

QUANTITATIVE INHERITANCE OF CALPASTATIN ACTIVITY AS AN
ASSESSMENT MEASURE FOR MEAT TENDERNESS
IN BRAHMAN STEERS

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ABSTRACT

Calpastatin activity at 24 h postmortem is inversely proportional to postmortem tenderization and accounts for a greater proportion of the variation in beef tenderness. Tenderness was determined by measurement of 24 h postmortem longissimus muscle calpastatin activity (CA) and Warner-Bratzler shear force after 7 and 14 d of steak aging. The quantitative inheritance of tenderness in Brahman steers was investigated phenotypically by Multiple Trait Derivative-Free Restricted Maximum Likelihood (MTDFREML) procedures and genotypically by using restriction fragment length polymorphisms (RFLP), a microsatellite marker, and DNA sequence analyses. In experiment 1, MTDFREML was used to obtain the genetic parameters of tenderness traits and their genetic and phenotypic correlations with carcass traits such as fat thickness, hot carcass weight, rib eye area, marbling score, and hump height. Contemporary group (CG) was defined as members that were exposed to the same environmental conditions with respect to year of birth and slaughter group. The statistical model included fixed effects of CG, random effects for animal, and residual effects with the covariable of slaughter age. Heritability estimates were 0.44 ± 0.17 , 0.30 ± 0.14 , and 0.21 ± 0.11 for calpastatin activity, d-7 shear force, and d-14 shear force, respectively. High heritability and additive genetic correlation estimates for CA and shear force indicated that genetic factors made a large contribution to the variation in tenderness and, therefore, tenderness traits would be reliable predictors not only for the rate of tenderization during the beef aging process but also for carcass characteristics.

In experiment 2, DNA samples were extracted for RFLP, microsatellite marker, and DNA sequence analyses from the CG of Brahman steers. No DNA polymorphisms were detected in a RFLP procedure conducted for a region between inhibitory domain I and inhibitory domain II of the calpastatin gene. However, microsatellite marker and DNA sequence analyses

conducted on the calpastatin promoter region and a region within the L-domain, respectively, revealed distinctive genetic polymorphisms within the calpastatin gene. These findings indicate that the polymorphic sites identified may reveal specific allelic diversity and such alleles may be used as a marker of tenderness for Brahman steer breeding.

CHAPTER 1

INTRODUCTION

Cattle improvement programs have focused on live animal growth traits since the 1940's. As consumers have become more diet conscious and more concerned with the palatability of meat, the desire to produce tender meat has increased. Morgan et al. (1991) stated that the single most important consumer component of beef palatability or eating quality is tenderness. In addition, because of the movement of the beef industry toward a more value based marketing system, providing this industry with high quality meats becomes more essential than ever.

Several studies have indicated that beef becomes less tender with the increase of Brahman inheritance (Crouse et al., 1989; Johnson et al., 1990). Wheeler et al. (1990) also stated that several sectors of the beef industry have expressed concern about the increased variability and lower tenderness associated with the increase of Brahman inheritance in beef cattle. O'Connor et al. (1997) recommended that composite cattle ideally should not be more than 3/8 Brahman breeding, as long as the other 5/8 consists of a breed(s) with high genetic potential for tender meat. Since then, the challenge has been to provide the beef industry with consistently tender meat from animals with Brahman inheritance.

One might ask why continue to use the Brahman breed in the commercial cattle industry when this breed appears to have a negative influence on the eating quality of beef? Why not replace the Brahman with another breed of cattle? The reason researchers continue to try to resolve the problem of Brahman tenderness is because of the importance of this breed in cross breeding and in crossbred commercial cow herds. Brahman first-year cows are more resistant to ecto and endo parasites, can tolerate the hot and humid climatic conditions of the southeast, and have excellent maternal ability and fertility (Damon et al, 1959; Turner, 1980). Because of these

attributes, it is estimated that at least 80% of the 15,000,000 beef cows in the southeast (1/3 of the U.S. cow populations) have some degree of Brahman inheritance. Critical aspects of beef palatability can be partially resolved by integrating the quantitative genetic inheritance of tenderness traits with molecular genetic basis of tenderness. Such integration may help in advancing genetic responses, establishing successful breeding programs, and identifying genetic markers that shed light on variability of tenderness within the Brahman breed.

Calpain, a calcium-dependent protease, has been recognized as a key player in postmortem tenderization of skeletal muscle (Koohmaraie, 1996). Calpastatin is a widely distributed endogenous inhibitor protein that specifically acts on calpain. The calpain system, and its inhibitor, calpastatin, is believed to be the primary proteolytic enzyme system involved in postmortem tenderization of aged beef (Koohmaraie et al., 1991). Koohmaraie (1996) indicated that the degradation of structural muscle proteins by calcium dependent neutral proteases is responsible for meat tenderization during postmortem storage of meat. Calpastatin activity at 24 h postmortem is inversely proportional to postmortem tenderization and accounts for a greater proportion of the variation in beef tenderness than any other single variable (Koohmaraie, 1994). Because of the significant role of calpastatin in tenderization, the present study focused primarily on calpastatin activity inheritance and its genotypic effect on meat tenderness. Multiple Trait Derivative-Free Restricted Maximum Likelihood procedures (Boldman et al., 1995) were used to: (1) estimate the additive genetic, environmental, and phenotypic variances for meat tenderness traits including calpastatin activity and Warner-Bratzler shear force, and (2) estimate the additive genetic and phenotypic correlations between calpastatin activity and carcass traits such as hot carcass weight, fat thickness, rib eye area, marbling score, and hump height. At the molecular level, RFLP, single-locus microsatellite-PCR, and DNA sequence analyses were used

to screen different DNA regions within the calpastatin gene in an attempt to establish a relationship between the observed calpastatin activity measurements and DNA polymorphisms. The presence of significant correlations between genetic markers (genotypes) within the promoter, coding, and non-coding DNA sequences of the calpastatin gene and calpastatin activity may increase fundamental knowledge about the magnitude of gene regulatory effect(s) that may influence calpastatin activity and allow the building of more realistic models for explaining phenotypic variations in meat tenderness. If a relationship between genetic marker(s) and meat tenderness can be established, then it will be possible to use marker-assisted selection for meat tenderness in Brahman cattle.

Therefore, the objectives of this study were to evaluate genetic parameters of meat quality traits and to classify the molecular basis of calpastatin activity in order to provide the beef industry with more palatable meat from steers containing Brahman inheritance.

CHAPTER 2

REVIEW OF LITERATURE

The exact mechanisms involved in the postmortem meat tenderization process and the nature of changes associated with improvement in tenderness are complex and not fully understood. The focus of this review is an attempt to clarify the factors affecting tenderness mechanisms, particularly the tenderness phases. The physiology, structure, and molecular genetics of calpastatin that explains how the endogenous calpains system is involved in meat tenderization, as well as genetic approaches for improving meat tenderness and the reason why calpastatin was chosen as a candidate gene for meat tenderness is discussed.

Factors Affecting Meat Tenderness

Research has shown that significant improvement in tenderness can be achieved by controlling the physiological processes that affect tenderness and processes that are influenced by environmental factors. These factors include carcass chilling (May et al., 1992), electrical stimulation (Nour et al., 1994), time on feed (Van Koevering et al., 1995), postmortem aging time (Huff-Lonergan et al., 1995), cooking method (Wulf et al., 1996a), vitamin D3 supplementation (Swanek et al., 1999), and end-point temperature (Wheeler et al., 1999). For example, Swanek et al. (1999) demonstrated that animals fed or injected with vitamin D3 for days before slaughter tended to have more tender meat.

The variation in tenderness is also due to the genetic variation, biological and physiological differences, biophysical changes during slaughter, and chemical differences created during post-mortem aging (Koohmaraie, 1996). In addition to these factors, the percentage of protein, fat, moisture, and collagen composition of meat may affect tenderness (Cross et al., 1973). In general, tenderness is affected by aging, extent of rigor, sarcomere length, proteolytic activity, and a variety of physiological and chemical factors that occur

during rigor mortis and postmortem (Pearson, 1987). Pre-rigor meat is tender but becomes progressively tougher as permanent cross-bridges form between myosin and actin (Pearson, 1987). Therefore, the variation of tenderness depends on the rate and extent of postmortem tenderization.

Rate and Extent of Meat Tenderization

Inconsistency in meat tenderness is due to a combination of the inability to produce routinely tender meat and the inability to identify carcasses that produce consistently tender meat and classify them accordingly.

At the time of slaughter, carcasses fail to undergo postmortem tenderization are classified as a tough meat. The toughness is affected by the development of rigor-mortis and the extent of tenderization process caused by the enzymatic break-down of the muscle contractile proteins (Jiang, 1998). Tenderization, as explained by Koohmaraie (1992a,b), also begins either at slaughter or shortly after slaughter, that results from the proteolysis of myofibrillar, denaturation of collagen, destruction of connectins, and disassociation of actinomyosin complex and the associated proteins. At the time of slaughter, Wheeler and Koohmaraie (1994) indicated that there were small variations in longissimus muscle tenderness, a muscle that possesses an intermediate shear force. In the next several hours after slaughter, there was a large decrease in sarcomere length (muscle shortening) that was accompanied by a large increase in shear force (toughness). Similarly, Ouali (1990) indicated that meat from some animals go through the tenderization process rapidly and may be consumed after one day aging, whereas other meat may be consumed after several days, and yet other meat would not be acceptable even after correct postmortem aging. These results indicate that sarcomere shortening is an important cause of meat toughening and animals tend to have similar tenderness levels due to the minor biochemical changes in meat at the time of

slaughter. The observed variations in tenderness are mainly due to the differences in the rate and extent of postmortem tenderization resulted from vigorous enzymatic and physiochemical reactions.

Physiology, Structure, and Molecular Genetics of Calpastatin

At the physiological level, calpastatin is an endogenous inhibitor of the calpains. Page et al. (2002) indicated that calpain degrades myofibrillar proteins under postmortem conditions and calpains appear to be the primary enzyme in the postmortem tenderization process. Calpain substrates include a variety of enzymes such as cytoskeletal proteins (Schoenwaelder et al., 1997), kinases and phosphatases (McGinnis et al., 1998), and epidermal growth factor receptors (Glading et al., 2000). The extent of physiological cleavage of these and other proteolytic proteins depends mainly on the presence and the activity of specific cell inhibitors. For example, calpastatin is one of the endogenous inhibitors that plays a key role in regulating calpains.

Many studies in livestock, particularly beef animals, have attempted to determine calpastatin's physiological role in tenderness, as well as the genetic components of the calpastatin gene (Killefer and Koohmaraie, 1994; Lonergan et al., 1995; Huff-Lonergan et al., 1996; Boehm et al., 1998). Levels of calpastatin vary considerably among species (Koohmaraie, 1991), breeds (Shackelford et al., 1994, 1995), and muscles (Geesink and Koohmaraie, 1999). An inverse relationship between calpastatin activity and tenderness in meat exists (Koohmaraie, 1994; Woodward et al., 2000). The relationship between calpastatin activity and muscle traits has also been reviewed in detail in livestock (Goll et al., 1998).

At the protein structural level, bovine calpastatin is a five-domain inhibitory protein (Figure 2.1) of a predicted molecular weight of 76 kDa (Killefer and Koohmaraie, 1994). Calpastatin is present in all tissues expressing calpains and in skeletal muscle. Calpastatin is

expressed at a higher level of activity than the calpains themselves. Of the five domains, the N-terminal leader (L) domain does not appear to have any calpains inhibitory activity (Emori et al., 1987), but may be involved in targeting or intracellular localization (Averna et al., 2001), while the other domains (I-IV) are highly homologous and are each independently capable of inhibiting calpains (Emori et al., 1987; Cong et al., 1998). Amino acid sequences for the calpastatin structural domains are given in Figure 2.2. Tompa et al. (2002) indicated that the inhibitory domains of calpastatin contain three highly conserved regions, A, B, and C, of which A and C bind calpain in a strictly Ca^{2+} -dependent manner but have no inhibitory activity, whereas region B inhibits calpain on its own. The presence of multiple calpastatin transcript isoforms suggested variation among isoforms in the choice and number of Cong et al. (1998) also found that the removal of the XL- and/or L-domains played a regulatory role by altering phosphorylation patterns on the protein. They reported that alternative promoters in bovine calpastatin led to the generation of different protein isoforms differing at the amino terminus. Differences in the N-terminal sequences deduced from the L-DNA domain might affect intracellular distribution of the inhibitors and efficiency of calpain-calpastatin interactions

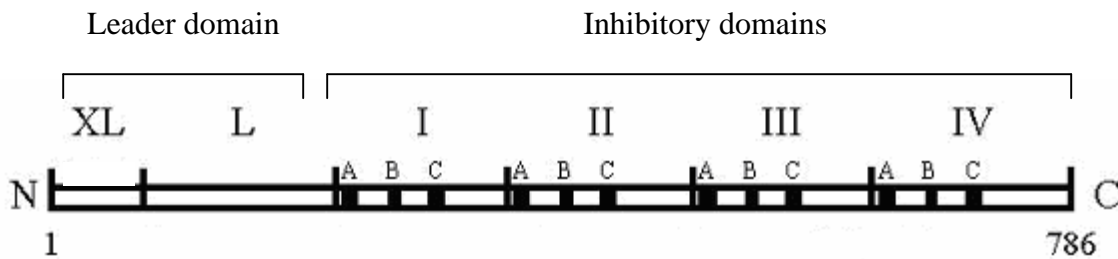


Figure 2.1. Structure of bovine cardiac calpastatin. Calpastatin is a five-domain inhibitory protein of 786 amino acids. It includes an N-terminal leader (XL and L) domain and four inhibitory domains (I - IV). The inhibitory domains of calpastatin contain three conserved regions, A, B, and C.

10	20	30	40	50	60
MNPTEAKAVK	TEPEKKPQSS	KPSVVHEKKT	QEVKPKHEHE	PKSLPKHSSD	TGVKHAPKEK
70	80	90	100	110	120
AVSKSSEQPP	SEKSTKPKTK	SQDKISGGGK	STVPAAAAAA	SAEPADKNKE	NKLLTSAVPA
130	140	150	160	170	180
ESKPSKPSGK	SDMDTALDDL	IDTLGEPPEM	KEDNTTYTGP	EVSDPMSSTY	IEELGKREST
190	200	210	220	230	240
PPPKYKELLN	KEEGIAGPPP	DSLKPLGPND	AIDALSSDFT	CSSLQLTTCS	PTADGKETEK
250	260	270	280	290	300
EKSTEEALKA	QSAGVIRSA	PPKEKRRKVE	EDTMTEQALQ	ALSASLGTRK	PEPELDPSSI
310	320	330	340	350	360
REVDEAKAKE	EKVKKCGEDE	ETVPSEYRLK	PATDKDGKPL	LPEAEKPKP	LSESELIDEL
370	380	390	400	410	420
SEDFDQSKPT	EKQSKPTEKT	EASPAAPYP	VAEDVPRTSM	CSLQSAPPTA	APAKGMVPDD
430	440	450	460	470	480
AVEALAGSLP	KKEADPEDGK	PVEDKVKEKA	KEEDRENFGE	KEETIPPDYR	LEEAKDKDGK
490	500	510	520	530	540
PLLKPEVKEP	LPPLSESVLL	DALSKDFTVP	SDTSSPQFED	AKLSAVVSEV	VSQTPAPTTQ
550	560	570	580	590	600
AAGPPPCAR	DNKELDDALD	QLSDTLGQRQ	PDPDENKVE	DKVKEKAKAE	HRDKLGERDD
610	620	630	640	650	660
TIPPKYQHLL	DDNKEGTPGK	PKDQRAQGIR	NCGEKPAGAQ	DPIDALSGDF	DSCPSTTETS
670	680	690	700		
TDTPKDKDKK	PASVPKHLGN	GKAKDSTKA	KEETSKPKAD	GKSTS	

Figure 2.2. Amino acid composition of calpastatin without the XL-domain (Killefer and Koohmaraie, 1994; Swiss-Prot Accession # P20811). Calpastatin includes the L-domain (1–148), inhibitory domain I (149–202), inhibitory domain II (292–344), inhibitory domain III (434–487), and inhibitory domain IV (571–624). inhibitory sites for calpain in the bovine (Cong et al., 1998) and mouse (Takano et al., 1999).

