level. Of those, 867 were associated with known genes. DAVID software was used to place 752 of these genes in the bovine specific pathway sets. Pathway analysis revealed a total of 15 significantly enriched pathways (Table 14). Focal adhesion, cell adhesion, and ECM-receptor pathways are all closely related functionally and were found to be significantly enriched.

Table 14. Significantly enriched pathways based on DAVID analysis

Term	Count	%	*P*-Value
Pathways in cancer	22	0.3	0.08
Regulation of actin cytoskeleton	18	0.2	0.01
Focal adhesion	15	0.2	0.07
Cell adhesion molecules (CAMs)	14	0.2	0.01
T cell receptor signaling pathway	11	0.1	0.04
Axon guidance	11	0.1	0.06
Tight junction	11	0.1	0.09
ECM-receptor interaction	9	0.1	0.03
B cell receptor signaling pathway	8	0.1	0.06

Table 14. Continued

Term	Count	%	*P*-Value
Autoimmune thyroid disease	7	0.1	0.02
Homologous recombination	6	0.1	0.01
Type I diabetes mellitus	6	0.1	0.04
Intestinal immune network for IgA production	6	0.1	0.09
Fructose and mannose metabolism	5	0.1	0.06
Allograft rejection	5	0.1	0.09

Enrichment is determined by 752 genes recognized by DAVID Bioinformatics overlaid on KEGG bovine pathways.

The pathways in cancer subset was not chosen due to the redundant role of many genes enriched in it that are important in normal muscle physiology and regulation.

GO analysis performed on the same data gave similar results with categories relating to cell adhesion, motility, and migration being highly enriched (Table 15).

Table 15. GO analysis based on microarray expression data

Term	Count	%	*P*-Value
Cell adhesion	26	0.3	0.002
Biological adhesion	26	0.3	0.002
Cell motion	18	0.2	< 0.001
Cell migration	16	0.2	< 0.001
Localization of cell	16	0.2	< 0.001
Cell motility	16	0.2	< 0.001
Hexose metabolic process	15	0.2	< 0.001
Monosaccharide metabolic process	15	0.2	0.001
Leukocyte activation	14	0.2	0.001
Cell activation	14	0.2	0.003
Lymphocyte activation	13	0.2	0.001
Glucose metabolic process	11	0.1	0.007
Antigen processing and presentation	10	0.1	0.010
Leukocyte differentiation	9	0.1	0.005

Table 15. Continued

Term	Count	%	*P*-Value
Skeletal system morphogenesis	8	0.1	0.006
Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	6	0.1	0.001

GO analysis performed by DAVID bioinformatics based on 752 genes.

DAVID analysis revealed 15 significantly enriched pathways in the bovine set. The focal adhesion pathway overlapped with the ECM-receptor and regulation of actin cytoskeleton pathways. Additionally focal adhesion is a factor in muscle cell adhesion, cell motion and adhesion, and muscle assembly. For these reasons genes in the focal adhesion pathway were the focus of follow up qRT-PCR verification.

Based on these expression and pathway analysis a total of 40 genes were selected for validation by qRT-PCR followup. For all qRT-PCR expression values results are normalized to the high shear force group: so results are shown as the fold ratio of expression of the low-WBSF group relative to the high-WBSF group (Table 16). Because of this, the mean expression for every gene in the high group is 1.0 and is not listed. For comparison, the NES group was analyzed in the same manner.

Table 16. mRNA relative expression levels for the ES and NES groups

	ES				NES			
Gene	mRNA Expression			*P*-value	mRNA Expression			*P*-value
	n = 12				n = 12			
18S	1	±	0.4	0.595	0.9	±	0.1	0.645
ACTA1	1.1	±	0.3	0.247	1	±	0.1	0.888
ACTN1	0.9	±	1.5	0.831	0.8	±	0.4	0.509
ACTN2	1	±	0.2	0.897	0.9	±	0	0.483
ACTN3	3.8	±	22.3	0.354	1.1	±	0.2	0.836
ACTN4	1.4	±	0.3	**0.003**	1.1	±	0.1	0.598
B2M	1.3	±	0.3	**0.019**	1.2	±	0.1	0.154
C4A	1.3	±	0.4	0.073	1.1	±	0.1	0.788
CAPN1	1.2	±	0.3	0.051	1	±	0.1	0.823
CAPN2	1	±	0.4	0.937	1	±	0.1	0.931
CAST	0.7	±	0.5	0.379	0.6	±	0.1	**0.05**
COL1A2	1.6	±	1	0.087	0.8	±	0.3	0.501
COL6A1	1.4	±	0.5	**0.037**	1.2	±	0.4	0.588
FLNA	0.8	±	2.7	0.619	0.6	±	0.9	0.282
FLNB	1.5	±	0.4	**0.005**	1.1	±	0.1	0.681
FLNC	1.6	±	1.4	0.191	1.9	±	0.5	0.103
IPO7	1.2	±	0.3	0.14	1.1	±	0.1	0.326
ITGA5	2.1	±	0.7	**0.003**	1.7	±	0.5	0.104
ITGA6	1.6	±	0.4	**0.006**	1	±	0.2	0.618
ITGA9	1.5	±	0.3	< **0.001**	1.1	±	0.1	0.529
ITGB1	1.1	±	0.9	0.669	1.5	±	0.3	0.084
ITGB4	1.7	±	0.7	**0.03**	1.1	±	0.2	0.774
ITGB5	1.5	±	0.7	0.113	1.1	±	0.3	0.686
ITGB6	1.3	±	0.5	0.278	1.2	±	0.2	0.223
LOC5181	1	±	0.3	0.877	1.1	±	0.1	0.382
MYH1	153	±	781.7	0.326	1.3	±	0.2	0.529
MYH2	2.1	±	3.2	0.172	1.7	±	0.3	0.222
NRAS	0.9	±	2	0.896	0.7	±	0.5	0.342
PRDX3	1.1	±	0.3	0.321	1	±	0.1	0.918
RPL19	1	±	0.1	0.229	1	±	0	0.316
RPL5	1	±	0.2	0.802	1	±	0	0.538
RPS20	1	±	0.2	0.877	1.1	±	0	0.134

Table 16. Continued

	ES		NES	
Gene	mRNA Expression	*P*-value	mRNA Expression	*P*-value
SHDA	1.1 ± 0.4	0.718	1.1 ± 0.1	0.619
TLN1	1.4 ± 3	0.537	0.9 ± 0.9	0.88
TLN2	1.2 ± 0.3	0.086	1.1 ± 0.1	0.593
UFM1	1.3 ± 0.3	**0.039**	1.1 ± 0.1	0.368
YWAHZ	1.3 ± 0.2	**< 0.001**	0.9 ± 0.1	0.553

Expression values were linearized then normalized to the geometric mean of *RPL5*, *RPL19*, and *RPS20*. Expression is presented as expression fold ratios of the low-WBSF group relative to the high-WBSF group; for example *ITGA5* is expressed 2.1 times higher in the low group than the high group among the population selected for extremes of ES residuals.

Several genes were considered for possible use as reference genes for normalization. Myosin heavy chain variant I (*MYH1*) expression was found to be so variable as to be completely unreliable as a reference gene. Two genes presented as potential reference genes in other studies were significantly different between ES groups: *B2M* (Perez et al., 2008) and *YWAHZ* (Goossens et al., 2005). Here, the reference genes used were *RPL5*, *RPL19*, and *RPS20*. All values given for qRT-PCR here were normalized to the geometric mean expression of these 3 genes.

All alpha-integrins assayed were significantly upregulated in the low ES group relative to the high. Beta-integrins were upregulated in the low ES group as well although only *ITGB4* was significant *(P* = 0.03). One collagen, *COL6A1*, was significantly upregulated in the low ES group as well. *COL1A2* was also upregulated in the low ES group, but it fell short of significance. This finding is interesting as all previous studies found that increased collagen was associated with increased toughness.

It is possible that an increase in collagen mRNA expression correlates to a higher turnover rather than a greater total amount of the protein. This transcript level would thus be associated with a lower percentage of mature collagen fibrils that are strongly correlated with increased toughness. Maturity of collagen fibers was not measured here.

