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EFFECTS OF CALLIPYGE LAMB GENOTYPE ON SKELETAL MUSCLE CHARACTERISTICS

By

John D. Heller

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ABSTRACT

EFFECTS OF CALLIPYGE LAMB GENOTYPE ON SKELETAL MUSCLE CHARACTERISTICS

By

John D. Heller

Callipyge (CLPG) lambs are leaner, heavier muscled, and more efficient at converting feed to body weight than non-CLPG lambs. The current study determined the effects of lamb genotype (NN, CN, NC, and CC where C= mutant CLPG allele, N = wild-type allele, paternal allele listed first) on skeletal muscle characteristics. This study confirms that lambs of the CN genotype have heavier longissimus dorsi (LD) and biceps femoris (BF) muscles compared to other genotypes (NN, NC, CC), whereas infraspinatus (IS) muscle weights were similar among genotypes. Muscle DNA and protein concentrations were generally not different among genotypes. However, protein:RNA ratios were lower and RNA:DNA ratios of LD muscles from CN and CC lambs were higher than those of NN and NC lambs. Type IIx myosin heavy chain (MHC) was 25% higher in CN lamb LD compared to LD of other genotypes. An increase in the proportion of type IIx MHC may explain the previously reported increase in percentage and size of type IIb muscle fibers in CLPG lamb LD. Western blot analysis revealed that desmin in myofibrils isolated from CN lamb LD appeared to degrade more slowly than desmin from myofibrils of NN lambs, when incubated with m-calpain *in vitro*. This suggests that desmin of CN lambs is more resistant to calpain cleavage than desmin of NN lambs. Collectively, these data provide evidence that changes in myosin isoform distribution and decreased susceptibility of desmin to proteolysis may contribute to muscle hypertrophy in CN lambs.

DEDICATION

"He said that men ought to remember those friends who were absent as well as those who were present."

Thales. vii., Diogenes Laertius. Circa 200 A. D.

This thesis was written in memory of those family members who have gone before me and those who are still with me.

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LIST OF ABBREVIATIONS

1°: Primary

2°: Secondary

Ab: Antibody

ALD: Aldolase

AP: Alkaline phosphatase

βAA: β- Adrenergic agonists

BF: Biceps femoris

BSA: Bovine serum albumin

CC: Homozygous callipyge, non-hypertrophied

CK: Creatine kinase

CLPG: Callipyge gene

CN: Heterozygous callipyge, hypertrophied

ddI: Double distilled water

DNA: Deoxyribonucleic acid

EDTA: Ethylenediamine tetra acetic acid

EN: Enolase

GDF-8: Growth differentiation factor-8

G3PDH: Glyceraldehyde-3-phosphate dehydrogenase

IS: Infraspinatus

kD: kiloDalton

LD: Longissimus dorsi

LDH: Lactate dehydrogenase

MHC: Myosin heavy chain

NC: Heterozygous callipyge, non-hypertrophied

NFDM: Non-fat dry milk

NN: Homozygous normal, non-hypertrophied

PAGE: Polyacrylamide gel electrophoresis

PGAM: Phosphoglycerate mutase

PGI: Phosphoglucose isomerase

PGM: Phosphoglucomutase

PHb: Phosphorylase b

PK: Pyruvate kinase

PMSF: Phenylmethylsulfonyl fluoride

PVDF: Polyvinylidene fluoride

REA: Ribeye area

RNA: Ribonucleic acid

RPM: Rotations per minute

SA: Serum albumin

SDS: Sodium dodecyl sulfate

TPI: Triose phosphate isomerase

INTRODUCTION

Growth and function of skeletal muscle, the most abundant tissue in the animal body, is essential for locomotion and therefore survival of animals. Skeletal muscle also provides a high quality source of protein and iron for human diets. Gaining a better understanding of skeletal muscle growth should allow researchers to both prevent and cure muscle atrophy from disease or injury and produce animal protein more efficiently.