(Takano et al., 1999). At the transcriptional level, Killefer and Koochmarai (1994) found that bovine longissimus muscle mRNA contained calpastatin mRNA isoforms of 3.8, 3.0, and 1.5 kb. They indicated that the level of mRNA isoforms seems to be differentially regulated. For example, treatment with β -adrenergic agonists increased the ratio of 3.0:3.8 kb calpastatin isoforms. The potential allelic variations in the calpastatin gene could influence the amount or activity of calpastatin that might contribute to the conspicuous variation in meat tenderness observed in beef cattle (Lonergan et al., 1995). It is also possible that the allelic variation will be reflected in the promoter rather than the coding region of the gene (Nonneman et al., 1999).

Cong et al. (1998) found that at least one cAMP-responsive element resided within 102 nucleotides of the transcription initiation point of the calpastatin gene. They suggested that cAMP-dependent protein kinase may be involved in regulation of both calpastatin gene transcription and the extent of calpastatin protein phosphorylation. In addition, Cong et al. (1998) also showed that alternative promoter usage of the bovine calpastatin gene can influence gene expression by influencing the level of transcription initiation and translation efficiency.

Genetic Improvement of Meat Tenderness

Because focusing on the genetic advance through mass selection and progeny testing is a long-term approach to reduce the variability within low heritable traits (Falconer and MaCkay, 1996), researchers have focused their attention on other physiological and environmental factors that may positively affect on tenderness such as time on feed, stress, postmortem aging time, pH level, cooking method, and end point temperature to reduce the variation in tenderness. Nevertheless, the major impacts of genetic variation on meat tenderness are well documented. For example, it is well known that the mean shear force value and its standard deviation are known to increase as the percentage of Brahman (*Bos indicus*) inheritance increases (Crouse et. al., 1989). Another example of genetic contribution is the

case of the callipyge phenotype in sheep. The callipyge condition is a recently identified phenotype in lambs, which has a major effect on carcass composition by increasing total muscle weight and decreasing meat tenderness (Koochmaraie et al., 1995; Duckett et al., 2000). Johnson et al. (1990) also suggested that differences in meat tenderness among breed types (Angus vs Brahman) due to the variability of genetic compositions may be explained at least partially by differences in postmortem proteolytic enzyme activity. Animal breeders accept that the rate of genetic improvement in a given trait is a function of the heritability of the trait, the generation interval, and the selection differential. There is evidence to suggest that significant improvement in the shear force measurement can be made to improve its accuracy (Wheeler et al., 1994, 1996). Improving the shear force accuracy may increase the heritability estimate for shear force. Thus, high heritability estimate would decrease the time is required to make an improvement in tenderness through selection.

It has been assumed by the geneticists that controlling the genetic background of the slaughter cattle population would help solve the beef industry's tenderness problem. It is clear that genetic background makes a significant contribution to the total variation in tenderness as tenderness varies among and within breeds (Crouse et. al., 1989; Koochmaraie 1992a,b; Wheeler et al., 1995). However, analyses indicated that genetic and environmental factors contribute equally to variation in tenderness (Wheeler et al., 1995). Most estimates indicate that, within a breed, additive gene effects control about 30% of the variation in beef tenderness (Koch et al., 1982). This 30% represents the heritability of tenderness within a breed. This implies that within a breed, 70% of the variation is explained by environmental and non-additive gene effects (dominance and epistasis). On the other hand, Shackelford et al. (1994) showed that approximately 65% of the variation in tenderness among cattle of all breeds was due to genetic effects and 35% was environmental.

It is apparent that the development of a method to predict meat tenderness requires a sound knowledge of the mechanisms that regulate meat tenderness. Mechanisms of postmortem meat tenderization have been the main subject of many studies and many laboratories have contributed to this knowledge base (Koohmaraie et al., 1988, 1987; Ouali, 1990, 1992; Koohmaraie 1992a,b, 1994; Goll et. al., 1998). These studies suggested that proteins of calpain-mediated proteolysis were responsible for improvement in meat tenderness during postmortem storage of carcasses. If the differences in the rate and extent of proteolysis of these specific proteins account for differences in the rate and the extent of tenderization, then the variation in the tenderness of meat at the consumer level could be controlled by controlling these mechanisms during carcass storage. Evaluating the genetic parameters of calpastatin activity would aid understanding of calpastatin inheritance within Brahman cattle and taking an advantage of such knowledge in establishing calpastatin as a candidate gene for molecular investigation.

Quantitative Inheritance of Calpastatin Activity and Shear Force

Classification of tenderness is important for the beef industry because of the need to identify consistently tender meat, and it would improve the current quality control methodology (i.e., the USDA quality grading system). Tenderness is typically measured objectively by the force required to shear cores from muscle using a Warner-Bratzler shear device. More recently, calpastatin activity has been used to assess meat tenderness in many breeding programs. For example, Ouali and Talment (1990) found that calpastatin activity at 24 h postmortem was highly related to meat tenderness in all species. It is generally accepted that the use of genetically correlated traits may enhance rates of genetic response in livestock via reduced generation intervals, increased selection differentials, and/or increased accuracy of selection (Falconer and Mckay, 1996; Bourdon, 1997). Therefore, over the last decades, considerable

effort has concentrated on the development of genetic models for meat quality traits that are facilitated by the use of more specialized and efficient algorithms for estimation of Least-Squares Analyses (Harvey, 1960), Variance and Covariance components (Henderson, 1977), Maximum Likelihood (Harville, 1977), Restricted Maximum Likelihood (REML, Patterson and Thompson, 1971), Expectation-Maximization (Dempster et al., 1977), Derivative-Free Restricted Maximum Likelihood (DFREML, Graser et al., 1987), and Multiple Trait Derivative-Free Restricted Maximum Likelihood (MTDFREML, Meyer, 1989). However, estimation of genetic parameters is always associated with the estimation of variance components. It is apparent that variance comprises not only the observation of meat tenderness but also the covariances that arise between meat tenderness and the other carcass traits.

Advances in statistical estimation methodologies have enhanced the ability of researchers to obtain estimates of additive genetic (co)variances from beef cattle data. MTDFREML methodology is a set of programs designed to obtain estimates of (co)variance components using mixed model analyses that include pedigree information for more precise estimation of genetic parameters. The development of MTDFREML methodology has triggered many recent studies in beef cattle aimed to identify traits that are genetically related to meat tenderness (Barkhouse et al., 1996; Crews and Franke, 1998; Ferreira et al., 1999; Lee et al., 2000; Woodward et al., 2000; Riley et al., 2003). Some of these studies showed that calpastatin activity measured at 24 h postmortem in Brahman steers is positively associated with muscle shear force, a physical objective measure of meat tenderness. Shackelford et al. (1994) reported that calpastatin activity was highly heritable (heritability = 0.65). In support, Bertrand et al. (2001) reported an average heritability estimate of 0.54 for calpastatin activity from multiple studies.

Kim et al. (1998) reported a genetic correlation of -0.95 between calpastatin activity and taste panel tenderness. In other studies (Whipple et al., 1990a,b; Shackelford et al, 1991a,b), it was determined that calpastatin activity at 24 h postmortem explains a large proportion of the variation in aged beef tenderness. Wulf et al. (1996b) also found that tenderness and calpastatin activity at genetic level were highly inversely correlated.

Shear force has been widely used as a direct measure of meat tenderness. The heritability estimates for shear force reported by Wheeler et al. (1996), Kim et al. (1998), and Robinson et al. (1998) were 0.37, 0.18, and 0.30, respectively. Genetic correlations of 0.50 and 0.68 between calpastatin activity measured 24 h postmortem and shear force were reported by Shackelford et al. (1994) and Kim et al. (1998), respectively. Riley et al. (2003) reported that the genetic correlation estimates between 24 h postmortem calpastatin activity and shear force measured after 7 and 21 d of aging were 0.73 and 1.00, respectively. Sherbeck et al. (1996) reported that the phenotypic correlation between calpastatin activity and shear force was 0.24. Therefore, the high and moderate heritability estimates for 24-h calpastatin activity and shear force, respectively, coupled with high genetic correlations and moderate phenotypic correlations between calpastatin activity and shear force, suggest that meat tenderness traits can be used to establish possible relationships between calpastatin activity (phenotypes) and polymorphic regions within the calpastatin gene (genotypes). Because calpastatin protein has been shown to be a contributor to postmortem tenderization of skeletal muscle (Koohmaraie, 1996), conducting genotyping techniques on calpastatin gene could hasten our ability to control some of the genetic aspects of meat tenderness. The genetic contribution to tenderness can be evaluated by molecular biotechnology techniques such as polymerase chain reaction, genome mapping for Quantitative trait loci (QTL) detection, nucleic acid marker systems, and/or the candidate gene approach.

Calpastatin as a Candidate Gene Approach

The candidate gene approach requires examination of the existing knowledge of the biochemical basis of meat tenderness. As discussed earlier, calpain-mediated proteolysis of key myofibrillar proteins is believed to be responsible for postmortem tenderization. Estimates of the relationship between calpastatin activity and meat tenderness vary, but up to 40% of the variation in beef tenderness is explained by calpastatin activity at 24 h postmortem (Koohmaraie, 1994). Such a high degree of association justifies using calpastatin as a candidate gene for predicting meat tenderness. It is most likely that more than one gene is involved in regulation of tenderness, and the candidate gene approach only allows for examination of one gene at a time.

Thus, the drawback for this analysis is that the factors affecting the expression of the gene of interest (e.g., calpastatin) could be separated by large non-coding sequences. Such regulatory factors may not be identified in a candidate gene approach. Furthermore, because a candidate gene approach depends mainly on calpastatin activity measurement, calpastatin activity must be accurately determined to establish a reliable phenotypic-genotypic association. Over-estimation or under-estimation of calpastatin activity due to environmental effects will cause misleading interpretation for any possible phenotypic-genotypic correlations.

This review has shown that the differences in the potential proteolytic activity of the calpain system results in differences in the rate and extent of postmortem tenderization. Nevertheless, calpastatin activity at 24 hr postmortem is highly related to beef tenderness after 14 days of postmortem aging (Koohmaraie, 1994, 1996). Therefore, the calpastatin gene was considered a candidate for molecular investigation to further the understanding of quantitative genetic inheritance of calpastatin activity and its role in the variability of meat tenderness within Brahman cattle.

Applications of Molecular Biotechnology

Advanced technologies including the use of marker assisted selection, artificial insemination, cloning, transcriptional and translational assays, and gene transfer are being researched. Their commercial applications have the potential to change the way we identify superior animals and the dissemination of their genes in the beef industry. In farm animals, molecular markers are utilized for several important applications. Among these applications are: using polymerase chain reaction (PCR) based techniques for comparing genomes, identification of a candidate gene(s) for particular QTL, and for the investigation of animal biodiversity and evolution at specific loci (Gustavo and Gresshoff, 1997). For example, RFLP and single strand confirmation polymorphism techniques have been shown to be effective tools to assess genetic variability for the bovine calpastatin locus in animal populations (Bishop et al., 1993; Cong et al., 1995; Lonergan et al., 1995).

RFLP analyses and DNA markers have proven to be useful tools in investigating alleles responsible for quantitative traits in meat tenderness (Cockett et al., 1995; Lonergan et al., 1995; Palmer et al., 1999). For example, Lonergan et al. (1995) used a 2.2-kb cDNA coding for domains (2 through 4) plus a 3' untranslated region of bovine skeletal muscle calpastatin as a probe for calpastatin RFLP. Although they found DNA polymorphisms by using the restriction enzymes *BamH I* and *EcoR I*, the polymorphic *EcoR I* and *BamH I* restriction sites within the bovine calpastatin locus could not detect DNA sequence substitutions responsible for the variation in calpastatin activity or tenderness of aged beef. However, examining candidate genes of individual steers can help identify markers whose allelic (haplotype) frequencies may correlate with the variability of calpastatin activity. If such polymorphisms exist, DNA-based markers can be established and such markers can be used as tools by animal breeders to establish marker-assisted selection for improved meat tenderness. However, such examination

requires pre-knowledge of the DNA sequence for candidate genes that presumably are responsible for the variation in meat tenderness. Identified QTL can subsequently be used directly in screening commercial herds for marker assisted breeding, as the markers may segregate with the trait of interest.

Microsatellite markers are based on either hybridization or PCR-based techniques (Karp and Edwards, 1997). The PCR-based technique includes simple sequence repeat (SSR) amplification, inter-SSR amplification, randomly amplified microsatellite polymorphism, and selective amplification of microsatellite polymorphic loci (Gustavo and Gresshoff, 1997). However, the lack of allelic information both in terms of dominance and assignment of alleles to loci is a limitation of arbitrarily amplified DNA. These problems are solved by PCR directed to a specific, single locus although PCR requires pre-knowledge of the simple sequence repeat (SSR) of the target or flanking target regions (Karp and Edwards, 1997). Nonneman et al. (1999), however, have reported highly informative microsatellite markers within the promoter regions of the calpastatin gene. Therefore, the sequence-tagged microsatellite site is a highly attractive marker because each primer pair is expected to identify a single allele in a specific locus. The length of the amplified fragment will vary according to the number of repeated residues $(CA)_n$. The presence of significant correlations between genetic markers (genotypes) within the promoter sequence and calpastatin activity may give a fundamental knowledge about the magnitude of gene regulatory effect(s). This knowledge will likely allow building more realistic models for explaining phenotypic variations in meat tenderness. Once a relationship between a genetic marker(s) and meat tenderness has been established, it will be possible to use marker assisted selection to improve tenderness in Brahman cattle and to integrate such markers into different breeding strategies.

In summary, to reduce toughness and the variation in tenderness in the Brahman breed, one must first understand the mechanisms involved in tenderization. If the causes of variation are identified, then it may be possible to manipulate the causes advantageously. Breeding programs aim to improve the genetic merit of individuals for beef quality and correlated traits with meat tenderness. Therefore, the important component of each breeding program is the identification of traits with the highest genetic variation (breeding values) that can be used as selection criteria for the next generations or for molecular studies.

CHAPTER 3

ESTIMATED GENETIC PARAMETERS FOR TENDERNESS IN BRAHMAN STEERS

Introduction

Improvement of meat quality is one of the top priorities of the beef industry. Morgan et al. (1991) considered meat tenderness a primary palatability characteristic for consumers. The problem of producing inconsistently tender meat needs resolution. Attempting to resolve the problem of variability of meat tenderness, many reports on the inheritance of meat tenderness traits, such as calpastatin activity and Warner-Bratzler shear force, have been published (Marshall, 1994; Bertrand et al., 2001; Riley et al., 2003). However, estimates of heritability, additive genetic correlations, and phenotypic correlations for meat tenderness traits within Brahman steers have been variable (Sherbeck et al., 1996; Crews and Franke, 1998; Riley et al., 2003). The differences in magnitude of heritability and genetic correlation estimates among carcass quality and growth traits may be attributed to differences in the degree of Brahman inheritance (Crews and Franke, 1998; Elzo et al., 1998), choice of genetic model (Ferreira et al., 1999), choice of covariables (Lee et al., 2000), and/or data structure (Norris et al., 2002). Ferreira et al. (1999) indicated that a full animal model that includes individual performance and pedigree information, would provide the beef industry with reliable estimates of genetic parameters and should result in improved genetic selection programs.

Although genetic parameters of carcass traits have been reported (Marshall, 1994; Crews and Franke, 1998; Elzo et al., 1998; Riley et al., 2003), the quest to predict new and better genetic parameters that explain significant variation in meat quality traits in Brahman cattle continues. Therefore, the objective of this study was to use a full animal model to estimate heritabilities, genetic correlations, phenotypic correlations, and breeding values of carcass traits,

including calpastatin activity and Warner-Bratzler shear force, to further our understanding of the inheritance of tenderness in cattle.