With the exception of calpastatin (*CAST*) no genes in this set were differentially expressed to a significant level between groups when based on NES residuals. Calpastatin was expressed at a lower level in those carcasses that responded most favorably in terms of WBSF to aging in the absence of electrical stimulation. Based on previous studies, this result is not surprising (Koohmaraie et al., 1988; Taylor et al., 1995b; Delgado et al., 2001; Geesink et al., 2006). Calpastatin expression was not significant in relation to WBSF following ES treatment, although μ-calpain (*CAPN1*) came close to significance *(P* = 0.051) and was upregulated in the low ES group (1.22), as might be expected.

Hapblock analyses was performed on the larger population (n = 196). A total of 15 unique hapblocks were examined for breed and parent of origin effects. Based on the literature (Koohmaraie, 1994; Taylor et al., 1995a; Casas et al., 2006; Geesink et al., 2006) the calpain system was selected and consisted of μ-calpain (*CAPN1*), m-calpain (*CAPN2*), calpain p94 (*CAPN3*), and the endogenous inhibiting element calpastatin (*CAST*). These were not significant in either the pathway or expression analysis in this study with the exception of *CAST* mRNA expression in NES. They are heavily cited as playing a role in postmortem beef tenderization in the literature. For this reason they were selected for haplotype analysis.

Muscle structural genes studied were α-actinin 2 and α-actinin 3 (*ACTN2* and *ACTN3*), and the myosin heavy chain components *MYH1* and *MYH2*. The actinin genes were significant in pathway analysis. The myosin heavy chain structures were a downstream component of the focal adhesion and ECM regulation pathways although those 2 were not found to be significant in terms of expression analysis, possibly due to variability in expression. *MYH1* and *MYH2* were located in close proximity to each other and are found within the same 1 megabase region so only one haplotype analysis was performed that encompassed both genes and surrounding elements (labeled here as *MYH1/2*).

Finally, components of the ECM and focal adhesions were analyzed. The majority of these were integrins (*ITGA5*, *ITGA6*, *ITGA9*, *ITGB1*, *ITGB4*, *ITGB5*, and *ITGB6*). Fibronectin 1 (*FN1*) was also included although it was not assayed for expression. Fibronectin is a signaling and structural component that interacts with various integrin dimers in the living tissue. *FN1* was not significant in the pathway or expression analysis. However, *ITGA5* and *ITGB1* form a complex that in muscle is a fibronectin receptor (Mayer, 2003). Among the calpains only *CAPN3* was linked to a difference in tenderness ($P = 0.04$). The paternally inherited Angus *CAPN3* hapblock was linked to an improvement in tenderness compared to the Nellore hapblock (WBSF ES residuals 0.06 and -0.08, respectively). The paternally inherited *CAST* hapblock approached significance ($P = 0.08$), with the Angus hapblock having an improved residual tenderness when compared to the paternally inherited Nellore hapblock (0.12 and -0.12, respectively). (Table 17).

Table 17. WBSF residuals grouped by hapblocks in the Calpain system

		Hapblock												Paternal							Maternal						
		NN			NA			AN			AA			NN/NA			AN/AA			*P*-value	NN/AN			NA/AA			*P*-value
CAST	n	51			49			51			45			100			96				102			94			
	ES	0.03	±	0.06	0.03	±	0.09	-0.04	±	0.06	-0.06	±	0.07	0.03	±	0.05	-0.05	±	0.05	0.25	0.00	±	0.04	-0.01	±	0.06	0.89
	NES	0.12	±	0.14	0.11	±	0.15	-0.02	±	0.12	-0.24	±	0.13	0.12	±	0.10	-0.12	±	0.09	0.08	0.05	±	0.09	-0.06	±	0.10	0.42
CAPN1	n	28			45			39			84			73			123				67			129			
	ES	0.00	±	0.07	0.03	±	0.08	-0.04	±	0.08	-0.01	±	0.05	0.02	±	0.05	-0.02	±	0.05	0.6	-0.02	±	0.06	0.00	±	0.04	0.7
	NES	0.26	±	0.21	-0.05	±	0.15	-0.13	±	0.14	0.00	±	0.10	0.07	±	0.12	-0.04	±	0.08	0.42	0.04	±	0.12	-0.02	±	0.08	0.7
CAPN2	n	44			63			45			44			107			89				89			107			
	ES	0.11	±	0.08	0.02	±	0.06	-0.07	±	0.07	-0.09	±	0.07	0.06	±	0.05	-0.08	±	0.05	0.06	0.02	±	0.05	-0.03	±	0.04	0.51
	NES	0.28	±	0.16	-0.03	±	0.12	-0.10	±	0.13	-0.13	±	0.12	0.10	±	0.10	-0.12	±	0.09	0.12	0.09	±	0.11	-0.07	±	0.09	0.23
CAPN3	n	42			63			44			47			105			91				86			110			
	ES	0.13	±	0.08	0.01	±	0.06	-0.07	±	0.08	-0.09	±	0.07	0.06	±	0.05	-0.08	±	0.05	**0.04**	0.03	±	0.06	-0.03	±	0.04	0.38
	NES	0.22	±	0.17	-0.01	±	0.12	-0.10	±	0.14	-0.10	±	0.12	0.09	±	0.10	-0.10	±	0.09	0.17	0.06	±	0.11	-0.05	±	0.09	0.45

ES and NES values are given as WBSF residuals, kg.

The different calpain hapblocks could not be linked significantly to differences in expression, as measured by qRT-PCR, of *CAPN1*, *CAPN2*, *CAPN3,* and *CAST* in the population assayed.

Two hapblocks from the focal adhesion pathway were significant. The *FN1* Angus hapblock was linked to an improvement in ES and NES tenderness compared to the Nellore hapblock when inherited paternally ($P < 0.01$ and $P = 0.02$, respectively). The *ITGA6* Nellore hapblock was linked to an improvement in ES tenderness when inherited maternally ($P = 0.03$). Additionally the *ITGB6* hapblock came close to significance ($P = 0.08$) with the Nellore being associated with an improved ES tenderness relative to the Angus hapblock. The *ITGA9* hapblock also approached significance ($P = 0.09$) with the Angus hapblock being associated with an improved ES tenderness relative to the Nellore hapblock. Other than the *FN1* hapblock no genes assayed in the focal adhesion pathway had any significant effect on NES residual tenderness (Table 18).

Table 18. WBSF residuals grouped by hapblocks in the focal adhesion and ECM pathways

		Hapblock												Paternal						Maternal					
		NN			NA			AN			AA			NN/NA			AN/AA		*P*-value	NN/AN			NA/AA			*P*-value
ITGB1	N	62			60			39			35			122			74			101			95			
	ES	0.07	±	0.06	0.00	±	0.06	-0.07	±	0.08	-0.07	±	0.08	0.03	±	0.04	-0.07	±	0.06	0.15	0.01	±	0.05	-0.02	±	0.05	0.60
	NES	-0.01	±	0.12	0.07	±	0.12	-0.21	±	0.16	0.14	±	0.16	0.03	±	0.08	-0.04	±	0.11	0.62	-0.09	±	0.10	0.09	±	0.10	0.18
ITGB4	N	37			50			55			54			87			109			92			104			
	ES	0.00	±	0.09	0.01	±	0.07	-0.02	±	0.06	-0.01	±	0.07	0.00	±	0.05	-0.01	±	0.05	0.85	-0.01	±	0.05	0.00	±	0.05	0.88
	NES	0.05	±	0.15	0.11	±	0.13	-0.07	±	0.14	-0.06	±	0.12	0.08	±	0.10	-0.07	±	0.09	0.27	-0.02	±	0.10	0.02	±	0.09	0.73
ITGB5	N	37			52			57			50			89			107			94			102			
	ES	-0.11	±	0.06	0.00	±	0.06	0.02	±	0.07	0.03	±	0.08	-0.04	±	0.04	0.03	±	0.05	0.32	-0.03	±	0.05	0.02	±	0.05	0.53
	NES	0.03	±	0.16	0.00	±	0.13	-0.05	±	0.14	0.03	±	0.12	0.01	±	0.10	-0.01	±	0.09	0.85	-0.02	±	0.11	0.02	±	0.09	0.80
ITGB6	N	53			48			40			55			101			95			93			103			
	ES	-0.10	±	0.06	-0.02	±	0.07	0.05	±	0.09	0.06	±	0.07	-0.06	±	0.04	0.06	±	0.05	0.08	-0.04	±	0.05	0.02	±	0.05	0.40
	NES	-0.01	±	0.13	0.03	±	0.15	-0.05	±	0.15	0.02	±	0.13	0.01	±	0.10	-0.01	±	0.10	0.92	-0.03	±	0.10	0.02	±	0.10	0.70
ITGA5	N	54			41			59			42			95			101			113			83			
	ES	0.01	±	0.08	-0.02	±	0.06	0.00	±	0.07	-0.02	±	0.05	0.00	±	0.05	-0.01	±	0.05	0.95	0.01	±	0.05	-0.02	±	0.04	0.69
	NES	-0.07	±	0.13	-0.13	±	0.15	0.07	±	0.12	0.13	±	0.14	-0.10	±	0.10	0.09	±	0.09	0.15	0.00	±	0.09	0.00	±	0.10	0.97
ITGA6	N	42			56			39			59			98			98			81			115			
	ES	-0.18	±	0.07	0.04	±	0.06	-0.01	±	0.08	0.07	±	0.07	-0.05	±	0.05	0.04	±	0.05	0.19	-0.09	±	0.05	0.06	±	0.04	**0.03**
	NES	-0.14	±	0.13	0.00	±	0.14	0.10	±	0.16	0.04	±	0.11	-0.06	±	0.10	0.06	±	0.09	0.37	-0.02	±	0.10	0.02	±	0.09	0.76
ITGA9	N	57			40			44			55			97			99			101			95			
	ES	0.08	±	0.07	0.02	±	0.09	-0.03	±	0.07	-0.09	±	0.06	0.05	±	0.05	-0.06	±	0.05	0.09	0.03	±	0.05	-0.04	±	0.05	0.29
	NES	0.03	±	0.11	-0.03	±	0.15	0.07	±	0.16	-0.06	±	0.13	0.00	±	0.09	0.00	±	0.10	0.95	0.05	±	0.10	-0.05	±	0.10	0.48
FN1	N	68			62			31			35			130			66			99			97			
	ES	0.10	±	0.06	0.05	±	0.06	-0.09	±	0.07	-0.22	±	0.08	0.07	±	0.04	-0.16	±	0.05	**< 0.01**	0.04	±	0.05	-0.05	±	0.05	0.21
	NES	0.04	±	0.12	0.19	±	0.12	-0.18	±	0.17	-0.25	±	0.15	0.11	±	0.08	-0.22	±	0.11	**0.02**	-0.03	±	0.10	0.03	±	0.10	0.68