Methods and Materials

Description of Data

Paternal half-sib groups of purebred Brahman male calves (2 to 25 calves per group) were purchased from purebred Brahman breeders throughout the state of Louisiana. Each producer was notified by a letter explaining the nature of the study and was asked to participate. Calves were spring born and weaned in the fall. Calves were purchased on the basis of weight and feeder calf score. Prices paid were based on average prices paid for similar calves in Louisiana auction markets the week the calves were delivered to Louisiana State University (LSU). Arrangements were made for the calves to be transported at weaning to the LAES Central Station Ben Hur Farm.

Five years of data were compiled for this study. Data were available on 97, 70, 73, 117, and 73 Brahman calves born in 1997, 1998, 1999, 2000, and 2001, respectively, and sired by 68 bulls. Calves were castrated, dehorned if necessary, given appropriate vaccinations and dewormed within two weeks after arrival. The herd health program was under the direction of the Large Animal Clinic in the School of Veterinary Medicine at Louisiana State University. Each calf was ear tagged and ear notched with an LSU identification number for permanent identification.

After processing (see above), steers were placed on re-growth common bermuda (*Cynadon dactylon*) and dalligrass (*Paspalum dilatatum*) pastures with access to about four pounds per head per day of a high roughage corn based diet (12% protein) and native hay. Ryegrass (*Lolium multiflorum*) became available for grazing in early December. Steers were

placed on ryegrass and stocked at approximately 650 to 700 pounds of calf per acre. Steers were observed daily for sickness and unsoundness. Steers were given a growth implant upon arrival at the feedlot. Steers from each study year were fed as a single group. The steers were fed until they reached an average of 0.4 inch backfat and a slaughter weight of approximately 1175 lb, as determined by the feed-yard personnel. Steers were slaughtered by Sam Kane Beef Processors (Corpus Christi, Texas). Steers were electrically stunned by high voltage (> 400 volts) during slaughter. Carcass data were collected at the plant by Dr. Joe Paschal and associates from Texas A & M Extension Center in Corpus Christi, Texas. A longissimus muscle tissue sample (15 g) was collected after a 24 h chill for measurement of calpastatin enzyme activity. In each study year, calpastatin assays were run by Dr. Georgina Whipple-Van Patter at Central Community College, Hastings, Nebraska, following the procedures of Whipple et al. (1990a) and Shackelford et al. (1994).

Traits Evaluated

The evaluated traits, numbers of observations, and statistical summary are given in Table 3.1. Carcass traits of interest include 24 h postmortem longissimus muscle calpastatin activity, Warner-Bratzler shear force after 7 and 14 d of steak aging, subcutaneous fat thickness of 12th rib, hot carcass weight, rib eye area, marbling score, hump height, and average daily gain. The data in each year were processed and managed for all animals by identical protocols and procedures to determine 24 h postmortem calpastatin activity and shear force values after 7 and 14 d of steak aging.

Measurement of Calpastatin Activity

The longissimus muscle activity of calpastatin was determined using methods similar to those described by Whipple et al. (1990a) and Shackelford et al. (1994). Five grams of muscle

Table 3.1. Descriptive statistics for carcass traits of Brahman steers.

Trait ^a	n	Mean	SD	CV
CA, units/g	355	4.51	1.22	26.98
WBS7, kg	428	4.57	1.21	26.56
WBS14, kg	427	3.86	0.86	22.26
FT, cm	430	0.88	0.42	47.35
HCWT, kg	430	336.45	36.89	10.96
REA, cm ²	430	85.98	8.72	10.14
MS ^b , units	430	390.30	61.68	15.80
HH, cm	420	19.36	3.30	17.03
ADG, kg	430	1.49	0.21	15.77

^aCA = muscle calpastatin activity after 24 h postmortem, WBS7 = Warner-Bratzler shear force after 7 d of steak aging, WBS14 = Warner-Bratzler shear force after 14 d of steak aging, FT = fat thickness of 12th rib, HCWT = hot carcass weight, REA = rib eye area, MS = marbling score, HH = hump height, and ADG = average daily gain.

^b200 to 299 = traces of marbling; 300 to 399 = slight marbling; 400 to 499 = small amount of marbling.

tissue were extracted in 30 mL of extraction buffer (150 mM Tris-HCl, 5 mM EDTA, pH 8.3, 0.2% β -mercaptoethanol, 100 mg/ml ovomucoid). Homogenization was carried out two times at 500 rpm for 30 seconds. The homogenate was centrifuged for 30 min at 12,000 x g. The supernatant was filtered through glass wool. After filtration, the supernatant volume was recorded for subsequent calculation of calpastatin activity. The supernatant was heated for 15 minutes at 98 °C to denature calpains, and then chilled on ice for 15 minutes. Calpastatin retains its activity under these conditions (Shackelford et al., 1994). The coagulated protein was

scrambled with a small glass rod to facilitate separation of the supernatant and pellet during centrifugation. In a two step centrifugation process, chilled aliquots of supernatant were centrifuged for 30 min at 1,500 x g with the supernatant transferred to 50-mL centrifuge tubes for a second centrifugation at 35,000 x g. The sample volume was brought up to 1 mL using an elution buffer (20 mM Tris-HCl, pH 7.35, 0.5 mM EDTA, 0.2% mercaptoethanol). Twenty microliters of partially purified standard m-calpain were added along with 930 μ L of assay media (100 mM Tris, pH 7.5 with 1 N acetic acid, 1 mM NaN_3 , 0.5% casein and 0.2% β -mercaptoethanol), followed by 50 μ L of 200 mM CaCl_2 . The assay reaction mixture was vortexed and then incubated for 1 h at 25°C. The reaction mixture was stopped by adding 2 mL of 5% trichloroacetic acid. The reaction mixture was centrifuged at 1,500 x g for 30 minutes. The A_{278} was determined on the supernatant, and the calpastatin activity was calculated using the methods of Koochmaraie (1990).

Measurement of Warner-Bratzler Shear Force

A wholesale loin section was obtained from the right side of each carcass and transported (2 °C) to the Louisiana State University Meat Laboratory. Vacuum-packaged steaks (2.54-cm-thick) were frozen (-20 °C) after 7 and 14 d of aging. These steaks were randomly assigned to either the 7 or 14 d ageing treatment group. For Instron Warner-Bratzler shear force determinations, aged samples were tempered and thawed at 4 °C for 24 h. Steaks were broiled to a final internal temperature of 70 °C on a Farberware Open-Hearth grill. After cooking, steaks were allowed to cool (24 h at 4 °C) and six cores of 1.25 cm in diameter were collected per steak. Cores were removed parallel to the direction of the muscle fibers for measurement of shear force. Each core was sheared using an Instron testing instrument equipped with a Warner-Bratzler attachment. Six shear force measurements were taken and the average of the measurements was

used as the shear force value for each steak. These procedures are consistent with guidelines established by the American Meat Science Association (AMSA, 1995).

Statistical Analysis

Data were analyzed using a software package titled Multiple Trait Derivative-Free Restricted Maximum Likelihood (MTDFREML; Boldman et al., 1995). This program was used to estimate (co)variance components using a full animal model and derivative-free restricted maximum likelihood. Four generation pedigrees of the Brahman steers were used to create a numerator relationship matrix. A total of 2155 animals were included in the numerator relationship matrix.

Each trait was first analyzed independently to obtain estimates of additive, environmental, and phenotypic variances. The animal model for single and multiple trait analyses included fixed effects for contemporary groups (see below), random effects for animal and residual effects, and a covariate for age of Brahman steers at slaughter. Contemporary group was defined as a group of steers that were born in the same year and harvested in the same group. There were 13 contemporary groups with an average of 33 steers per group. The age of steer at slaughter (average = 545 d) was included as a linear covariate. Estimation of the genetic parameters in this model involved partitioning phenotypic covariances between relatives into its components using the degree of relationship between animals. A linear mixed animal model was written as follows:

$$\mathbf{Y}_i = \mathbf{X}_i \mathbf{b}_i + \mathbf{Z}_i \mathbf{u}_i + \mathbf{e}_i \quad \text{where,}$$

\mathbf{y} is a vector of observations for the i^{th} trait; \mathbf{X} is the known incidence matrix relating observations to the respective fixed effects of the i^{th} trait; \mathbf{b} is a vector of fixed effects (contemporary group) of the i^{th} trait; \mathbf{Z} is a known incidence matrix relating observations to the

respective random effects of the i^{th} trait; \mathbf{u} is a vector of random effects of the i^{th} trait; and \mathbf{e} is a vector of residual effects of the i^{th} trait. The expected means and the variances of the single trait analyses model were assumed to be:

$$\mathbf{E} \begin{pmatrix} \hat{y} \\ \hat{\mathbf{u}} \\ \hat{\mathbf{e}} \end{pmatrix} = \begin{pmatrix} \mathbf{X}\mathbf{b} \\ \mathbf{0} \\ \mathbf{0} \end{pmatrix}; \quad \text{Var} \begin{pmatrix} \hat{\mathbf{u}} \\ \hat{\mathbf{e}} \end{pmatrix} = \begin{pmatrix} \mathbf{A}\sigma_a^2 & \mathbf{0} \\ \mathbf{0} & \mathbf{I}_n\sigma_e^2 \end{pmatrix} \text{ where,}$$

\mathbf{A} is the additive numerator relationship matrix among animals, σ_a^2 is the direct additive genetic variance, \mathbf{I}_n is identity matrices of order equal to the number of animals, and σ_e^2 is the residual variance. This assumption was extended to include two-trait analyses as follows:

$$\text{Var} \begin{pmatrix} \hat{\mathbf{u}}_1 \\ \hat{\mathbf{u}}_2 \\ \hat{\mathbf{e}}_1 \\ \hat{\mathbf{e}}_2 \end{pmatrix} = \begin{pmatrix} \mathbf{A}\sigma_{a1}^2 & \mathbf{A}\sigma_{a1}\sigma_{a2} & \mathbf{0} & \mathbf{0} \\ \mathbf{A}\sigma_{a1}\sigma_{a2} & \mathbf{A}\sigma_{a2}^2 & \mathbf{0} & \mathbf{0} \\ \mathbf{0} & \mathbf{0} & \mathbf{I}_1\sigma_{e1}^2 & \mathbf{0} \\ \mathbf{0} & \mathbf{0} & \mathbf{0} & \mathbf{I}_2\sigma_{e2}^2 \end{pmatrix} \text{ where,}$$

\mathbf{A} is the additive numerator relationship matrix among animals, σ_a^2 is the direct additive genetic variance, $\sigma_{a1}\sigma_{a2}$ is the covariance for the additive genetic effects between compared traits, \mathbf{I}_1 and \mathbf{I}_2 are identity matrices of order equal to the number of animals; and σ_e^2 is the residual variance.

Prior values for variances were used as starting values and the program solved for the best variances through iterations. To avoid the convergence to a local maximum of variance component estimates in MTDFREML algorithm, values from several sources (Sherbeck et al., 1996; Crews and Franke, 1998; Riley et al., 2002) were used as starting values with convergence 1×10^{-6} . The (co)variance components from analyses were used as a prior for the next run with convergence criterion 1×10^{-9} . This process was repeated until minus twice the logarithm of the likelihood for two successive restarts differed by less than 1×10^{-4} . A minimum of ten cold starts

was used to ensure the convergence to the same estimates. No changes were seen in (co)variance components after using different starting values.

Results and Discussion

Heritabilities and Genetic (Co)variances

Estimates of additive genetic, environmental, and phenotypic variances from a full animal model are given in Table 3.2. The additive genetic, environmental, and phenotypic variances obtained in this study were more variable than those obtained from a multiple-trait sire model by Barkhouse et al. (1996), a two-trait animal model by Crews and Franke (1998), or a full animal model by Riley et al. (2002).

Heritabilities for tenderness and carcass traits are also given in Table 3.2. The heritability estimates for all carcass traits were significantly different from zero ($P < 0.05$). Estimates of heritability (\pm SE) for calpastatin activity, shear force after 7 d of steak aging, and shear force after 14 d of steak aging were 0.44 ± 0.17 , 0.30 ± 0.14 , and 0.21 ± 0.11 , respectively. The heritability estimate of 24 h postmortem muscle calpastatin activity agreed closely with average estimates from many studies as summarized by Kim et al. (1998), was higher than the estimates of 0.15, 0.30, and 0.07 presented by O'Connor et al. (1997), Robinson et al. (1998), and Riley et al. (2003), respectively, and was lower than the estimate (0.65) of Shackelford et al. (1994). The high heritability estimates for calpastatin activity and shear force observed in this study suggest that these meat tenderness assessment traits should be responsive to selection in Brahman cattle. In addition, moderate heritability estimates from studies of crossbred *Bos taurus* cattle (Gregory et al., 1995) and crossbreds with some *Bos indicus* (O'Connor et al., 1997; Elzo et al., 1998) have suggested that tenderness traits would be responsive to selection programs. However, others have reported lower estimates of heritability in tenderness traits (Van Vleck et al., 1992;

Table 3.2. Estimated additive genetic (σ_a^2), environmental (σ_e^2), and phenotypic (σ_p^2) variances and heritabilities ($h^2 \pm SE$) for carcass traits of Brahman steers.

Trait ^a	σ_a^2	σ_e^2	σ_p^2	$h^2 \pm SE$
CA	0.37	0.47	0.84	0.44* \pm 0.17
WBS7	0.30	0.72	1.02	0.30* \pm 0.14
WBS14	0.12	0.46	0.58	0.21* \pm 0.11
FT, cm	0.05	0.08	0.13	0.38* \pm 0.17
HCWT, kg	479.05	373.65	852.70	0.56* \pm 0.15
REA, cm ²	35.07	33.28	68.35	0.51* \pm 0.16
MS ^b , unit	1186.51	1977.27	3163.79	0.38* \pm 0.16
HH, cm	4.06	6.58	10.64	0.38* \pm 0.16
ADG, kg	0.01	0.03	0.04	0.33* \pm 0.14

^aCA = 24 h postmortem muscle calpastatin activity, WBS7 = Warner-Bratzler shear force after 7 d of steak aging, WBS14 = Warner-Bratzler shear force after 14 d of steak aging, FT = fat thickness of 12th rib, HCWT = hot carcass weight, REA = rib eye area, MS = marbling score, HH = hump height, and ADG = average daily gain;

^b200 to 299 = traces of marbling; 300 to 399 = slight marbling; 400 to 499 = small amount of marbling.

* Value is significantly different from zero ($P < 0.05$).

Barkhouse et al., 1996; Wulf et al., 1996b). The heritability estimate for shear force at d 7 of steak aging was nearly 50% higher than the estimate shear force after 14 d of steak aging. The magnitude of reduction in heritability between shear force after 7 and 14 d of steak aging may be due to the increase of environmental influences on tenderness during the steak aging.

Interestingly, the magnitude of reduction in heritability estimates between shear force at d 7 and d 14 was consistent to the reduction of heritability of shear force observed by O'Connor et al.

(1997) from 7 to 14 d of beef aging. The heritability of calpastatin activity was higher than those of shear force at d 7 and d 14 indicating that selection based on calpastatin activity may be more promising. Similar results were reported by Shackelford et al. (1994).

Woodward et al. (2000) reported the possibility of obtaining calpastatin activity from live-animal tissue samples other than longissimus muscle to predict end product tenderness. Biopsies (approximately 10 g) were obtained surgically two days before slaughter from the supraspinatus muscle from contemporary purebred Angus bulls and steers. The authors reported that there were high phenotypic correlation estimates between sensory panel tenderness and antemortem calpastatin activity ($r = -0.46$) and between 24 h postmortem calpastatin activity and antemortem calpastatin activity ($r = 0.78$). Therefore, it may be possible to change calpastatin activity rapidly, and subsequently, improve meat tenderness in beef cattle (Brahman) through selection by using muscle samples from live animals.