ES and NES values are given as WBSF residuals, kg.

No integrin hapblocks used here were associated with any differences in integrin mRNA expression. Nellore hapblocks were associated with a general increase in integrin expression however this trend was not universal or significant.

Of the remaining muscle component hapblocks only paternally inherited *ACTN3* had any significant impact on tenderness (Table 19). A paternally inherited Angus *ACTN3* hapblock is associated with a lower ES ($P = 0.02$). No other muscle component hapblocks approached significance for either ES or NES residual tenderness. No muscle component hapblocks could be linked significantly to any differences in mRNA expression, as measured by qRT-PCR, for the muscle component genes assayed.

ACTN3 is a muscle specific structural protein found only in fast twitch muscle fibers. Fiber type differences have been associated with differences in eating quality in beef previously (Calkins et al., 1981; Chikuni et al., 2010) as well as proteolytic capacity (Burniston et al., 2005a; Burniston et al., 2005b; Kocturk et al., 2008; McMillan and Quadrilatero, 2011). Haplotype differences in the *ACTN3* gene only affected tenderness when inherited paternally.

Table 19. WBSF residuals grouped by hapblocks in the muscle structural component pathways

		Hapblock												Paternal							Maternal						
		NN			NA			AN			AA			NN/NA			AN/AA			value	NN/AN			NA/AA			value
ACTN2	n	50			46			45			55			96			100				95			101			
	ES	-0.06	±	0.07	0.01	±	0.05	0.07	±	0.09	-0.03	±	0.07	-0.03	±	0.04	0.02	±	0.06	0.53	0.00	±	0.05	-0.01	±	0.04	0.86
	NES	0.02	±	0.12	0.08	±	0.16	-0.17	±	0.11	0.05	±	0.15	0.05	±	0.10	-0.05	±	0.10	0.47	-0.07	±	0.08	0.06	±	0.11	0.33
ACTN3	n	43			62			44			47			105			91				87			109			
	ES	0.13	±	0.08	0.02	±	0.06	-0.09	±	0.07	-0.09	±	0.07	0.07	±	0.05	-0.09	±	0.05	**0.02**	0.02	±	0.06	-0.02	±	0.04	0.54
	NES	0.23	±	0.17	-0.04	±	0.12	-0.08	±	0.13	-0.08	±	0.12	0.07	±	0.10	-0.08	±	0.09	0.26	0.07	±	0.11	-0.06	±	0.09	0.33
MYH1/2	n	32			48			61			55			80			116				93			103			
	ES	0.05	±	0.10	-0.02	±	0.06	0.03	±	0.07	-0.07	±	0.06	0.01	±	0.05	-0.01	±	0.05	0.73	0.04	±	0.06	-0.04	±	0.04	0.24
	NES	0.03	±	0.15	-0.15	±	0.14	0.04	±	0.12	0.06	±	0.14	-0.07	±	0.10	0.05	±	0.09	0.37	0.04	±	0.09	-0.03	±	0.10	0.60

ES and NES values are given as WBSF residuals, kg.

To verify that gene expression differences could be tied to protein differences a western blot analyses was performed on a subset (n = 8) of the samples used in the qRT-PCR study. For the western blot analyses samples 8208, 8133, 7123, and 7115 were selected from the tender group (mean ES WBSF residuals of -0.98) and samples 8156, 7232, 8157, and 8050 were selected from the tough group (mean ES WBSF residuals of 1.43). These 2 groups were significantly different in WBSF ES residuals, but not in WBSF NES residuals (Table 20).

Table 20. Mean tenderness values for sample subset used in western blot analyses

	High	Low	P-value
N	4	4	
ES Residuals, kg	1.43	-0.98	< 0.001
NES Residuals, kg	-0.33	-0.48	0.832

A total of 8 animals were used for protein quantification based on extremes of ES residuals. The NES residuals for these animals is also listed for comparison purposes. Mean values are listed.

For the protein expression assay ITGA6 was selected based on qRT-PCR results and hapblock analysis. Additionally ITGB6 was chosen because it was significantly different in the hapblock analysis. And finally CAPN1, CAPN2, and CAST were selected based on their established role in tenderness from the literature despite not being found significant in any previous analyses performed on these samples. Based on

western blot assays, only the ITGA6 protein concentration was shown to be different between groups ($P = 0.045$) (Figure 2).

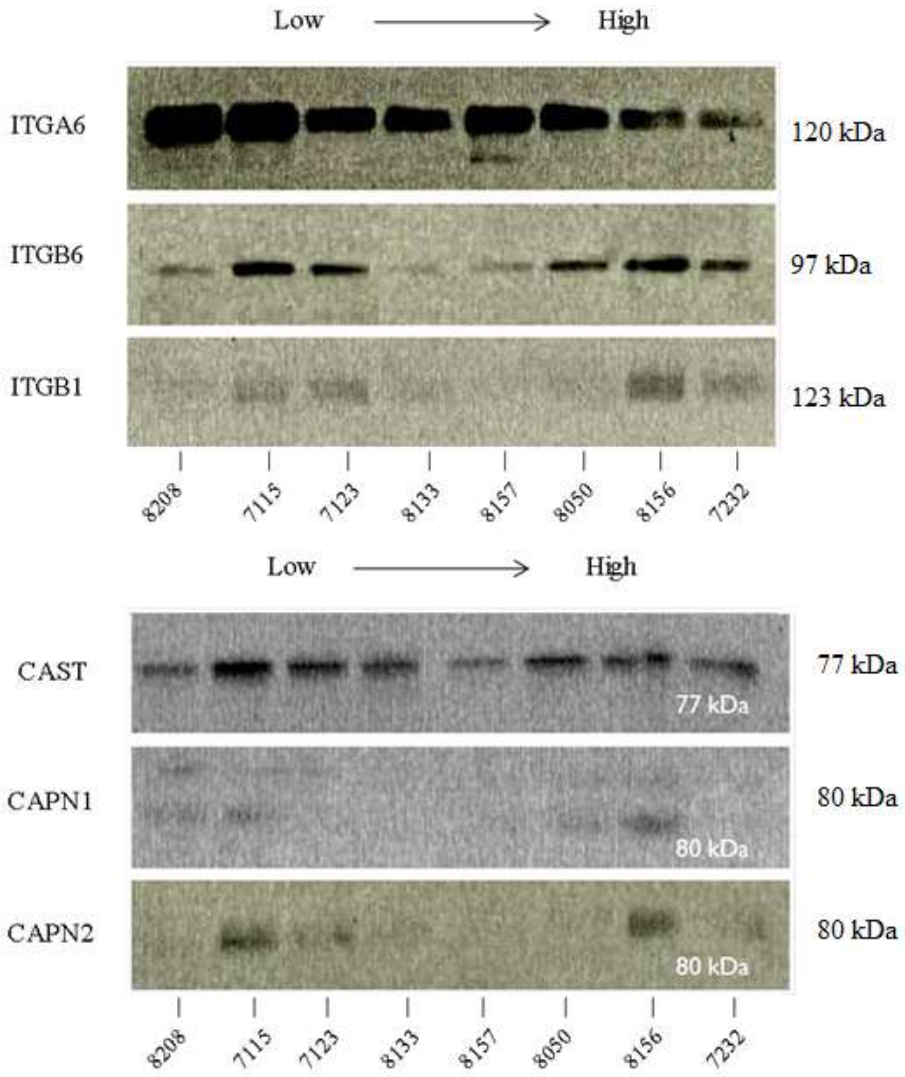

Figure 2. Western blot analyses on samples grouped by WBSF ES residuals. A 5% polyacrylamide stacking gel and 10% resolving gel was used. Picture was taken with a Gel Doc XR and ChemicDoc XRS imager.

The tender group had a background corrected expression level 1.8 fold higher than the tough group (Table 21). This was roughly the same expression difference as was found among the mRNA. Protein expression levels for the other genes measured do not seem to closely match mRNA expression, possibly due to post transcriptional regulation or protein degradation.

Table 21. Background corrected mean fluorescence as determined by western blot analysis

		High	Low	Low/High	*P*-value
Protein Expression	CAPN1	83,312 ± 7299	69,422 ± 3476	0.8	0.137
	CAPN2	103,334 ± 2948	100,843 ± 4271	1.0	0.648
	CAST	116,740 ± 4489	118,315 ± 4229	1.0	0.807
	ITGA6	81,237 ± 18413	149,840 ± 20002	1.8	**0.045**
	ITGB1	94,742 ± 6956	85,622 ± 1210	0.9	0.244
	ITGB6	44,066 ± 4276	50,586 ± 4174	1.2	0.317
mRNA Expression	*CAPN1*	1.0 ± 0.1	1.4 ± 0.2	1.5	0.052
	CAPN2	0.9 ± 0.1	1.1 ± 0.1	1.2	0.111
	CAST	0.4 ± 0.1	0.5 ± 0.2	1.3	0.597
	ITGA6	0.9 ± 0.1	1.6 ± 0.2	1.8	**0.019**
	ITGB1	0.6 ± 0.4	0.9 ± 0.2	1.5	0.563
	ITGB6	0.7 ± 0.4	1.7 ± 0.3	2.4	0.108

Values represent the mean protein fluorescence for the four samples selected for western blot analysis. Units given for protein fluorescence are optical density. Quantitative RT-PCR results are given for these four as a comparison.

Discussion

The purpose of this study was to examine genetic differences between beef cattle at the time of slaughter related to the ultimate response to tenderness following electrical stimulation postmortem. To account for environmental factors that might influence this trait a mixed model was used that incorporated calving group and slaughter date as fixed class effects, sire and family nested within sire as random effects, and age as a linear covariate. Using raw shear force values as input residuals were generated for both the ES and NES groupings. The selection of animals represented extremes of tenderness following 14 d of aging for those carcass halves subjected to ES. Animals representing extremes of response to aging without ES (NES) were included to account for genes that influenced tenderness that were not responsible for the ES response.

Using a systems approach that integrated genomic, expression, and protein information it was possible to discover a pathway and several unique genes that played a significant role in response to electrical stimulation in this mixed herd structure. Pathway analysis found significant enrichment in focal adhesion, actin cytoskeleton regulation, ECM interaction, and cell adhesion pathways. Zhang et al. (2011) found enrichment of the cell adhesion and ECM pathways partially explained the difference in tenderness between male and female Qinchuan cattle.

Quantitative RT-PCR results for the 24 samples assayed for gene expression showed significant differences in expression in components of these pathways between the two electrically stimulated tenderness groups. *ACTN4, COL6A1, FLNB, ITGA5, ITGA6, ITGA9,* and *ITGB4* were all significantly upregulated in the low WBSF residual-

group (1.4, 1.5, 1.4, 2.1, 1.6, 1.5, and 1.7 fold, respectively) compared with the high-WBSF residual group. With the exception of *CAST* (1.6 fold higher in the tough group) no genes were expressed differently at a significant level between groups selected for NES tenderness.

Higher expression of *CAST* leading to increased toughness by suppression of calpain-induced proteolysis is well supported by the literature (Whipple et al., 1990a; Delgado et al., 2001; Hope-Jones et al., 2010). What was interesting is that *CAST* expression failed to even approach significance in animals selected for extremes of tenderness following ES. This indicates that ES interacts with, or overshadows, the effects of calpain-induced proteolysis in tenderization.

Hapblock studies showed that breed differences in *CAPN1, CAPN2,* and *CAST* did not play a role in ES tenderness. The *CAST* Angus hapblock was associated with an improvement in NES tenderness although it fell short of significance ($P = 0.08$). Commercial genetic tests for tenderness are based on differences in the *CAPN1*, *CAPN2*, and *CAST* genes (Gruber et al., 2011). However, our data suggests breed differences in the *CAPN1*, *CAPN2*, and *CAST* genes would make a poor predictor of aged tenderness following ES, although an Angus *CAPN3* hapblock was associated with an improvement in ES tenderness ($P = 0.04$).

As a measure of ES tenderness, hapblocks encompassing components of the focal adhesion pathway were found to be more useful than hapblocks encompassing *CAPN1*, *CAPN2*, and *CAST*. The *ITGA6* and *FN1* genes both showed significant breed of origin effects for ES tenderness. The Nellore *ITGA6* was associated with a reduction

in ES residual tenderness by 0.15 kg when inherited maternally. The Angus *FN1* hapblock was associated with a reduction in ES residual tenderness by 0.23 kg and a reduction of NES residual tenderness by 0.33 kg when inherited paternally ($P < 0.01$ and $P = 0.02$, respectively). The *ITGB6* hapblock had a similar effect (reduction of ES residual tenderness by0.12 kg for the maternal Nellore hapblock)) to the *ITGA6* hapblock although it fell short of significance ($P = 0.08$). *ITGB6* mRNA expression levels were not significantly different between groups ($P = 0.278$).

Protein analysis of a subset of samples revealed that the ITGA6 protein was present at different levels between tenderness groups. Concentrations of ITGA6 protein in muscle samples of the more tender ES group were 1.8 fold greater than in the tougher ES group ($P = 0.045$). Gene expression between these two groups was also 1.8 fold higher in the more tender group ($P = 0.019$). ITGB6 protein expression was not significantly different between groups. And the CAPN1, CAPN2, and CAST proteins did not differ significantly in concentration between ES tenderness groups ($P = 0.137$, 0.648, and 0.807, respectively). In the mRNA expression studies the CAPN1, CAPN2, and CAST genes were also not found to vary significantly between ES tenderness groups ($P = 0.051$, 0.937, and 0.379, respectively).

Components of the focal adhesion pathway have not, to our knowledge, been previously known to affect tenderness in beef. Integrins and their role in tenderness have been studied somewhat in pork. Pigs grouped by tenderness had a focal adhesion pathway that was significantly enriched (Cherel et al., 2012). Integrin degradation was

also linked to the formation of drip channels and increased drip loss in pigs (Lawson, 2004).

The role of *ITGA6* in influencing response in tenderness to ES has not been explored in any livestock species. Several of other hapblocks (*ACTN3*, *FN1*, and *ITGB6*) were also assayed that have the potential for positive selection within mixed herds. These represent novel markers for gene assisted selection for improved tenderness in beef cattle.

CHAPTER IV

SUMMARY

A number of gene haplotype blocks including *ACTN3*, *CAPN2*, and *FN1* all showed significant influences on efficiency (as measured by RFI ($_{NRC}$)) with a parent and breed of origin effect. With the exception of *FN1* none of these hapblocks were shown to affect tenderness (*ACTN3* only was significant for NES tenderness when inherited paternally, whereas it played a role in efficiency only when inherited maternally).