Literature reports on genetic parameters for growth and quality carcass traits based on steers with Brahman inheritance have been published (Sherbeck et al., 1996; Crews and Franke, 1998; Elzo et al., 1998; Riley et al., 2002). In our study, heritability estimates (\pm SE) were 0.56 ± 0.15 , 0.56 ± 0.15 , 0.51 ± 0.16 , 0.38 ± 0.16 , and 0.38 ± 0.16 for fat thickness, hot carcass wt, rib eye area, marbling score, and hump height, respectively. The heritability estimates for the carcass traits were within the range (0.31 to 0.68) reported by Marshall (1994), relatively similar to the estimates obtained by Riley et al. (2002), and generally larger than the heritability estimates reported by Crews and Franke (1998) for groups of Brahman steers differing in fractions of Brahman inheritance.

The estimates of genetic, environmental, and phenotypic covariances among traits are given in Table 3.3. The additive genetic covariances estimated between carcass tenderness traits

(24 h postmortem muscle calpastatin activity and shear force after 7 and 14 d of steak aging) and other carcass traits are higher and in opposite direction than the respective environmental covariances. These findings suggest that there were limited pleiotropic effects of the gene(s) for tenderness and carcass traits. Tenderness and carcass traits may be controlled by different genes which respond differently to the environment. The findings also suggest that the environmental influences could have confounding effects on the additive genetic advance that might be gained by direct selection in breeding programs.

Additive Genetic Correlations

Animal breeders use genetic correlation estimates to predict correlated responses in unselected traits due to direct selection on other traits. These responses are most likely due to the result of pleiotropy and/or genetic linkage. The estimates of genetic correlation can be useful to predict indirect changes with selection and are often required in selection indices (Falconer and MaCkay, 1996). The estimates of genetic and phenotypic correlation among carcass traits are given in Table 3.4. As expected, the genetic correlation estimates among tenderness traits were very high. Results suggested that selection for reduced 24 h postmortem calpastatin activity would reduce shear force values, or vice versa, and that there are high positive pleiotropic effects between the two traits. The high genetic correlation between calpastatin activity and shear force values showed that calpastatin activity had a role in regulating postmortem tenderization during the aging process. The results in this study supported the findings of Wiegand and Kriese-Anderson (2001) who found that calpastatin activity measured at the conclusion of aging time (d 2 and d 7 postmortem) were significantly correlated with shear force measured at d 2 and d 7 postmortem. Shackelford et al. (1994) and O'Connor et al. (1997) also observed moderate to high genetic correlations between calpastatin activity and shear force. The estimate of genetic

Table 3.3. Estimation of additive genetic (Cov_{a1a2}), environmental (Cov_{e1e2}), and phenotypic (Cov_{p1p2}) covariances for carcass traits of Brahman steers.

Compared traits ^a	$Cov_{a1,a2}$	$Cov_{e1,e2}$	$Cov_{p1,p2}$
CA, WBS7 (units/g x kg)	0.260	0.010	0.270
CA, WBS14 (units/g x kg)	0.170	0.030	0.200
CA, FT (units/g x cm)	-0.080	0.030	-0.050
CA, HCWT (units/g x kg)	-7.610	5.240	-2.370
CA, REA (units/g x cm ²)	-1.380	1.120	-0.260
CA, MS ^b (units/g x units)	-8.380	4.740	-3.640
CA, HH (units/g x cm)	-0.240	0.180	-0.060
CA, ADG (units/g x kg)	-0.010	0.020	0.010
WBS7, WBS14 (kg ²)	0.160	0.050	0.210
WBS7, FT (kg x cm)	-0.100	0.090	-0.010
WBS7, HCWT (kg ²)	-5.010	4.740	-0.270
WBS7, REA (kg x cm ²)	-0.230	0.640	0.410
WBS7, MS (kg x units)	0.500	-8.650	-8.150
WBS7, HH (kg x cm)	-0.410	0.280	-0.130
WBS7, ADG (kg ²)	-0.010	-0.002	-0.010
WBS14, FT (kg x cm)	-0.030	0.020	-0.010
WBS14, HCWT ((kg ²)	-3.310	2.530	-0.780
WBS14, REA (kg x cm ²)	0.400	0.380	0.780
WBS14, MS (kg x units)	0.120	0.130	0.250

(table 3.3 continued)

WBS14, HH (kg x cm)	-0.070	0.220	0.150
WBS14, ADG (kg ²)	-0.006	0.010	0.004
FT, HCWT (cm x kg)	0.550	2.160	2.710
FT, REA (cm ³)	0.000	-0.140	-0.140
FT, MS (cm x units)	1.860	0.610	2.470
FT, HH (cm ²)	-0.090	0.340	0.250
FT, ADG (cm x kg)	-0.003	0.010	0.008
HCWT, REA (kg x cm ²)	56.420	39.600	96.020
HCWT, MS (kg x units)	360.930	4.460	356.470
HCWT, HH (kg x cm)	-12.410	37.310	24.900
HCWT, ADG (kg ²)	1.330	2.170	3.510
REA, MS (cm ² x units)	29.920	-7.180	22.740
REA, HH, (cm ³)	-4.060	4.700	0.640
REA, ADG (cm ² x kg)	0.020	0.300	0.310
MS, HH (units x cm)	9.790	18.360	38.150
MS, ADG (units x kg)	1.000	0.590	1.580
HH, ADG (cm x kg)	-0.104	0.260	0.160

^aCA = 24 h postmortem muscle calpastatin activity, WBS7 = Warner-Bratzler shear force after 7 d of steak aging, WBS14 = Warner-Bratzler shear force after 14 d of steak aging, FT = fat thickness of 12th rib, HCWT = hot carcass weight, REA = rib eye area, MS = marbling score, HH = hump height, and ADG = average daily gain;

^b200 to 299 = traces of marbling; 300 to 399 = slight marbling; 400 to 499 = small amount of marbling.

correlation for calpastatin activity with the shear force at d 7 of steak aging for the present study was similar to the estimate reported by Riley et al. (2003).

The results of high heritability and genetic correlation between calpastatin activity and shear force in present Brahman steers suggest that the selection for decreased calpastatin activity would have a favorable effect on tenderness. Furthermore, the genetic correlation estimates of calpastatin activity with fat thickness, hot carcass weight, rib eye area, marbling score, and hump height were -0.16, -0.60, -0.61, -0.38, and -0.44, and -0.20, respectively, indicating that many of the genes that resulted in decreased calpastatin activity also resulted in additional desirable carcass traits. Low to moderate negative genetic correlations between meat tenderness traits and average daily gain (ADG) were found. A similar negative association between calpastatin activity and ADG was reported by Shackelford et al. (1994). Interestingly, the moderate negative genetic correlation observed between hump height and meat tenderness traits found herein suggest that Brahman steers with greater hump height tend to be tenderer than steers with less hump height. Thus, Brahman phenotypic appearance may be informative as an indication for tenderness.

In an attempt to integrate the inheritance of calpastatin activity with cell metabolism, growth, and function, it seems that the high genetic correlation estimates observed between calpastatin activity and carcass traits strongly support the hypothesis that calpastatin activity is indirectly related to carcass traits, fat deposition, and tenderness. For example, Johnson and Guttman (1997) pointed out that calpain was likely involved in the processing of numerous enzymes and cytoskeletal components, thereby linking their activity to a variety of intracellular events. However, observations of tenderness were not affected by the administration of BSA-estradiol (Crouse et al., 1987; Hunt et al., 1991), growth hormone (Fabry et al., 1987), glucocorticoids (Corah et al., 1995), or androgenic hormones (Gerken et al., 1995) in cattle. It seems that the physiological and biochemical mechanisms linking calpastatin activity to carcass

Table 3.4. Estimated additive genetic and phenotypic correlations^a for carcass traits of Brahman steers.

Trait ^b	CA	WBS7	WBS14	FT	HCWT	REA	MS	HH	ADG
CA, units/g		0.30	0.28	-0.13	-0.09	-0.03	-0.07	-0.02	0.14
WBS7, kg	0.78		0.27	-0.03	-0.01	0.05	-0.14	-0.04	-0.02
WBS14, kg	0.77	0.93		-0.03	-0.03	0.12	0.01	0.06	0.09
FT, cm	-0.60	-0.82	-0.34		0.25	-0.05	0.12	-0.20	0.24
HCWT, kg	-0.61	-0.42	-0.46	0.11		0.40	0.22	0.26	0.69
REA, cm ²	-0.38	-0.07	0.19	0.00	0.44		0.05	0.02	0.31
MS ^c , units	-0.44	0.03	0.01	0.23	0.49	0.15		0.21	0.08
HH, cm	-0.20	-0.37	-0.10	-0.20	-0.29	-0.35	0.14		0.65
ADG, kg	-0.16	-0.19	-0.15	-0.12	0.55	0.03	0.25	-0.43	

^agenetic correlation estimates are below the diagonal of blank cells; phenotypic correlation estimates are above the diagonal of blank cells.

^bCA = muscle calpastatin activity after 24 h postmortem, WBS7 = Warner-Bratzler shear force after 7 d of steak aging, WBS14 = Warner-Bratzler shear force after 14 d of steak aging, FT = fat thickness of 12th rib, HCWT = hot carcass weight, REA = rib eye area, MS = marbling score, and HH = hump height; ADG = average daily gain.

^c200 to 299 = traces of marbling; 300 to 399 = slight marbling; 400 to 499 = small amount of marbling.

traits are required further investigation. The results of the present study also implied that the selection in favor of fat thickness, hot carcass weight, rib eye area, or marbling would be successful in breeding programs owing to an expected correlated reduction in calpastatin activity and, therefore, increase of tenderness. Gwartney et al. (1996) indicated that heifer carcasses from a high marbling group had better ratings for muscle fiber tenderness and overall tenderness than carcasses from a low marbling group of heifers.

Thus, selection for increased tenderness may result in increased fatness, something that may be undesirable. For example, Splan et al. (1998) showed that calving difficulty had favorable genetic correlations with measures of carcass tenderness and indicated that selection for some traits expressed in one sex of beef cattle may result in undesirable responses in traits expressed in the opposite sex. Therefore, caution should be exercised in breeding programs when calpastatin activity is used as selection criteria to improve tenderness. Overall, no unfavorable genetic correlations were identified in this study and it seems that calpastatin activity is a suitable predictor for improving not only tenderness but also carcass traits.

Based on genetic correlation estimates for shear force, improvement in shear force would be associated with decreased longissimus muscle calpastatin activity and increased 12th rib fat thickness and hot carcass weight and would have little or no effects on rib eye area and marbling score. In addition, genetic correlation estimates for shear force after 7 and 14 d of steak aging with fat thickness and hot carcass weight averaged -0.58 and -0.46, respectively. This means that as fat thickness or hot carcass weight increased, the amount of force needed to cut through the steak decreased. In essence, steaks from carcasses with more fat thickness or hot carcass weight were tenderer.

Phenotypic Correlations

Estimated phenotypic correlations for meat tenderness and growth carcass traits are also given in Table 3.4. Although the phenotypic correlation estimates were of moderate magnitude, the estimates were higher than those reported by Riley et al. (2003). It seems that the decrease of phenotypic correlation between calpastatin activity and shear force measurements is associated with the sharp decrease of phenotypic correlation between calpastatin activity and carcass traits. Estimates of phenotypic correlation for calpastatin activity with fat thickness, hot carcass weight,

rib eye area, marbling score, and hump height were -0.13, -0.09, -0.03, -0.07, and -0.02, respectively. Such estimates indicated that increases in carcass traits were associated with a slight phenotypic decrease in calpastatin activity. In addition, phenotypic correlation estimates of shear force with carcass traits ranged from 0.12 to -0.14 and averaged -0.004, indicating that there was little or no phenotypic association between carcass traits and shear force. In agreement, Elzo et al. (1998) observed similar ranges of phenotypic correlation estimates (0.16 to -0.16) between shear force and carcass traits. The small phenotypic correlation estimates between tenderness and carcass traits is likely due to the magnitude of environmental effects. It appears that environment influences between these traits cancel out the overall phenotypic (co)variation that tends to result from negative genetic correlations. The presence of high positive environmental correlations and high negative genetic correlation between tenderness and carcass traits indicates that these traits are controlled by different genes. Therefore, any significant genetic response that may result from direct selection to the correlated traits is likely due to linked genes rather than pleiotropic effects. Such results indicate that environmental covariances contribute larger to (co)variation in tenderness. Thus, it may be more efficient to improve tenderness in Brahman steers by indirect selection for 24 h postmortem calpastatin activity under appropriate controlled management and processing programs.

The estimates of the equations for the single regression of shear force after 7 and 14 d of steak aging (Y_{wbs7} , and Y_{wbs14} , respectively) on 24 h postmortem calpastatin activity (X) as calculated by the sire model for all of Brahman steer data were: $Y_{wbs7} = 0.28 X + 3.34$ and $Y_{wbs14} = 0.19 X + 3.16$ that explained 34% and 30%, respectively, of the variation. All regression parameters for both equations were significantly different from zero ($P < 0.05$). The correlations between the predicted and actual shear force after 7 and 14 d of steak aging were 0.58 and 0.55,

respectively. These data suggest that 24 h postmortem muscle calpastatin activity for slaughter steers can be accurately used to predict shear force after 7 and 14 d of steak aging.

Breeding Values of Brahman Steers for Market Purposes

Sire evaluation is a method of characterizing the breeding values of bulls based on progeny test information. The genetic merit of the offspring is dependent on the genetic makeup or breeding value of its parents. For this reason, the expected difference between the progeny of a bull and the original population is one-half the breeding value of that bull. In this study, genetic predictions were computed based on a best linear unbiased prediction procedure using a single-trait animal model. The predicted breeding values ranged from -0.72 to 1.2 units/g for 24 h postmortem muscle calpastatin activity and -0.75 to 0.91 kg for shear force after 7 d of steak aging (Table 3.5). Elzo et al. (1998) reported that the ranges of shear force breeding values for Angus, Brahman, and Brangus beef sires mated to dams ($\frac{1}{2}$ Angus $\frac{1}{2}$ Brahman) were from -2.68 to -1.26, -0.52 to 0.82, and -2.84 to -0.32 kg, respectively. Barkhouse et al. (1996) also reported that the range in predicted breeding values of sires for market animal tenderness was small and from -0.34 to 0.32 kg for market animal shear force. They found that the *Bos taurus* crosses had the most desirable breeding values and the *Bos indicus* crosses had the highest (less desirable) breeding values for shear force. Prediction and the accuracies of the breeding values are not only due to the decrease in number of progeny records within a sire but also due to the moderate heritability estimates observed for 24 h postmortem muscle calpastatin activity and shear force after 7 d of steak aging. Bourdon (1996) indicated that expected breeding values are determined by a number of factors, including the heritability of the trait, the number of records on the animal and its relatives, and the number of records on genetically correlated traits. Based on the estimates of breeding values from mixed model analysis, essentially the same bulls, listed in

Table 3.5. Estimates of expected breeding value (EBV) and accuracy (ACC) for Brahman sires derived from varying numbers of Brahman steer (progeny) records for 24 h postmortem muscle calpastatin activity and shear force after 7 d of steak aging.

Calpastatin Activity			Shear Force		
Sire #	EBV	ACC	Sire #	EBV	ACC
High Tenderness					
675776	-0.72	0.81	657770	-0.75	0.50
701487	-0.55	0.67	701487	-0.61	0.59
588945	-0.54	0.61	728763	-0.50	0.50
657770	-0.51	0.57	739591	-0.50	0.65
669699	-0.51	0.69	716854	-0.49	0.58
715234	-0.48	0.58	727631	-0.42	0.63
736360	-0.47	0.51	669699	-0.37	0.61
675284	-0.38	0.62	660155	-0.31	0.52
739591	-0.35	0.63	736360	-0.31	0.44
Low Tenderness					
687740	0.35	0.58	687740	0.30	0.52
750384	0.35	0.48	715953	0.31	0.48
692228	0.36	0.75	750381	0.34	0.46
700339	0.37	0.77	689781	0.43	0.55
699736	0.43	0.63	692228	0.45	0.69
672282	0.46	0.77	664333	0.46	0.58
634573	0.58	0.68	702545	0.48	0.60
674911	0.73	0.77	673981	0.56	0.75
689781	0.81	0.63	646690	0.65	0.48

Table 3.5, would be selected for either a combined reduction or increase in 24 h postmortem muscle calpastatin activity and shear force after 7 d of steak aging. The use of such animals with the lowest estimated breeding values for tenderness traits will result in genetic improvement in the population (i.e. average performance of the progeny generation will be improved over the average performance of the parental generation). Application of such a model for sire evaluation may help beef producers make timely, accurate decisions about bull selection based on their predicted breeding values.

Conclusion

Caution is necessary when interpreting these results because of the relatively small number of animals involved. Nevertheless, moderate to high estimates of heritability were observed for tenderness traits and carcass traits in the present Brahman cattle. The high estimate of heritability observed for muscle calpastatin activity indicated that this trait would respond to selection in the Brahman breed. Calpastatin activity and steak shear force also had moderate to high negative genetic correlation estimates with several carcass traits that are of economic importance in beef cattle. Such findings suggest genetic interdependency among these traits. It is apparent that muscle calpastatin activity after 24 h postmortem is highly related to beef tenderness after 7 or 14 d of postmortem steak aging. It is also clear that unless the pre- and particularly the post-slaughter conditions are carefully controlled, then genetic variation can be overshadowed by variation due to environmental effects. Therefore, calpastatin activity under appropriate controlled management and processing procedures becomes a useful physiological indicator trait that could be used to enhance rates of genetic change for tenderness or carcass traits.

CHAPTER 4

RELATIONSHIP OF RFLP, A MICROSATELLITE MARKER, AND DNA SEQUENCE ANALYSES WITH REGIONS OF THE CALPASTATIN GENE IN BRAHMAN STEERS

Introduction

The Ca⁺²-dependent protease calpain has been implicated as a major cause of the degradation of skeletal muscle during postmortem tenderization (Geesink and Koohmaraie, 1999). Evidence in beef cattle has shown that the levels of calpastatin, the inhibitory component of the system, measured 24 h after slaughter can be used to predict the degree of tenderization achieved at the end of the normal finishing period of steers (Goll et al., 1998). Although several genetic markers and QTLs affecting meat quality traits have been investigated in cattle (Lonergan et al., 1995; Keele et al., 1999; Stone et al., 1999; Rexroad III et al., 2001), the quest continues to identify markers that explain significant variation in these traits of economic importance. The structure of the calpastatin protein indicates that this protein contains five domains, four of which are inhibitory repetitive domains, and the last one corresponding to the N-terminal region of the protein (L-domain) lacks inhibitory activity (Emori et al., 1987; Takano et al., 2000). Research has shown that the L-domain has enzymatic regulatory properties (Killefer and Koohmaraie, 1994; Cong et al., 1998) and variable modulations for calpain inhibitory activity (Killefer and Koohmaraie, 1994) due to different post-transcriptional and post-translational modifications.

In attempts to clarify the source of quantitative variation of the calpastatin activity, the present study was conducted to screen regions of the calpastatin gene that could be responsible for the combined variation in 24 h postmortem muscle calpastatin activity and shear force after 7 d of steak aging. RFLP was used to investigate a region located between inhibitory domain I and

inhibitory domain II of the calpastatin gene. A microsatellite marker within 1.4 kb upstream of the initiation site for the promoter region of the calpastatin gene was investigated as well as the relationship between any possible polymorphic genotypes and 24 h postmortem calpastatin activities. DNA and deduced amino acid sequence alignments for the region within the L-domain were also investigated. The primary objective of the experiments was to identify novel molecular genetic markers, together with advances in the area of quantitative genetics that may lead to the development of marker assisted selection programs for improved meat tenderness.

Methods and Materials

Brahman Steer Population

Genetic parameters for 24 postmortem muscle calpastatin activity and Warner-Bratzler shear force after 7 d of steak aging for Brahman steers were discussed in Chapter 3. Identifying genetic markers for quantitative traits such as tenderness relies on the pedigree structure of the population and on the recording of useful data. Therefore, a mixed model analysis was conducted to verify the validation of using either 24 h postmortem muscle calpastatin activity or shear force after 7 d of steak aging as phenotypic responses for molecular investigation. Carcass and tenderness data of Brahman steers ($n = 430$) sired by 68 bulls were collected over 5 yr (1997 through 2001). The data in each year were processed and managed for all animals by identical protocols and procedures to determine 24 h postmortem muscle calpastatin activity and shear force after 7 and 14 d of steak aging. To reduce any possible existence of environmental influences on calpastatin activity and shear force measurements, DNA samples were extracted from individuals within specific contemporary groups. A contemporary group was defined as members that were exposed to the same environmental conditions with respect to year of birth, slaughter group, and age at slaughter. An attempt was made to compare two contrasting

tenderness groups of individuals derived from similar contemporary groups. One group was identified as having a combined reduction in 24 h postmortem muscle calpastatin activity and shear force after 7 d of steak aging (LCA) and the other group was sampled for a combined increase in 24 h postmortem muscle calpastatin activity and shear force after 7 d of steak aging (HCA). It was hoped that the possible occurrences of distinctive DNA polymorphisms between LCA and HCA groups may lead to the identification of specific DNA regions within the calpastatin gene that may relate to the variability of tenderness. Descriptive statistics for 24 h postmortem muscle calpastatin activity and shear force after 7 and 14 d of steak aging for the overall Brahman steer population and the LCA and HCA groups are given in Table 4.1.

Measurements of Tenderness Traits

The methods used to determine 24 h postmortem longissimus muscle calpastatin activity and Warner-Bratzler shear force after 7 and 14 d of steak aging for Brahman steers were discussed in Chapter 3.

DNA Extraction

Twenty-five μL of each thawed white blood cell (WBC) sample was digested by gentle mixing with 400 μL of a lysis buffer (10 mM Tris-HCl, 10.95% Saccharose, 1% Triton X-100, 5 mM MgCl_2). The mixture was centrifuged at 21 x g for 10 min and the pellet was resuspended in 200 μL DNA extraction buffer (0.01 M Tris, 0.005 M EDTA, 0.5% SDS). Proteinase K (20 μg) was added to the resuspended solution and incubated overnight at 50 °C. After incubation, 20 μL of 5 M NaCl was added to each sample. The samples were then extracted twice using 200 μL of 1:1 phenol-chloroform solution and centrifuged at 21 x g for 2 min. After centrifugation, the supernatant was transferred to labeled culture tubes and mixed with 100% cold ethanol (2x volume of supernatant). The DNA was then pelleted by centrifugation at 21 x g for 5 min,

Table 4.1. Descriptive statistics for 24 h postmortem muscle calpastatin activity (CA) and Warner-Bratzler shear force after 7 and 14 d (WBS7 and WBS14, respectively) of steak aging for the overall Brahman steer population and for steers sampled for either a combined reduction (LCA) or increase (HCA) in 24 h postmortem muscle calpastatin activity and shear force after 7 d of steak aging.

Group	Trait	n	Max	Min	Mean	STD	CV
Brahman Population	CA	355	9.20	1.80	4.51	1.22	26.98
	WBS7	428	9.90	2.00	4.57	1.21	26.56
	WBS14	427	7.00	1.70	3.86	0.86	22.26
LCA	CA	13	2.50	1.83	2.21	0.21	9.62
	WBS7	13	5.94	1.96	3.24	1.17	35.27
	WBS14	13	4.33	2.93	3.45	0.48	14.01
HCA	CA	17	5.90	3.97	4.85	0.51	10.69
	WBS7	17	6.67	2.73	4.46	1.12	25.24
	WBS14	17	5.05	3.14	3.99	0.62	15.55

washed twice with 2 mL 70% ethanol, dried at 30 °C for 5 min, and resuspended in 200 µL Tris (10 mM, pH 8.3). For microsatellite and DNA sequence analyses, DNA was extracted as follows: seven µM of WBC were diluted and vortexed in 1 mL of distilled water and then incubated at room temperature for 20 min. The diluted WBC was centrifuged at 21 x g and 970 µM of the supernatant was removed. One hundred seventy µM of 5% chelex-20 were added to the remained 30 µM of centrifuged WBC and incubated at 58 °C for 30 min. The chelex-DNA mixture was vortexed and incubated at 98 °C for 8 min. The mixture was then vortexed and then centrifuged at 21 x g for 3 min. The supernatant (180 µM) was stored at -20 °C and used for PCR

amplification. The concentrations of DNA samples were quantified according to the instructions of DyNA Quant™ 200 Fluorometer Manual (Hoeffer Pharmacia Biotech Inc., San Francisco, CA).

Source and Description of Primers

Primers were designed for specific DNA regions from a published DNA sequence of the bovine calpastatin genome. These regions were between the inhibitory domain I and inhibitory domain II (NCBI, Accession # AF117813), the bovine calpastatin promoter region (Cong et al., 1998; Nonneman et al, 1999), and the bovine calpastatin exons five and six within the L-domain (NCBI, Accession # AY008267). These primers were used to amplify products from Brahman steer genomic DNA of approximately 560, 640, and 250 bp, respectively.

The primer sequences used to amplify the region between inhibitory domain I and inhibitory domain II of the calpastatin gene were as follows: forward primer: 5'- TGG GGC CCA ATG ATG CCAT-3', and reverse primer: 5'GGT GGA GCA GCA CTT CTT CTG-3'. Primer sequences for microsatellite marker analysis were as follows: forward primer: 5'-GTA AAG CCG CAC AAA ACA CAC CCA GG-3', and reverse primer: 5'-CCT GGA CCC TCT GGA TGA GGA AGC GG-3'. In microsatellite analyses, the length of the amplified fragment will vary according to the number of repeated residues (CA)_n. Allelic frequencies were also calculated for the individuals based upon observed genotypic classifications. Statistical analyses (ANOVA) were performed within each region separately, and across the calpastatin gene. The primer sequences used to amplify exons five and six within L-domain were as follows: forward primer: 5'- ATG AGA AAA AAA CCC AAG AAG -3', and reverse primer: 5'- CTT TGG TTT TGT TGA TTT CTC-3'.

DNA Amplification and Sequence Analyses

The amplified products were examined by gel electrophoresis to confirm the exact size of polymerase chain reaction (PCR) amplification (Figure 4.1). A mixture of DNA *Taq* and *Pfu* polymerases was used in PCR elongation process to exhibit a high fidelity proofreading activity that minimizes the number of PCR-induced mutations when amplifying PCR products. A total of 35 cycles was adapted for denaturation at 94 °C / 45 sec, annealing at 57 °C / 45 sec, and elongation at 72 °C / 45 sec. The annealing temperature was modified to increase the stringency of PCR reaction and based on the primer's melting temperature used in the amplification process. A mixture (25 µL) was used for PCR amplifications and consisted of the following: 40-50 ng of purified genomic DNA, 10% of 10X PCR buffer (100 mM Tris, 15 mM MgCl₂, and 500 mM KCl), 4% of 10 mM dNTP, 4% of 15 pmol of forward and reverse primers, 0.2 unit of *pfu* DNA polymerase, and 0.8 unit of *Taq* DNA polymerase. DNA from the LCA and HCA groups of Brahman steers were amplified separately for auto-sequencing. Amplification products were purified with a Qiagen DNA purification kit (Qiagen Inc., Valencia, CA 91355) and DNA auto-sequencing was performed at GeneLab (Louisiana State University, School of Veterinary Medicine, Baton Rouge, LA 70803). The purified PCR-products were analyzed on a 3.5% agarose gel or a combination of agarose and synergelTM (Diversified Biotech, Boston, MA 02132) to get a functional equivalent of high concentration of 6% agarose gels. Electrophoretic steps were performed at 100-125 V. Tris-Acetic-EDTA gel buffer (1x) was used as a running buffer (1x buffer: 40 mM Tris base, 40 mM acetic acid, 1 mM EDTA). PCR products were mixed with one-fifth of the final reaction volume (25 µL) of loading dye (10% formamide, 4% bromophenol blue, 4% orange G, 4% xylene cyanole). Samples were loaded at room temperature. Wells within the gel were rinsed with running buffer and 6 µL of each sample was

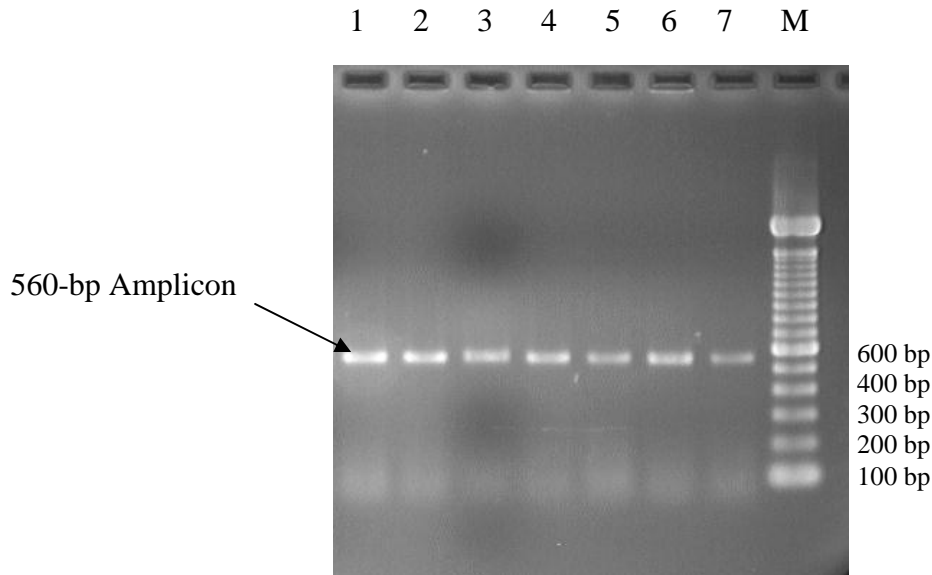


Figure 4.1. DNA samples were extracted from Brahman steers characterized with either a combined reduction or increase in 24 h postmortem muscle calpastatin activity and shear force after 7 d of steak aging. DNA amplicons (≈ 560 bp) from exons five and six of the calpastatin gene were amplified and electrophoresed on a 3.5% agarose gel. The amplicons were visualized by ethidium bromide. M= 100 bp DNA marker.

loaded per well. To detect DNA polymorphisms, a series of restriction digestions was conducted for 2-3 h at optimal temperatures with *EcoRI*, *TaqI*, *MfeI*, *SphI*, *NcoI*, *BamHI*, *MseI*, *ApoI*, and *HhaI* restriction enzymes that have the following recognition sites: (G[^]AATT), (T[^]CGA), (G[^]AATTG), (GCATG[^]C), (C[^]CATGG), (G[^]GATCC), (R[^]AATTY), (T[^]TAA) and (GCG[^]C), respectively. Restriction fragments were separated on fine resolution of agarose and SynergelTM gels and visualized by ethidium bromide or SYBR green.

Creating Consensus DNA Sequences

A consensus approach was used to generate a consensus sequence from heterogeneous DNA sequences (Rogozin and Kolchanov, 1992; Morgante et al., 1997). The simplest approach to determine a consensus sequence is to count the number of the same DNA bases that align in a

given column. This method is referred to as the majority method (Staden, 1982). If the majority base count is above a given fractional threshold of the total base count, then the given base is called unambiguous (A, C, G, or T); otherwise, the consensus is called as the appropriate ambiguous base (combination of A, C, G, and/or T). The main purpose of the method is to construct a set of consensus sequences matching all hotspot positions. DNA sequences for LCA and HCA groups were separately aligned in CLUSTAL X (Thompson et al., 1997) to build the consensus call. The consensus sequences for LCA and HCA groups were generated from 12 and 17 DNA sequences, respectively (Appendices A and B). The consensus call was created by the program CONSENSUS (EMBL, Heidelberg, Denmark; www.bork.embl-heidelberg.de) with a minimum threshold of 75% to remove all the ambiguous calls. For example, the counts for the first site in DNA sequences of HCA group (appendix B) were 2 and 15 nucleotide bases for cytosine and guanine, respectively. At this site, the guanine count (15 out of 17 DNA sequences = 88%) was greater than the 75% threshold and, therefore, the consensus call for the first nucleotide site in the sequence of the HCA group was guanine. The two consensus sequences of LCA and HCA groups were then aligned with the program CLUSTAL X (Thompson et al., 1997) to identify any possible polymorphic sites.