Selection for feed efficiency is desirable but runs the risk of increasing toughness (McDonagh et al., 2001). Here I have presented a number of breed hapblock variants that, when inherited from the optimal parent, can positively affect feed efficiency while having apparently no significant impact on tenderness.

In addition, we have identified a number of gene variants that can be used in a selection process to improve tenderness without apparent effects on feed efficiency. *ITGA6* has shown statistically significant expression levels (protein and mRNA) as well as genomic differences that make it possible to identify breed variants.

A number of other functionally related muscle genes (*ACTN4*, *COL6A1*, *FLNB*, *ITGA5*, *ITGA9*, and *ITGB4*) were all significantly differentially expressed between tenderness groups. In addition to *ITGA6* the *FN1* and *ACTN3* hapblocks influenced tenderness with both a breed and parent of origin effect.

Only the *FN1* and *ACTN3* hapblocks were found to be significant for both efficiency and ES tenderness. However both were only significant for efficiency when inherited maternally and significant for ES tenderness when inherited paternally. That they function differently for these two traits based on parent of origin opens up possible breeding opportunities to select for both traits simultaneously.

These data present a novel set of genetic factors that influence the intertwined traits of efficiency and tenderness. These results support a potential method by which selection of cattle for improved tenderness may be achieved without sacrificing productivity. This information could be beneficial to producers and consumers alike.

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APPENDIX

A-1. Pedigree and model input data on animals selected for efficiency ($RFI_{(NRC)}$ residuals) study.

ID	BYS	SYS	SIRE	DAM	$RFI_{(NRC)}$ residuals	DOF, d	DDMI, kg	Efficiency Group
7011	S04	F05	297J	431H	-4	146	7.4	Efficient
7020	F04	S06	297J	431H	5.8	150	11.9	Inefficient
7021	S05	F06	297J	431H	-8	131	8.1	Efficient
7112	S04	F05	297J	760H	-0.6	144	8.3	Average
7115	S04	F05	297J	760H	0.3	148	8.4	Average
7122	F04	S06	297J	760H	0.2	147	8.1	Average
7135	S05	F06	297J	760H	-6.4	132	9.9	Efficient
7136	S05	F06	297J	760H	-6.3	133	10.8	Efficient
7202	S03	F04	432H	511G	-3.8	146	8.7	Efficient
7215	S04	F05	432H	511G	-0.3	144	7.1	Average
7217	S04	F05	432H	511G	4	146	10.5	Inefficient
7222	S05	F06	432H	511G	-4.6	130	6.9	Efficient
7223	S05	F06	432H	511G	-4.8	129	5.4	Efficient
7305	F03	S05	432H	732H	-0.6	133	7.7	Average
7503	S03	F04	437H	728H	-0.4	146	9	Average
7504	S03	F04	437H	728H	5	146	11.9	Inefficient
7519	F04	S06	437H	728H	4.7	147	11.7	Inefficient
7523	S05	F06	437H	728H	-3.8	133	9.9	Efficient
7606	F03	S05	551G	664J	0.1	134	8.3	Average
7702	F03	S05	551G	787G	6	134	10.4	Inefficient
7705	F03	S05	551G	787G	5.6	135	10.1	Inefficient
7706	F03	S05	551G	787G	5	133	11.2	Inefficient
7707	F03	S05	551G	787G	7.1	133	9	Inefficient
7709	F03	S05	551G	787G	6.2	135	10.1	Inefficient
7712	S04	F05	551G	787G	3.5	144	10.1	Inefficient
7716	S05	F06	551G	787G	-3.6	131	8.6	Efficient
7730	S05	F06	551G	787G	-5.2	129	8.4	Efficient

A-1. Continued

ID	BYS	SYS	SIRE	DAM	$RFI_{(NRC)}$ residuals	DOF, d	DDMI, kg	Efficiency Group
7732	S05	F06	551G	787G	-6.4	133	6.8	Efficient
8005	F03	S05	551G	429H	0.3	135	9.4	Average
8019	F04	S06	551G	429H	-0.1	148	9.4	Average
8033	F04	S06	551G	429H	0	147	8.9	Average
8035	F04	S06	551G	429H	0.1	149	7.2	Average
8037	F04	S06	551G	429H	3.8	149	9	Inefficient
8040	F04	S06	551G	429H	-3.7	150	5.9	Efficient
8045	F05	S07	551G	429H	0.5	151	7.9	Average
8122	S04	F05	437J	636H	0.2	148	9.9	Average
8141	S05	F06	437J	636H	-6.2	132	8.5	Efficient
8142	S05	F06	437J	636H	-4.3	132	8.4	Efficient
8144	S05	F06	437J	636H	-6.6	131	6.4	Efficient
8146	S05	F06	437J	636H	-0.2	131	10	Average
8147	F05	S07	437J	636H	4.5	151	10.3	Inefficient
8148	F05	S07	437J	636H	0.3	151	8.6	Average
8150	F05	S07	437J	636H	6.9	151	12.2	Inefficient
8151	F05	S07	437J	636H	3.6	152	10.2	Inefficient
8204	F05	S07	432H	559J	0.5	151	11.4	Average
8209	F05	S07	432H	559J	-0.2	152	9.6	Average
8308	F04	S06	437J	637H	4.5	148	11.2	Inefficient
8312	S05	F06	437J	637H	-6.4	130	8.7	Efficient
8323	S05	F06	437J	637H	-4.2	132	8	Efficient
8326	F05	S07	437J	637H	4	152	10.8	Inefficient
8328	F05	S07	437J	637H	3.7	151	11.2	Inefficient
8407	S05	F06	551G	911H	-4.1	131	7.2	Efficient
8410	S05	F06	551G	911H	-0.1	130	8.9	Average
8421	F05	S07	551G	911H	5.8	151	8	Inefficient

A-2. Microarray results of efficiency study.

Name	*P*-value	Fold Change	Description
BF074284	0.0024	0.41	Unidentified transcripts on BTA10 position 27141957-27141274
CB533071	0.0025	0.45	Unidentified transcripts on BTA19 position 44123200-44122637
AW417048	0.0033	0.49	Unidentified transcripts on BTA8 position 24418273-24418926
XM_582513	0.0036	2.3	Bos taurus similar to Homo sapiens CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2 (CTDSP2)
XM_604268	0.0038	2.35	Bos taurus similar to Homo sapiens carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9 (CHST9)
CB441016	0.0043	2.29	Unidentified transcripts
NM_174344	0.0069	0.56	Bos taurus heat shock 70 kD protein 3 (HSPA3)
XM_867100	0.0072	1.71	Bos taurus similar to Homo sapiens homeobox D1 (HOXD1)
AV606331	0.0073	5.57	Bos taurus similar to Homo sapiens synaptotagmin IV (SYT4)
NM_174773	0.0075	1.57	Bos taurus creatine kinase, muscle (CKM)
CB538551	0.0085	0.48	Unidentified transcripts on BTA16 position 51799535-51799051
XM_593936	0.0091	2.72	Bos taurus similar to Homo sapiens Fanconi anemia, complementation group D2 (FANCD2), transcript variant 1
BM030863	0.0092	1.68	Unidentified transcripts
CB447356	0.0097	1.69	Bos taurus similar to Homo sapiens v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) (MAF), transcript variant 1
XM_869454	0.0101	0.63	Bos taurus similar to Homo sapiens hypothetical protein MGC72075
NM_001034034	0.0111	1.52	Bos taurus glyceraldehyde-phosphate-dehydrogenase (GAPDH)
XM_584289	0.0113	1.85	Bos taurus similar to Homo sapiens B-cell CLL/lymphoma 6 (zinc finger protein 51) (BCL6), transcript variant 1