Results and Discussion

Restriction Fragment Length Polymorphisms

The RFLP technique was defined earlier for the bovine calpastatin gene (Bishop et al., 1993). RFLP was conducted mainly for a specific bovine calpastatin region between inhibitory domain I and inhibitory domain II of calpastatin gene (NCBI, Accession # AF117813).

Unfortunately, no DNA polymorphisms were detected in 50 samples of Brahman steers using the restriction enzymes *EcoR* I, *Taq* I, *Mfe* I, *Sph* I, *Nco* I, *BamH* I, *Mse* I, *Apo* I, and *Hha* I. Similar

patterns of restricted DNA fragments were visualized on the gel. The results showed that there was no evidence that allelic polymorphisms, digested by the particular restriction enzymes, were detected within the inhibitory domains I and II of the calpastatin gene of these Brahman steers. Perhaps significant polymorphisms may be detected by using additional restriction enzymes other than those used herein.

Microsatellite Marker Analysis

The mutational mechanism causing microsatellite loci to be hyper-variable is not completely understood. It is thought to be caused generally by a process referred to as DNA strand slippage that occurs primarily during DNA replication (Levinson and Gutman, 1987). The bovine calpastatin promoter region is contained in a cytosine-adenine repeat (CA)_n, which is located 1.4 kb upstream of the initiation site of the calpastatin gene (Nonneman et al., 1999). For microsatellite analysis, the sequence-tagged microsatellite site in the calpastatin promoter region was a highly attractive marker because a primer pair can identify a single haplotype in a specific locus. Amplification within the calpastatin promoter region revealed three distinctive genotypes. Eighty five DNA samples from contemporary groups of Brahman steers were used in sequence-tagged microsatellite analysis.

The polymorphism was a (CA)_n repeat that identified two alleles with lengths of approximately 250 and 254 bp (Figure 4.2). Brahman steers with both alleles were assigned the AB genotype, whereas those possessing only the A or B alleles were assigned the AA or BB genotypes, respectively. The genotypic frequencies for AA, AB, and BB are given in Table 4.2. The genotypic frequencies for heterozygous genotypes (AB) were most abundant in these Brahman steer samples. Least-squares mean (\pm SE) estimated for 24 h postmortem muscle calpastatin activity and shear force after 7 and 14 d of steak aging are also given in Table 4.2.

There were no significant differences in the means of shear force after 7 and 14 d of aging among AA, AB, and BB genotypes ($P > 0.05$). There were also no significant differences in the means of 24 h postmortem muscle calpastatin activity among AA, AB, and BB genotypes ($P > 0.05$) except for the marginal ($P = 0.067$) genotype difference between AA and AB. The genotype AA tends to have lower numerical values in muscle calpastatin activity, d-7 shear force, and d-14 shear force when it was compared to the numerical values of genotypes AB and BB. Although such a finding would be important for understanding the inheritance patterns of calpastatin activity, there were 15 individuals with AB genotypes that had muscle calpastatin activity less than the average of the muscle calpastatin activity for individuals with AA genotypes.

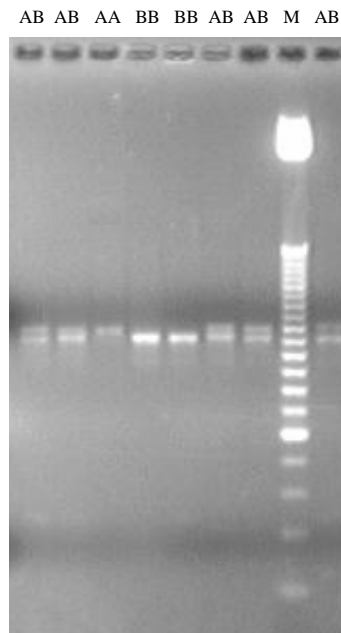


Figure 4.2. Microsatellite band patterns of Brahman steers after DNA was amplified from 1.4 kb upstream initiation site of the bovine calpastatin gene. DNA samples with both bands were assigned the AB genotype, whereas those possessing only the A or B band were assigned the AA or BB genotypes, respectively. DNA bands (≈ 250 bp) were electrophoresed on a mixture of 0.7% agarose and 2.65% SynergelTM gels and stained with ethidium bromide for visualization. M= 25 bp DNA marker.

Cong et al. (1998) indicated that the deletion of the calpastatin promoter sequence between nt -1667 and -944 increased promoter activity more than 2-fold. Dobi and Agoston (1998) showed that calcium within its physiological intranuclear concentration can induce a specific concentration-dependent conformational change of the DNA specific to the (AC)_n repeat. The authors also found that calcium affected protein-DNA interactions by regulating secondary modifications such as phosphorylation of various transcription factors with consequences for gene transcription. Therefore, it is likely that the polymorphism in the (AC)_n repeat within the microsatellite promoter region of the calpastatin gene may partially contribute to the variation of muscle calpastatin activity among Brahman steers. Selection and breeding of individuals with AA genotypes is recommended because this may improve tenderness by increasing the allelic frequencies of “A” allele. The finding also indicates these variable regions reveal specific allelic diversity and they are now considered as distinctive simple sequence repeats (SSRs) for

Table 4.2. Least-squares mean (\pm SE) 24 h postmortem muscle calpastatin activity¹ (CA) and Warner-Bratzler shear force after 7 and 14 d (WBS7, and WBS14, respectively) of steak aging for Brahman steers related to the tagged-sequence microsatellite analyses. DNA samples with both bands were assigned the AB genotype, whereas those possessing only the A or B band were assigned the AA or BB genotypes, respectively.

Genotype	n	Least-squares mean ^{a,b} (\pm SE)			Genotypic Frequency
		CA	WBS7	WBS14	
AA	9	3.65 ^a \pm 0.38	4.60 ^a \pm 0.44	3.18 ^a \pm 0.77	0.11
AB	65	4.42 ^b \pm 0.14	4.79 ^a \pm 0.17	4.06 ^a \pm 0.29	0.76
BB	11	4.30 ^{a,b} \pm 0.34	4.82 ^a \pm 0.40	4.03 ^a \pm 0.69	0.13

¹Calpastatin activity was reported as units of activity per gram of tissue.

^{a,b}Means within a column with no common superscripts are different ($P < 0.07$).

Brahman animal breeding with little intra-specific polymorphism. Furthermore, the reproducibility of SSRs is such that they can be efficiently used by different laboratories to produce consensus data, which makes them useful for animal breeding projects and they may result in successful polymorphisms for many beef cattle breeds.

Comparative DNA Sequence Analysis

The average Kimura two-parameter distance (Kimura, 1980) for the LCA group sequences was 0.117, and the corresponding value for the HCA group was 0.097. Conversely, the net between-group distance, corrected for the within-group distance, was 0.003. This suggests that there were no discernible differences among the DNA sequences of LCA and HCA groups. In addition, the overall transition/transversion ratio was 0.53, which was much lower than for most eukaryotic genes (Shao et al., 2000). The observed ratio is close to the expected value (1/2) if substitutions were occurring randomly along the DNA molecule. Although this random-substitution pattern can result from a high mutation rate, it can also be produced by a high frequency of sequencing artifacts (Hoyle and Higgs, 2003).

A neighbor-joining tree was also constructed based on the distances estimated from Kimura-two parameter model. No phylogenetic structure was observed for the DNA sequences from LCA and HCA groups. Instead, the result was a 'star phylogeny' with short internal branches and long terminal branches. This pattern is expected either with a high mutation rate or a high frequency of sequencing artifacts (Hoyle and Higgs, 2003). These results also suggest that there is no real genetic divergence between LCA and HCA alleles.

The Consensus DNA and Amino Acid Sequence Analyses

Although the DNA sequences showed considerable sequence heterogeneity due to the sequencing artifacts, a consensus approach with high threshold identity was used to combine the

data sets of the DNA sequence into two consensus sequences for LCA and HCA groups. The consensus sequences exhibited an average similarity of 99% to the sequenced units. The region sequenced was 580 bp in length, including 156 bp of exon sequence (part of exon 5 and part of exon 6) and 424 bp of an intron sequence. Amino acid sequences inferred from the consensus nucleotide sequences for LCA and HCA groups were used for comparative amino acid sequence analyses. The alignment of consensus DNA and amino acid sequences corresponding to a genomic region in calpastatin L-domain for LCA and HCA groups of Brahman steers is given in Figure 4.3. There were six sites polymorphic for nucleotide substitutions, three in exons and three in the intronic region (Figure 4.3). Interestingly, all the nucleotide substitutions observed in the coding region were transition whereas all of those observed in the intron were transversion. The overall similarity in the DNA coding sequences was 98 % between LCA and HCA groups (Figure 4.3). Alignment of the consensus nucleotide sequences revealed high conservation of the sequence, particularly in the protein-coding region (identity of 153 out of 156 nucleotide bases). In addition to the notable sequence similarity in the coding region of the two contrasting tenderness groups, the calpastatin non-coding region (intron) showed high sequence similarity between LCA and HCA groups except for polymorphisms located at positions 117, 211, and 444 (Figure 4.3). The consensus DNA sequence similarity between the two contrasting tenderness groups in the non-coding region was 99%. The observed polymorphisms in non-coding DNA sequences for the LCA and HCA groups suggest that these polymorphic sites may have a variable effect on the splicing mechanisms that may act on the calpastatin gene transcripts. Furthermore, the overall similarity in the deduced amino acid sequences was 96 % between the two contrasting tenderness groups (Figure 4.3). The amino acid composition for LCA and HCA groups is also given in Figure 4.3. Comparison of the amino acid composition deduced from the

	Exon 5										E	K	K	T	Q	E/G	V	K	P	K		
LCA	GGG	CAG	CGA	AGC	CCT	TTT	TGT	GAG	AAA	AAA	ACC	CAA	GAA	GTA	AAG	CCA	AAG				51	
HCA	GG					
	<u>E H T E</u>																					
LCA	GAA	CAC	ACA	GAG	GTA	GGT	AAC	CAG	TTA	TTG	GGT	CAG	GAG	GAT	CCT	AAG	AGG				102	
HCA					
LCA	AAG	CCT	TTT	TAT	CTG	TGC	CCT	TTA	TTG	GCT	TTA	AGG	CTA	AAA	TTT	CGC	AAC				153	
HCA	T					
LCA	CCC	CCT	TTT	GAA	CCT	TTA	AAT	CCC	ACC	TAT	AGC	GGC	TGT	TTT	TTG	TTT	TGT				204	
HCA					
LCA	TGA	AAA	GAA	GGC	ACC	TCT	CTT	GGG	TTT	CAT	GGG	GTC	TAT	CAT	TTG	GCT	TTA				255	
HCA	C					
LCA	GAT	TAG	TTT	GCT	GAA	CAT	GGG	GGG	GAG	GGC	ACT	CTT	TGT	TTA	AAA	TTT	TTT				306	
HCA					
LCA	ACC	CAG	CAG	GGA	ACT	TCC	TCC	AAG	TCA	AGC	AAG	ACT	AAT	TCA	AGC	TTT	TCG				357	
HCA					
LCA	CAT	TAC	CTC	CTG	AAA	CCA	CAC	AGT	GTT	ATG	AAT	TGT	TTC	ATA	CTC	CCA	AAT				408	
HCA					
LCA	AAA	TGC	CAG	CCT	TGG	TTG	GAC	TTT	TGT	TAA	CCC	CAA	GGG	GTG	TAT	GCG	TGT				459	
HCA	T					
	Exon 6		<u>P K S L P K H S S D T G S K H</u>																			
LCA	TTT	CAG	CCA	AAA	AGC	CTA	CCC	AAG	CAC	TCA	TCA	GAT	ACA	GGA	AGC	AAG	CAT				510	
HCA					
	<u>A P K E K A V S K S S E Q P P S E</u>																					
LCA	GCT	CCT	AAG	GAA	AAA	GCC	GTT	TCC	AAA	TCA	AGT	GAG	CAG	CCA	CCA	TCA	GAG				561	
HCA					
	<u>K S/L T K P K</u>																					
LCA	AAA	TCA	ACA	AAA	CCA	AAG	G															580
HCA	...	T															

Figure 4.3. Consensus DNA sequence alignment and corresponding deduced amino acid sequences for a genomic region in the calpastatin L-domain between two contrasting tenderness groups of Brahman steers sampled for either a combined reduction (LCA) or increase (HCA) in 24 h postmortem muscle calpastatin activity and shear force after 7 d of steak aging. Dots represent identity with LCA group. Polymorphic sites are in a bold.

corresponding consensus nucleotide sequences of the LCA group with that obtained from the HCA group revealed that the amino acid composition deduced from exons five and six of the bovine calpastatin gene lacked cysteine, phenylalanine, isoleucine, methionine, asparagine, arginine, tryptophan, and tyrosine. However, the deduced amino acid sequences between the two contrasting tenderness groups were highly conserved. In addition, comparison of the amino acid compositions of the entire bovine skeletal muscle calpastatin protein with that of bovine cardiac calpastatin (Otsuka and Goll, 1987) and those derived from other species (Emori et al., 1987; Takano et al., 1988; Lee et al., 1992; Cong et al., 1998) revealed that substantial differences can be present in the relative levels of many amino acids, whereas the levels for tryptophan, methionine, cysteine, and phenylalanine were consistently low (Table 4.3). The variability of amino acid composition between the two contrasting tenderness groups of Brahman steers, and the variation of amino acid composition among species could explain the variation of meat tenderness within these Brahman steers and among species as well. In the present study, amino acid alignment also revealed two amino acid substitutions at positions six and 48 encoded from exons five and six, respectively. The two deduced amino acid sequences were analyzed by Neural Network Analysis (Blom et al., 1999) to predict potential serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins. The amino acid sequence analyses showed that a few individuals from the LCA group possessed six potential serine phosphorylation sites at positions 22, 27, 37, 39, 40, 45, and 48 whereas many individuals of the HCA group had five serine phosphorylation sites at positions 22, 27, 37, 39, 40, and 45. However, the determination of calpastatin activity was measured *in vitro* in the absence of phosphorylation. Therefore, the significance of this finding is still not clear. The difference in the total number of serine residues in the L-domain may explain the variability of calpastatin activity *in vivo*. Takano et al. (1999)

Table 4.3. Comparison of the amino acid composition deduced from consensus nucleotide sequences of two groups of Brahman steers sampled for either a combined reduction (LCA) or increase (HCA) in 24 h postmortem muscle calpastatin activity and shear force after 7 d of steak aging with that obtained from the amino acid analysis of the calpastatin L-domain from different resources and species.

Amino Acid	LCA	HCA	Bovine ¹	Bovine ²	Ovine ³	Swine ⁴	Rabbit ⁵	Human ⁶
	Times Found	Times Found	Times Found	Times Found	Times Found	Times Found	Times Found	Times Found
Alanine (A)	2	2	2	2	2	4	4	5
Cysteine (C)	0	0	0	0	0	0	0	0
Aspartic acid (D)	1	1	1	1	1	1	1	2
Glutamic acid (E)	7	6	7	7	7	7	7	7
Phenylalanine (P)	0	0	0	0	0	0	0	0
Glycine (G)	1	2	1	1	0	1	4	2
Histidine (H)	3	3	3	3	3	3	4	2
Isoleucine (I)	0	0	1	0	0	0	0	0
Lysine (K)	13	13	12	13	13	11	11	11
Leucine (L)	1	2	1	1	0	1	0	1
Methionine (M)	0	0	0	0	0	0	0	0
Asparagine (N)	0	0	0	0	0	1	1	1
Proline (P)	7	7	7	7	8	6	5	5
Glutamine (Q)	2	2	2	2	3	2	1	4
Arginine (R)	0	0	0	0	1	2	3	1
Serine (S)	9	8	9	8	8	7	4	8

(table 4.3 continued)

Valine (V)	2	2	2	3	2	2	1	1
Threonine (T)	4	4	4	4	4	4	6	2
Tryptophan (W)	0	0	0	0	0	0	0	0
Tyrosine (Y)	0	0	0	0	0	0	0	0

¹Cong et al., 1998; ²Killefer and Koohmaraie, 1994; ³Mainelli et al., 1995; ⁴Takano et al., 1988; ⁵Emori et al., 1987; ⁶Lee et al., 1992.

showed that the amino acid sequences of the L-domain contained potential phosphorylation sites for protein kinase C and protein kinase A. They indicated that these sequences were conserved among the mouse, human, and bovine calpastatin genes. It has been reported that phosphorylation of calpastatin increased the calcium requirement for inhibition of calpain (Averna et al., 1999), decreased calpastatin solubility (Averna et al., 2001), and increased the cleavage of L-domain from the four inhibitory domains (Cong et al., 1998). Therefore, the variability of the potential serine phosphorylation sites within the L-domain of calpastatin protein may help in developing an understanding of the regulation and variability of 24 h postmortem calpastatin activity among Brahman steers. The results also showed that amino acids deduced from exons five and six of the HCA group have more basic residues (R, K, and H) than those sampled for the LCA group (Table 4.4). The presence of one or more positive amino acid residues within L-domain of the calpastatin protein from the HCA group may be responsible for the variability of 24 h postmortem muscle calpastatin activity within Brahman breed.

Conclusion

As a tool for understanding the genetic aspects of carcass traits, phenotypic data (muscle calpastatin activity and steak shear force) and pedigree information may be combined to screen

Table 4.4. Distribution of charged residues of the amino acids deduced from exons five and six of the bovine calpastatin L- domain between two groups of Brahman steers sampled for either a combined reduction (LCA) or increase (HCA) in 24 postmortem muscle calpastatin activity and shear force after 7 d of steak aging.

Domain-L	No. of Amino Acid Residues	Arg (R)	Lys (K)	His (H)	Asp (D)	Glu (E)	Sum of Charge Values ^a
LCA							
Exon 5	14	0	4	1	0	4	+1
Exon 6	38	0	9	2	1	3	+7
HCA							
Exon 5	14	0	4	1	0	3	+2
Exon 6	38	0	9	2	1	3	+7

^aCharge values were calculated from the numbers of residues by calculating Arg, Lys, and His as +1, and Asp and Glu as -1.

for genes with major effects on calpastatin activity. However, the polymorphic sites detected for the (AC)_n repeat within the promoter region of the calpastatin gene and the deduced amino acid sequences of L-domain may partly contribute to the variations of 24 h postmortem muscle calpastatin activity among Brahman steers. However, this conclusion should remain guarded since intense investigation is required. Such analyses in large and environmentally controlled populations would be able to identify calpastatin genotypes that may relate to 24 postmortem muscle calpastatin activity variation and tenderness.

CHAPTER 5

SUMMARY AND IMPLICATIONS

The widespread use of MTDFREML for genetic evaluation in livestock has led to significant improvement in many genetic parameters for traits of economic importance. This improvement can further be enhanced by increasing the accuracy of genetic parameters through inclusion of pedigree information in the genetic evaluation models. Although data used in this study were limited to provide strong recommendations, the large amount of genetic variation among individuals, plus moderate to high genetic correlations between 24 h postmortem muscle calpastatin activity and tenderness measurements suggests genetic improvement in tenderness would be successful through selection. By monitoring muscle calpastatin activity in the early postmortem period (24 h), it is possible to predict whether or not a carcass will fail to tenderize adequately, and this has the potential to be incorporated into meat quality assurance schemes. In addition, a negative association found between calpastatin activity and carcass traits indicates that selection for low 24 h postmortem muscle calpastatin activity under controlled management may result in improved carcass traits such as fat thickness, hot carcass weight, rib eye area, and marbling score. Selection for reduced calpastatin activity concomitant with careful crossing of complementary breeds can overcome some of the quality defects in the carcasses produced from Brahman cattle. In this study, the finding of the strong genetic correlation between calpastatin activity and shear force should lead to extensive molecular research on the role of this protein in the tenderization process.

The current knowledge of the structure of the calpastatin gene suggests that the presumed link between the calpastatin gene and tenderness may be explained at the molecular level. The RFLP technique conducted on DNA sequences amplified from the bovine calpastatin gene

located between inhibitory domain I and inhibitory domain II was not useful to identify polymorphic fragments in Brahman steers. Perhaps significant polymorphisms may be detected by using additional restriction enzymes other than those used herein.

Overall, the possibility of observing genetic polymorphisms of the SSR in the promoter region of calpastatin gene and the significant correlations between the polymorphic genotypes with its respective phenotypes (24 h postmortem muscle calpastatin activity) indicate that the use of SSR analysis as a DNA marker for genetic improvement of 24 h postmortem calpastatin activity may provide a promising approach for improving tenderness. Therefore, SSR analysis may present a faster and less expensive method to predict 24 h calpastatin activity. Analyses of additional breeds would be required to determine the effect and frequency of alleles within the promoter region that influence meat quality traits.

Future research to elucidate the expression of genotypic polymorphisms may reveal improved breeding strategies for controlling unwanted variation in 24 h postmortem muscle calpastatin activity. Such studies could also lead to improved breeding programs, more accurate testing methods, and the possibility of identifying other DNA markers associated with tenderness.

DNA sequence analysis of the L-domain of the calpastatin gene may identify the involvement of this region in regulation of muscle degradation during postmortem storage and its potential in marker-assisted breeding. On the basis of the DNA polymorphism observed in this study, it could be speculated that the variability of calpastatin activity among Brahman steers is possible due to the presence of variable numbers of potential serine phosphorylation sites within the L-domain of calpastatin, which indicates that posttranslational phosphorylation may promote changes in the functional properties of calpastatin or may promote different cellular responses in

the calpastatin molecules. Future studies to assess the possible variation and impact of the calpastatin gene on tenderness may allow address of the possible mechanisms on how calpastatin is more clearly involved in the process of postmortem tenderization.

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APPENDIX A

MULTIPLE SEQUENCE ALIGNMENTS FOR BRAHMAN STEERS SAMPLED FOR A
 COMBINED REDUCTION IN 24 h POSTMORTEM MUSCLE CALPASTATIN
 ACTIVITY AND SHEAR FORCE AFTER 7 d OF STEAK AGING
 (SITE NUMBERS ARE WRITTEN IN THE END OF EACH LINE)

```
#LOW1  GGGCAGCGAA  GCCCTTTTTG  TGAGAAAAAA  ACCCAAGAAG  TAAAG [ 45]
#LOW2  .....
#LOW3  .....
#LOW4  .....A...
#LOW5  .....
#LOW6  .....GG...C.
#LOW7  .....CT...A...T...
#LOW8  .....G...A...GG...
#LOW9  C...G...G...
#LOW10 .....A...GG...
#LOW11 .....-...GG...
#LOW12 .....-T...G...-
```

```
#LOW1  CCAAAGGAAC  ACACAGAGGT  AGGTAACCAG  TTATT-GGGT  CAGGA [ 90]
#LOW2  .....-...A...
#LOW3  .....-...
#LOW4  .....C...T...T...G--...-...
#LOW5  .....A..T...A...--...-...
#LOW6  -...C.C...T...C...T...C...--...
#LOW7  .....-...A..
#LOW8  .....C..G...G...A.C...--...
#LOW9  .....C...G...G...T...C...
#LOW10 .....C...T...--...
#LOW11 .....A..C...--...
#LOW12 .....G..AG...A...A-C..C T...
```

```
#LOW1  GGATCCTAAG  AGGAAGCCTT  TTTATCTGTG  CC-CTTTATT  GGCTT [135]
#LOW2  .....
#LOW3  .....
#LOW4  ..C...T...C...T...C...
#LOW5  T..G...G...A.A...A...-A.C...AT..
#LOW6  .....A...A.A...G.A...
#LOW7  ..C...T...T...C...-...
#LOW8  .....-...-...
#LOW9  .A.C...G...-...T.A...
#LOW10 .....T...T.C...--...
#LOW11 .....T...-...G...
#LOW12 ..C..A...G...A..A...T G.C...C...T..G. [135]
```

#LOW1	TAAGGCTAAA	ATTTGCAAC	CCCC-CTTTT	GAACCTTTAA	ATCC-	[180]
#LOW2--	[180]
#LOW3--	[180]
#LOW4T..	.A.....-A	.T.-.....	.G.A.C....	T.T.C	[180]
#LOW5G	..A.....G	..A.-T....	..T.A.....	G...A	[180]
#LOW6C...G...---	[180]
#LOW7	..G.....	T...-T....	C...A...T-	[180]
#LOW8A.....	T...-.....	-...-	[180]
#LOW9C.....-	...A.....-	[180]
#LOW10--C.G.	A.....c-	[180]
#LOW11	G.....C.-T....-	[180]
#LOW12	.T...C...-...	...A.....	T...C...	..T.C	[180]

#LOW1	CACCTATAGC	GGCTGTTTTT	TGTTTTGTTG	AAAAGAAGGC	-ACCT	[225]
#LOW2C.....	-....	[225]
#LOW3C.....	-....	[225]
#LOW4C.-G	C....	[225]
#LOW5G.AGT.....	T.....	C...A	[225]
#LOW6A.....	.T.....-A.T	[225]
#LOW7T..C..G.....	-....	[225]
#LOW8	..A.....	T..C.....	...G.....A..	[225]
#LOW9A..C..	...G.....	-....	[225]
#LOW10	T.....	-....	[225]
#LOW11G..	-....	[225]
#LOW12	..A.....-C...	-....	[225]

#LOW1	CTCTTGGGTT	TCATGGGGTC	TATCATTG	CTTTAGATTA	GTTTG	[270]
#LOW2A..	[270]
#LOW3	[270]
#LOW4	..T.....	.T.....T	[270]
#LOW5	..T.....CGTA..A..	T..G.	[270]
#LOW6T.....	...G..A..A..	...T	[270]
#LOW7G.....	...A....	...G.	[270]
#LOW8	..G...T..	[270]
#LOW9A..	[270]
#LOW10T.....	[270]
#LOW11C.G.....T	[270]
#LOW12G.....	C....	[270]

```

#LOW1 CTGAACATGG GGGGG-AGGG CACTCTTTGT TTAAAATTTT TTACC [315]
#LOW2 ..... -..... [315]
#LOW3 ..... -..... [315]
#LOW4 ..T..... -.....T..... A.... [315]
#LOW5 ..C..... -...T --..... A..... --.. [315]
#LOW6 G..... -A.. .T.G..A. ....T..A .A... [315]
#LOW7 ..-..... -.....G.. .....A ..... [315]
#LOW8 ..-.....T. ....-G... ..... -... [315]
#LOW9 T.-G....- . ....-G... ..T..... -..... [315]
#LOW10 ..-G..... -..... [315]
#LOW11 ....--..T. ....G.... ..... ..C.. [315]
#LOW12 .....T.... ...A-...A .-..... .G..... [315]

```

```

#LOW1 CAGCAGGGAA CTCCTCC-- AAGTCAAGCA AGACTAATTC AAGCT [360]
#LOW2 ..... -- ..... [360]
#LOW3 ..... -- ..... [360]
#LOW4 .G-....T.. .....-- C.AGTC.... ....C.C.. [360]
#LOW5 .C-.C..T.. .....T.-- ..... [360]
#LOW6 T.-..... ..T.CA ..... [360]
#LOW7 .....T.... .....-- .G..... [360]
#LOW8 ..-..... .....-- .....-..... .C..... [360]
#LOW9 .....T.... .....-A -G..... .C..... [360]
#LOW10 ....-..... ..G....-- ..... [360]
#LOW11 ....-..... .....-- -...T..... .TG..... [360]
#LOW12 .....GCT.-- .G..A..... [360]

```

```

#LOW1 TTTCGCATTA CCTCCTGAAA CCACACAGTG TTATGAATTG TTTCA [405]
#LOW2 ..... [405]
#LOW3 ..... [405]
#LOW4 ..... [405]
#LOW5 ....C..... ..G.T.. G....-G..T ..... [405]
#LOW6 .....T..... ..... [405]
#LOW7 ....C..... ..G.... .T..C..... [405]
#LOW8 ..... [405]
#LOW9 .....A. G.T..G... [405]
#LOW10 ..... [405]
#LOW11 ....C..... [405]
#LOW12 C...C..... ..T.... [405]

```

```

#LOW1 TACTCCCAA TAAATGCCAG CCTTGG-TTG GACTTTTGTT AACCC [450]
#LOW2 ..... -..... [450]
#LOW3 ..... -..... [450]
#LOW4 ..... -..... .C... [450]
#LOW5 ..... A..... -..... T... [450]
#LOW6 ..... T..G..-..... [450]
#LOW7 ..... A..... -..... A... [450]
#LOW8 ..... A..... T..G.. AA... T... [450]
#LOW9 ..... G.C...G... [450]
#LOW10 ..... -..... .C... [450]
#LOW11 ..... -..... [450]
#LOW12 ..... A..... -G... [450]

```

```

#LOW1 CAAG-GGGTG TATGCGTGTT TTCAGCCAAA AAGCCTAC-C CAAGC [495]
#LOW2 ..... -..... [495]
#LOW3 ..... -..... [495]
#LOW4 ..... T..... .A..T...-G [495]
#LOW5 ..... C..... A. -.. [495]
#LOW6 ..... -..... [495]
#LOW7 A.T.-..... -A.C..... - G..C. [495]
#LOW8 ..... T..... C..... -...C. [495]
#LOW9 ..T.-..... G..... C..... [495]
#LOW10 ..... -..... G [495]
#LOW11 ..... -..... [495]
#LOW12 .G.-..... T..... .A...A-... [495]

```

```

#LOW1 ACTCATCAGA TACA-GGAAG CAAGCATGCT CCTAAGGAAA AAGCC [540]
#LOW2 ..A..... -..... G..... [540]
#LOW3 ..... -..... [540]
#LOW4 ..... -..... [540]
#LOW5 CA..... -..... G.....C [540]
#LOW6 ..... -..... T..... [540]
#LOW7 ..... -..... [540]
#LOW8 .T..... G-..... G..T.-- [540]
#LOW9 ..... -..... [540]
#LOW10 ..... -..... [540]
#LOW11 ..... -..... [540]
#LOW12 .....G GC.G-...G. ..G..T.C.C ..... -.G. [540]

```

```

#LOW1  GTTTCCAAAT CAAGTGAGCA GCCACCATCA GAGAAATCAA CAAAA [585]
#LOW2  .....C A...C [585]
#LOW3  ..... [585]
#LOW4  ..... [585]
#LOW5  .....T.. [585]
#LOW6  ..... T...G.G..C .G.G..T.TC CT.....TCG AC--- [585]
#LOW7  ..... [585]
#LOW8  ..... [585]
#LOW9  ..... [585]
#LOW10 ..... [585]
#LOW11 ..... T..... ..C..CCTC ...G..... .C.T. [585]
#LOW12 C...T.C... ..... .....T.. [585]

```

```

#LOW1  CCAAAGGGGT T----- [604]
#LOW2  .A..G..... .----- [604]
#LOW3  .....----- [604]
#LOW4  .....----- [604]
#LOW5  .....TT. .GGGGATAA [604]
#LOW6  ----- [604]
#LOW7  .....T... ----- [604]
#LOW8  .....----- [604]
#LOW9  .....A.. .GG----- [604]
#LOW10 .....----- [604]
#LOW11 TTCC..T... G----- [604]
#LOW12 .....G CCTTTTGG- [604]

```

APPENDIX B

**MULTIPLE SEQUENCE ALIGNMENTS FOR BRAHMAN STEERS SAMPLED FOR A
COMBINED INCREASE IN 24 h POSTMORTEM MUSCLE CALPASTATIN
ACTIVITY AND SHEAR FORCE AFTER 7 d OF STEAK AGING
(SITE NUMBERS ARE WRITTEN IN THE END OF EACH LINE)**

```

#HIGH1  GGGCAGCGAA  GCCCTTTTTG  TGAGAAAAAA  ACCCAAGGGG  TAAAG  [ 45 ]
#HIGH2  .....
#HIGH3  .....
#HIGH4  .....T.....
#HIGH5  .....T.....
#HIGH6  .....--.....
#HIGH7  ...GT.G...  ....A.A...
#HIGH8  .....
#HIGH9  .....-...  .A.....
#HIGH10 C.....  .....  .....  .A..AA...
#HIGH11 C.....
#HIGH12 .....G...  .....  .....-..AA...
#HIGH13 .....  .....T...  .....AA...--
#HIGH14 .....  .....T...  ...G..A...
#HIGH15 .....
#HIGH16 .....  .....T...  .....-..AA...
#HIGH17 .....