A-2. Continued

Name	*P*-value	Fold Change	Description
XM_584950	0.0115	0.6	Bos taurus similar to Homo sapiens SP100 nuclear antigen (SP100)
XM_613093	0.0126	0.67	Bos taurus similar to Homo sapiens cathepsin S (CTSS)
hmm239033	0.0127	1.72	Bos taurus similar to PREDICTED: Homo sapiens KIAA1394 protein (KIAA1394)
hmm169308	0.0133	0.53	Bos taurus similar to Homo sapiens lysozyme (renal amyloidosis) (LYZ)
XM_881933	0.0134	0.59	Bos taurus similar to Homo sapiens Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse) (MEIS2), transcript variant g
NM_001035304	0.0137	1.52	Bos taurus similar to Homo sapiens NDRG family member 2 (NDRG2), transcript variant 2
CB171289	0.0211	0.66	Bos taurus similar to Homo sapiens MAD2 mitotic arrest deficient-like 1 (yeast) (MAD2L1)
BE667389	0.0212	1.55	Bos taurus similar to Homo sapiens Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4 (CITED4)
NM_174542	0.0231	0.64	Bos taurus gamma-aminobutyric acid (GABA) A receptor, alpha 3 (GABRA3)
NM_001038117	0.0238	0.64	Bos taurus similar to Homo sapiens coiled-coil domain containing 52 (CCDC52)
XM_867923	0.0243	0.65	Bos taurus similar to Homo sapiens insulin-like growth factor 1 (somatomedin C) (IGF1)
XM_867687	0.0245	0.46	Bos taurus similar to Homo sapiens mesenchymal stem cell protein DSC54 (LOC51334)
XM_591494	0.0292	0.49	Bos taurus similar to Homo sapiens dynactin 6 (DCTN6)
XM_590109	0.0303	1.53	Bos taurus similar to Homo sapiens pyruvate kinase, muscle (PKM2), transcript variant 1
XM_866591	0.0305	0.63	Bos taurus similar to Homo sapiens TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa (TAF15), transcript variant 1

A-2. Continued

Name	*P*-value	Fold Change	Description
NM_173940	0.0306	1.98	Bos taurus myxovirus (influenza) resistance 1, (murine homolog) (MX1)
BE237035	0.0324	1.53	Bos taurus similar to Homo sapiens sarcoglycan, delta (35kDa dystrophin-associated glycoprotein) (SGCD), transcript variant 1
XM_611974	0.0325	0.61	PREDICTED: Bos taurus TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1 (TBC1D1), partial mRNA.
NM_001034328	0.0325	1.89	Bos taurus similar to Homo sapiens odd-skipped related 2 (Drosophila) (OSR2)
EE943676	0.0342	0.58	Unidentified transcripts on BTA29 position 6143082-6144010
NM_173985	0.0343	0.63	Bos taurus allograft inflammatory factor 1 (AIF1)
BE757901	0.0347	1.66	Bos taurus similar to Homo sapiens retinoid X receptor, alpha (RXRA)
NM_174094	0.0349	1.8	Bos taurus inhibin, alpha (INHA)
CB468423	0.0349	0.62	Bos taurus similar to Homo sapiens potassium large conductance calcium-activated channel, subfamily M, beta member 4 (KCNMB4)
XM_582508	0.036	1.66	Bos taurus similar to Homo sapiens timeless homolog (Drosophila) (TIMELESS)
XM_615433	0.036	1.51	Bos taurus similar to Homo sapiens guanine nucleotide binding protein (G protein), beta 5 (GNB5), transcript variant 2
XM_596652	0.0379	1.98	Bos taurus similar to Homo sapiens eEF1A2 binding protein (DKFZp434B1231)
NM_174014	0.0391	0.67	Bos taurus CD69 antigen (p60, early T-cell activation antigen) (CD69)
XM_865690	0.0393	0.63	Bos taurus similar to Homo sapiens V-set and immunoglobulin domain containing 4 (VSIG4)
XM_613380	0.0439	0.63	Bos taurus similar to Homo sapiens CD163 molecule (CD163), transcript variant 1
XM_598942	0.0442	1.64	Bos taurus similar to Homo sapiens leucine rich repeat neuronal 6A (LRRN6A)

A-2. Continued

Name	*P*-value	Fold Change	Description
NM_001038158	0.0443	1.55	Bos taurus similar to Homo sapiens myeloid leukemia factor 1 (MLF1)
NM_001038579	0.045	0.61	Bos taurus similar to Homo sapiens family with sequence similarity 33, member A (FAM33A)
BF654881	0.0453	1.77	Bos taurus similar to Homo sapiens neural precursor cell expressed, developmentally down-regulated 4 (NEDD4), transcript variant 2
AV592606	0.0456	1.57	Unidentified transcripts on BTA1 position 77843182-77842501
AW307950	0.0457	1.67	Bos taurus similar to Homo sapiens serine/arginine repetitive matrix 1 (SRRM1)
XM_587120	0.0464	1.64	Bos taurus similar to Homo sapiens ring finger protein 122 (RNF122)
XM_870939	0.0465	0.53	Bos taurus similar to Homo sapiens sphingosine kinase 1 (SPHK1), transcript variant 1
NM_174210	0.0471	2.92	Bos taurus uncoupling protein 3 (mitochondrial, proton carrier) (UCP3)
XM_581386	0.0472	1.52	Bos taurus similar to Homo sapiens cysteine and histidine-rich domain (CHORD)-containing 1 (CHORDC1)
XM_868860	0.0478	0.49	Bos taurus similar to Homo sapiens cytokine inducible SH2-containing protein (CISH)
XM_882229	0.0513	2.51	Bos taurus similar to Homo sapiens actinin, alpha 3 (ACTN3)

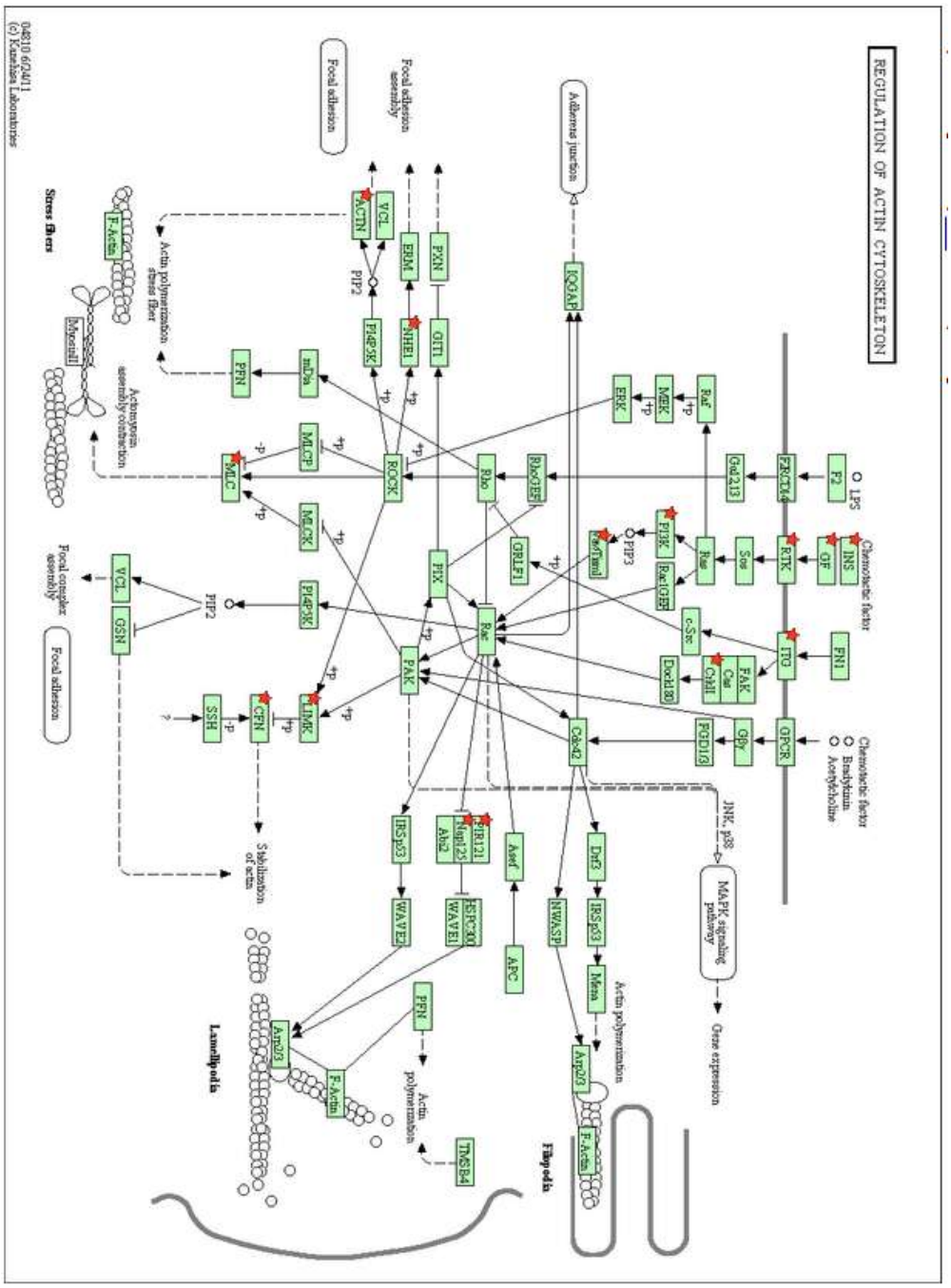
Fold change is given as inefficient group divided by efficient group.