```

```

#HIGH1  CCAAAGGAAC  ACACAGAGGT  AGGTAACCAG  TTATT-GGGT  CAGGA  [ 90 ]
#HIGH2  .....
#HIGH3  .....  .....  .....  G...-C...  ...G [ 90 ]
#HIGH4  .....  .....  .....  ...C.-...C  ..A.. [ 90 ]
#HIGH5  .....
#HIGH6  .....-.....  .....-A...  .T... [ 90 ]
#HIGH7  .....  .....  .....A  .....-..... [ 90 ]
#HIGH8  .....T...  .....  .....T...  .....-..C... [ 90 ]
#HIGH9  .....  .....G  .....A...  .....-..... [ 90 ]
#HIGH10 .....T...
#HIGH11 .....
#HIGH12 .....C.T...  .....T.-  ..T.....  .....-..T... [ 90 ]
#HIGH13 .....
#HIGH14 .....  .....G..G...  ...C.-..... [ 90 ]
#HIGH15 .....  ..C.-...C  .....T...  .....-..... [ 90 ]
#HIGH16 .....  .....T.C...  .....-..... [ 90 ]
#HIGH17 .....  .....T  G...-..... [ 90 ]

```

```

#HIGH1  GGATCCTAAG AGGAAGCCTT TTTATCTTTG CC-CTTTATT GGCTT [135]
#HIGH2  ..... [135]
#HIGH3  .....G. [135]
#HIGH4  C....GC... ..-..... [135]
#HIGH5  .....-C..... [135]
#HIGH6  T.T.....-C..... [135]
#HIGH7  .....C... ..-..... [135]
#HIGH8  -.....T... A.A..... AT-A..... [135]
#HIGH9  ....A.....C.G.. -A..T.. [135]
#HIGH10 .....T... .T...C.. -...G.. [135]
#HIGH11 -.....T-.....G [135]
#HIGH12 -..C.....C.....-..... [135]
#HIGH13 -A.....A.....A. [135]
#HIGH14 C.....G.....-..... [135]
#HIGH15 ...C.....-TCC.-.. .T... [135]
#HIGH16 .....-..... [135]
#HIGH17 ....T......T.....C.....G.. ..-..... [135]

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#HIGH1  TAAGGCTAAA ATTTCGCAAC CCCC-CTTTT GAACCTTTAA ATCC- [180]
#HIGH2  .....-...A. ....- [180]
#HIGH3  .....-.....T ..... [180]
#HIGH4  .....-.....A.. ....- [180]
#HIGH5  .....A.-. ....- [180]
#HIGH6  G.....-.....C..... [180]
#HIGH7  .....A.-. ....- [180]
#HIGH8  .....-.....A.G T..A......T.- [180]
#HIGH9  .....T .A.....-...G [180]
#HIGH10 ..G.....-.....T ..... [180]
#HIGH11 .....-.....-A... .G..... [180]
#HIGH12 .....G.T.....-.....G.A..... [180]
#HIGH13 .....-..C.T .....-C..... [180]
#HIGH14 .....T......T.-.....T ..... [180]
#HIGH15 .T......T.....-.....A.....T...- [180]
#HIGH16 ..G.....-..C. T...-T..... [180]
#HIGH17 .....A.....-..A..... [180]

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#HIGH1  CACCTATAGC  GGCTGTTTTT  TGTTTTGTTG  AAAACAAGGC  -ACCT  [225]
#HIGH2  .....      .....A..      .....      .....      .....      -....  [225]
#HIGH3  .....      .....      .....      .....      .....      -....  [225]
#HIGH4  .....G..G      .....      .....      .....      .....      -....  [225]
#HIGH5  .....      .....      .....      .....      .....      -....  [225]
#HIGH6  .....      .....      .....T...  T.....      .....      -....  [225]
#HIGH7  .....      .....      .....      .....      .....      -....  [225]
#HIGH8  .....      .C.....      .....      ...G...T.  -...A  [225]
#HIGH9  .....      T.....      .T.....      ...T..G...  -.TA.  [225]
#HIGH10 .....      .....      .....      .....      .....      -....  [225]
#HIGH11 .....      ...T.....      .....      .....      .....      -....  [225]
#HIGH12 .....      T.....C      .....      .....      .....      -....  [225]
#HIGH13 .....G..      .....C      ..A.....      ...G..T..  -....  [225]
#HIGH14 .....      .....      .....      .....      ...G.....  -....  [225]
#HIGH15 ...A..G..G      .....      .T.....T      .....      -...A  [225]
#HIGH16 .....      ...T.....      .....      .....      .....      -....  [225]
#HIGH17 .....      .....      .....      .....      .....      -....  [225]

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#HIGH1  CTCTTGGGTT  TCATGGGGTC  TATCATTTGG  CTTTAGATTA  GTTTG  [270]
#HIGH2  .....      .....      .....      .....      .....      [270]
#HIGH3  .....      .....      ..G.....      .....T      .....  [270]
#HIGH4  .....C.....      .....      .....      .....      .....  [270]
#HIGH5  .....      .....      .....G.....      .....      .....  [270]
#HIGH6  .....      .....      .....      .....      .....T....  [270]
#HIGH7  .....      .....      .....      .....      .....      [270]
#HIGH8  .....      .....      .....      .....A.....      .....  [270]
#HIGH9  ...G.....      .....      .....A.....      ...A.....      .....  [270]
#HIGH10 ..G.....      .....      ..G.....      .....      .....  [270]
#HIGH11 .A.....      .T.....G.      .....      G...G.....      .....  [270]
#HIGH12 .....      .....      .....      .....      .....      [270]
#HIGH13 .....C.....      .....G.....      ..G.....      .....      ..C..  [270]
#HIGH14 .....      .....      .....      .....      .....      [270]
#HIGH15 ..T.....GA      ...G.....      .....      ...A..A.  T....  [270]
#HIGH16 .....      .T.....      .....      .....      .....      [270]
#HIGH17 .....C.....      .....      .....      .....      ...G.  [270]

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#HIGH1 CTGAACATGG GGGGG-AGGG CACTCTTTGT TTAAAATTTT TTACC [315]
#HIGH2 ..... - ..... [315]
#HIGH3 ..... - ..... [315]
#HIGH4 ..... - ..... T ..... G ..... A ..... [315]
#HIGH5 ..... - A ..... [315]
#HIGH6 ..... A ..... - ..... [315]
#HIGH7 ..T ..... - ..... C ..... A ..... [315]
#HIGH8 ..... - ..... T ..... A ..... [315]
#HIGH9 G..T..G.. ..A.-..... ..T.C.. .....G A..... [315]
#HIGH10 ..... - ..... [315]
#HIGH11 ..... -G..... ..G..... [315]
#HIGH12 ..... - ..... [315]
#HIGH13 GC..... - ..... [315]
#HIGH14 -..... ..G..... [315]
#HIGH15 ....C..... -G..... ..C..... ..C..... [315]
#HIGH16 .....T..... - ..... [315]
#HIGH17 ...G..... - ..... G..... G..... [315]

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#HIGH1 CAGCAGGGAA CTCCTCC-- AAGTCAAGCA AGACTAATTC AAGCT [360]
#HIGH2 ..... -- ..... [360]
#HIGH3 ..... -- ..-.-C..... [360]
#HIGH4 ..... G...-A ..-.-..... T..... [360]
#HIGH5 ..... -- ..-..G.-..... [360]
#HIGH6 ..... -- ..-..... T..... [360]
#HIGH7 ..... -- ..-..... [360]
#HIGH8 ..... -- ..--TC..... [360]
#HIGH9 ..A...-..... ..CT.-- ..A.TCGCA. ....CA. .... [360]
#HIGH10 ..... T...-A ..-..... ..C..... [360]
#HIGH11 ..... A..... -- ..-..... [360]
#HIGH12 ..... TG..... -- ..-..... ..A..... [360]
#HIGH13 ..C..... -- ..-G..... [360]
#HIGH14 ..... -C ..-..... [360]
#HIGH15 ..A..T-A.. ..G.T...-- ..-T.-... T.....A. ..A.. [360]
#HIGH16 ..... -- ..-..... ..-..C..... [360]
#HIGH17 ..... T.-- ..-..... [360]

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#HIGH1	TTTCGCATTA	CCTCCTGAAA	CCACACAGTG	TTATGAATTG	TTTCA	[405]
#HIGH2	[405]
#HIGH3T.....A.	G.....	[405]
#HIGH4A.G.....	[405]
#HIGH5T	[405]
#HIGH6C.....-	[405]
#HIGH7	[405]
#HIGH8	[405]
#HIGH9	..C...A..T..	GA...GT..-	[405]
#HIGH10	..A.....	[405]
#HIGH11	..GC.....	[405]
#HIGH12	[405]
#HIGH13G.....	[405]
#HIGH14A.	[405]
#HIGH15	A.....T	[405]
#HIGH16T.....	[405]
#HIGH17	[405]

#HIGH1	TACTCCCAAA	TAAATGCCAG	CCTTGG-TTG	GACTTTTGT	AACCC	[450]
#HIGH@-	[450]
#HIGH3-	[450]
#HIGH4A.....-	[450]
#HIGH5A.....	...A..-G.T..	[450]
#HIGH6	...C.....-	[450]
#HIGH7-	[450]
#HIGH8	G.....-	[450]
#HIGH9AA.....---AT..	.C....	[450]
#HIGH10-	[450]
#HIGH11	..A.....-	[450]
#HIGH12G..	G.....-	[450]
#HIGH13C.....	...A..-G.	[450]
#HIGH14-	.G.....	[450]
#HIGH15	...C.A.....G..-A.....	-.....	[450]
#HIGH16-	T.....	[450]
#HIGH17A.....	.G.....-G..	[450]

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#HIGH1  CATG-GGGTG  TATGCGTGTT  TTCAGCCAAA  AAGCCTAC-C  CAAGC  [ 495 ]
#HIGH2  ..A.-.....  .....      .....      .....-G  .....  [ 495 ]
#HIGH3  ..A.-.....  .....      --.....      .....-  .....  [ 495 ]
#HIGH4  ....-.....  .....      ...G.....  .....-  ..G..  [ 495 ]
#HIGH5  ....-.....  .....      .....      -.....-  .....  [ 495 ]
#HIGH6  ....-.....  .....      ...C...C..  .....-  .....  [ 495 ]
#HIGH7  ....-.....  .....      .....      .....-  .....  [ 495 ]
#HIGH8  .G.-.....  .....      .....      .....-  G....  [ 495 ]
#HIGH9  ....-.....  .....      .AT.....  .G.G.....  ..-..  [ 495 ]
#HIGH10  ....-.....  .....      .....C  ..A.....  .....  [ 495 ]
#HIGH11  ....-.....  .....      .....      .....-  .....  [ 495 ]
#HIGH12  .GAT-.....  .....A....  ..TC.....  ....T...-  .....  [ 495 ]
#HIGH13  ..AT-.....  .....      ....C.....  .....-  .....  [ 495 ]
#HIGH14  ....-...A.  .....      .....      .....-  .....  [ 495 ]
#HIGH15  ....-.....  .....      ...T.....  ....T...-G  .....  [ 495 ]
#HIGH16  ....-.....  .....      .....      .....-  .....  [ 495 ]
#HIGH17  ....-.....  .T...A--..  .AT...T...  .....-  GG...  [ 495 ]

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#HIGH1  ACTCATCAGA  TACA-GGAAG  CAAGCATGCT  CCTAAGGAAA  AAGCC  [ 540 ]
#HIGH2  .....      .....      .....      .....      .....  [ 540 ]
#HIGH3  .....      .....      .....C  .....      .....  [ 540 ]
#HIGH4  C.....      .....      .....      .....      .....  [ 540 ]
#HIGH5  .....      .....G.  .....      .....      .....  [ 540 ]
#HIGH6  .....T...  .....      .A.....  .A.....  .....  [ 540 ]
#HIGH7  .....      .....      .....      .....      -...  [ 540 ]
#HIGH8  .....C..  .....C..  .....G..  .A.....  -...  [ 540 ]
#HIGH9  ...AG.....  .....      .....      .....      .....  [ 540 ]
#HIGH10  .....      .....G.  .....      .....      .....  [ 540 ]
#HIGH11  .....      .....      .....T  .....      .....  [ 540 ]
#HIGH12  .T.....      .....      .....      .....      .....  [ 540 ]
#HIGH13  .....      .....      ...T...  .....A.  .....  [ 540 ]
#HIGH14  A.....      .....      .....      -.....      .....  [ 540 ]
#HIGH15  .....      .....      .....A  .....T.  .....  [ 540 ]
#HIGH16  .....      ...G-.....  .....      .....      -...  [ 540 ]
#HIGH17  .....      .....G.....  .....      .....T.  .....  [ 540 ]

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#HIGH1  GTTTCCAAAT CAAGTGAGCA GCCACCATCA GAGAAATTAA CAAAA [585]
#HIGH2  .....C.. ..... [585]
#HIGH3  .....C... .....AC.. ..... [585]
#HIGH4  ..... [585]
#HIGH5  ..... T..G.G..C CG.C..T.TC CTTT..GG.. .CC.T [585]
#HIGH6  ..... .GG..... [585]
#HIGH7  .....C.. ..... [585]
#HIGH8  ..... [585]
#HIGH9  ..... T..G.G..C CT.C..TC.T TCTTGGGGC. .CT.G [585]
#HIGH10 ..... ----- - [585]
#HIGH11 .....CC.. ..... [585]
#HIGH12 .....C.. ..... [585]
#HIGH13 .....C ..... [585]
#HIGH14 .....C ..... [585]
#HIGH15 ..... [585]
#HIGH16 ..... [585]
#HIGH17 ..... [585]

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#HIGH1  CCAAAG---- - [604]
#HIGH2  .....---- - [604]
#HIGH3  .....G--- - [604]
#HIGH4  .....G--- - [604]
#HIGH6  T.CC..G--- - [604]
#HIGH5  .....G--- - [604]
#HIGH7  .....G--- - [604]
#HIGH8  .----- - [604]
#HIGH9  T.C...G--- - [604]
#HIGH10 ----- - [604]
#HIGH11 .....---- - [604]
#HIGH12 .....---- - [604]
#HIGH13 .....---- - [604]
#HIGH14 .....---- - [604]
#HIGH15 .....---- - [604]
#HIGH16 .....---- - [604]
#HIGH17 .....G--- - [604]

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VITA

Fuad Mohammad T. Odeh, was born November 26, 1969, in Kuwait, to Mr. and Mrs. Mohammad Odeh. He received the Bachelor of Science degree, in Feb., 1997 majoring in Animal Production from College of Agriculture at Jordan University of Science and Technology (JUST), Irbid, Jordan. Then he worked as a Research and Teaching Assistant for two years in the Dept. of Animal Production at JUST, Jordan. The Master of Science degree in animal, dairy, and poultry sciences (Breeding and Genetics) was conferred in May, 2001 from Louisiana State University (LSU). He pursued further graduate study at LSU in June, 2001, to pursue the doctor of philosophy degree in animal science with emphasis on beef cattle breeding and genetics and minor in Biological Sciences.