A-3. Pedigree and model input data on animals selected for tenderness (WBSF ES residuals) study.

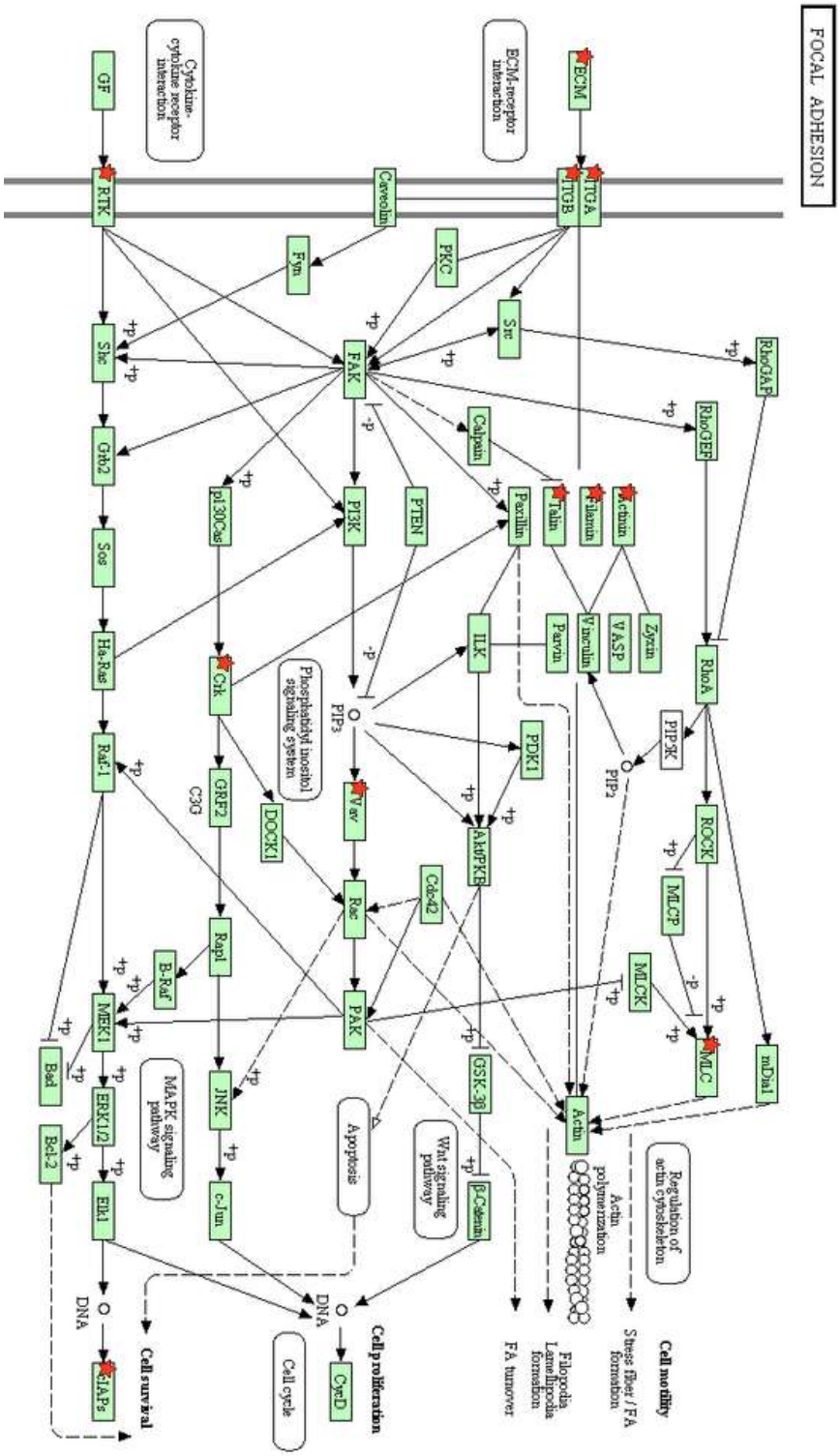
ID	BYS	SYS	Age, d	Sire	Dam	Tenderness Group (ES Residuals)	Tenderness Group (NES Residuals)	WBSF (ES)	Predicted WBSF (ES)	Residuals (ES)	WBSF (NES)	Predicted WBSF (NES)	Residuals (NES)
8125	S04	F05	524	437J	636H		High	2.5	2.2	0.3	4.4	2.7	1.7
7127	F04	S06	563	297J	760H		High	2.4	2.4	0	5.5	3.4	2.1
8035	F04	S06	569	551G	429H		High	2.5	2.4	0.1	5.1	3.4	1.8
7123	F04	S06	581	297J	760H	Low		1.6	2.4	-0.8	2.4	3.6	-1.2
7126	F04	S06	578	297J	760H	High		3.5	2.4	1.1	3.4	3.6	-0.1
8133	F04	S06	571	437J	636H	Low		1.6	2.4	-0.8	3.5	3.6	0
7112	S04	F05	553	297J	760H		Low	2.6	2.4	0.2	2.7	4	-1.3
7215	S04	F05	529	432H	511G		High	2.8	2.4	0.3	7.1	4	3.1
8303	S04	F05	545	437J	637H		Low	2.4	2.4	-0.1	2.5	4	-1.4
7736	S06	F07	547	551G	787G		Low	2.4	2.6	-0.2	4.4	6.1	-1.7
8159	S06	F07	550	437J	636H		High	2.8	2.6	0.2	7.8	6.1	1.7
7232	F05	S07	548	432H	511G	High		4.6	2.6	2	3.9	3.8	0.1
8148	F05	S07	568	437J	636H		High	2.8	2.6	0.1	5.9	3.8	2.1
8208	F05	S07	566	432H	559J	Low	Low	1.5	2.6	-1.2	2.6	3.8	-1.2
8328	F05	S07	575	437J	637H		Low	2.3	2.6	-0.3	2	3.8	-1.8
8004	F03	S05	571	551G	429H	Low	Low	2	2.6	-0.6	2.1	3.7	-1.6
8010	F03	S05	490	551G	429H	High		3.8	2.6	1.1	4	3.7	0.3
8113	S04	F05	537	437J	636H	High		3.7	2.7	1.1	3.5	3.7	-0.1
7742	S06	F07	549	551G	787G		High	2.7	2.7	0.1	5.2	3.3	1.9
8314	S05	F06	551	437J	637H	Low	Low	2.3	2.7	-0.4	2.2	3.7	-1.5
7738	S06	F07	544	551G	787G		Low	3.2	2.8	0.4	2.7	4	-1.3
7021	S05	F06	555	297J	431H		High	3.7	2.8	0.9	6.2	3.8	2.4
7728	S05	F06	533	551G	787G	Low	High	2.2	2.8	-0.6	5.1	3.8	1.4
7731	S05	F06	523	551G	787G	Low		1.9	2.8	-0.9	3.2	3.8	-0.6
8146	S05	F06	543	437J	636H	High		4	2.8	1.2	3.3	3.8	-0.4
7005	F03	S05	583	297J	431H	Low		2.2	2.8	-0.6	3.6	3.4	0.2
7009	F03	S05	576	297J	431H	High		4.6	2.8	1.8	3	3.4	-0.4

A-3. Continued

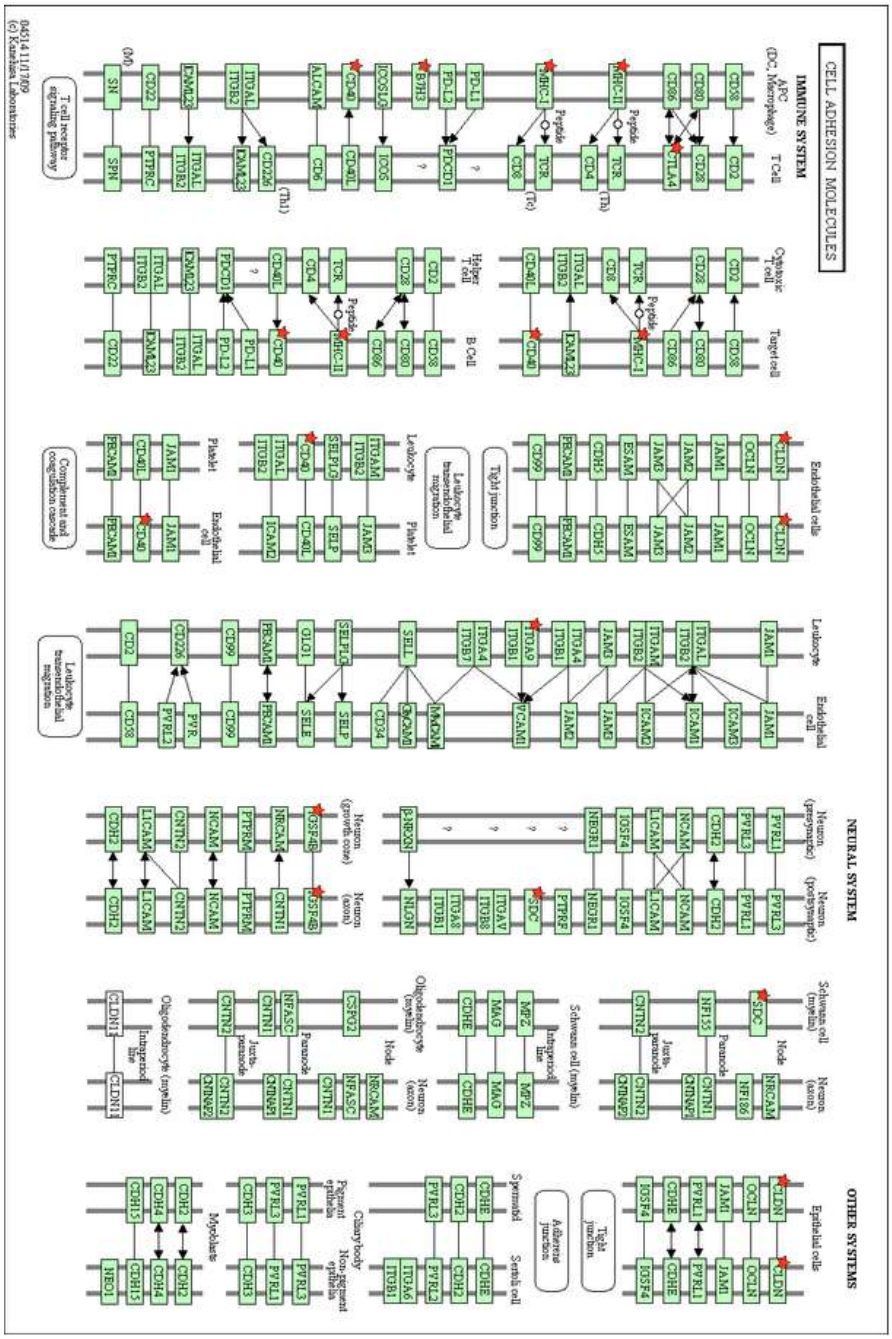
ID	BYS	SYS	Age, d	Sire	Dam	Tenderness Group (ES Residuals)	Tenderness Group (NES Residuals)	WBSF (ES)	Predicted WBSF (ES)	Residuals (ES)	WBSF (NES)	Predicted WBSF (NES)	Residuals (NES)
7115	S04	F05	551	297J	760H	Low		1.7	2.8	-1.1	3.6	3.1	0.5
8115	S04	F05	535	437J	636H	Low		2.3	2.8	-0.5	3.1	3.1	0
8156	S06	F07	566	437J	636H	High	Low	4.4	3	1.4	3.5	4.9	-1.5
7101	S03	F04	567	297J	760H	High	Low	4	3	1	2.7	4.3	-1.6
7303	S03	F04	558	432H	732H		High	2.6	3	-0.4	6.4	4.3	2
7715	F04	S06	569	551G	787G	High		4.1	3	1.1	4.8	4	0.8
8019	F04	S06	589	551G	429H		High	2.9	3	-0.1	6.9	4	2.9
8306	F04	S06	567	437J	637H		Low	2.7	3	-0.3	2.2	4	-1.8
7730	S05	F06	523	551G	787G	High		4.2	3.1	1.1	3.2	4	-0.8
8419	F05	S07	543	551G	911H	Low		2.4	3.5	-1.1	4.7	4.6	0.1
8420	F05	S07	543	551G	911H		High	4.3	3.5	0.8	5.6	4.6	1
7142	S06	F07	572	297J	760H	Low		2.5	3.5	-1	6.8	6.1	0.7
8157	S06	F07	562	437J	636H	High		4.7	3.5	1.1	5.4	6.1	-0.8
8050	S06	F07	565	551G	429H	High		4.9	3.7	1.2	5.4	4.5	0.9
8053	S06	F07	563	551G	429H		Low	3.6	3.7	-0.1	3.1	4.5	-1.4



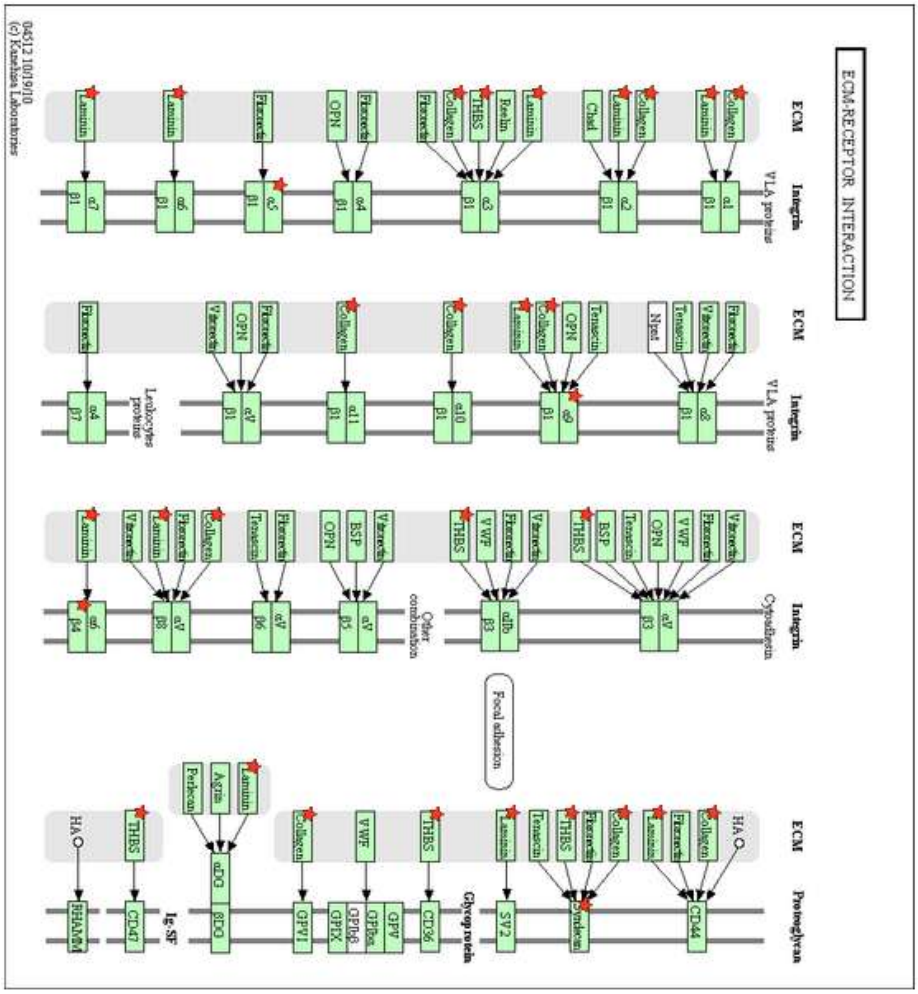
A-4. Regulation of actin cytoskeleton pathway based on KEGG pathways produced by DAVID Bioinformatics.



A-5. Focal adhesion pathway based on KEGG pathways produced by DAVID Bioinformatics.



A-6. Cell adhesion molecules pathway based on KEGG pathways produced by DAVID Bioinformatics.



A-7. ECM Receptor Interaction Pathway based on KEGG pathways produced by DAVID Bioinformatics.