A ram with a heritable, heavy muscled phenotype was first noticed in 1983. This phenotype resulted from a novel genetic mutation. The gene responsible for the mutation has been named callipyge (CLPG, Cockett et al., 1994). Phenotypic CLPG lambs have 11% more muscle mass, 23% less fat, and are more efficient converters of feed to lean than phenotypic non-CLPG lambs (Freking et al., 1998a,b; Jackson et al., 1997a). Increases in production efficiency and muscle mass are of great interest to the livestock industry. Phenotypic CLPG lambs also have larger legs and loins than their normal counterparts, which result in more desirable carcasses. However, CLPG lamb chops are less tender and flavorful than lamb chops from non-CLPG lambs (Cockett et al., 1994; Shackelford et al., 1998). Today's standard consumer of lamb does not consider these traits desirable. Overcoming these obstacles would allow CLPG lamb to meet consumer demands for larger, leaner portions of meat while satisfying demands for a tender, flavorful product.

The biological mechanisms resulting in muscle hypertrophy due to the CLPG condition are unknown. Additional research is needed to address both the

CLPG muscle growth phenomenon and meat quality issues associated with the mutation. An improved knowledge of muscle development in CLPG lambs will allow for development of strategies to more efficiently produce meat from sheep and other species. This information may also shed light on potential management practices to make CLPG lamb more palatable.

The CLPG mutation is an example of paternal polar overdominance, an inheritance pattern rarely seen in nature. This mode of inheritance restricts expression of the characteristic CLPG muscle hypertrophy to only heterozygous lambs inheriting the mutant CLPG allele from their sire.

Most previous experiments have evaluated CLPG lambs based solely on phenotype. This means that up to 66% of the categorized lambs in the non-CLPG group may be carrying the mutant CLPG allele. The objectives of the current study are to examine the effects of lamb genotype and/or phenotype on skeletal muscle DNA, RNA, and protein concentrations, indices of muscle fiber type, and susceptibility of myofibrillar proteins to calpain-induced proteolysis.

LITERATURE REVIEW

Skeletal Muscle Growth

Skeletal muscle development occurs as a result of distinct prenatal and/or postnatal events. Muscle fiber hyperplasia, an increase in muscle fiber number, occurs during prenatal development. Postnatal muscle growth results from muscle fiber hypertrophy (Pearson and Dutson, 1991).

Embryonic formation of muscle fibers from mononucleated cells is called myogenesis (Stockdale, 1992). Myogenesis begins with the formation of myoblasts, in the myotomes of somites. Myoblasts are derived from mesenchymal stem cells that become committed to the myogenic lineage. Myoblasts proliferate, differentiate and fuse together to form multinucleated myotubes. During differentiation, myoblasts permanently withdraw from the cell cycle and begin to express muscle-specific genes. The first myotubes that develop as elongated structures are termed "primary". Myotubes develop with centrally located nuclei. Myotubes then begin to accumulate myofibrillar proteins, such as actin and myosin, and assemble them into organized contractile units. As myotubes mature nuclei migrate to the periphery of the cell and become displaced by myofibrils, which are the organelles of muscle contraction. These myofibrils align to give skeletal muscle fibers their typical striated appearance.

Secondary muscle fiber formation occurs late in embryogenesis and throughout fetal development. Secondary myotubes result from myoblast proliferation, differentiation and fusion at the periphery of primary myotubes.

Numerous secondary myotubes form around each primary myotube. These myotubes also undergo myofibrillogenesis and maturation into muscle fibers, as described for primary muscle fibers. Skeletal muscle fiber numbers typically do not change after birth in livestock species. As previously stated, postnatal growth is considered to result primarily from hypertrophy, or growth through an increase in cell size (Pearson and Dutson, 1991).

Muscle fiber hypertrophy occurs throughout prenatal and postnatal growth. Hypertrophy of muscle fibers is associated with increases in both DNA and protein. Since muscle fiber nuclei are incapable of DNA synthesis, the accumulation of muscle fiber DNA results from proliferation, differentiation and incorporation of satellite cells (Mauro, 1961; Moss and LeBlond 1971). Satellite cells, which lie between the sarcolemma and the basement membrane of muscle fibers, undergo proliferation and differentiation in response to external stimuli, such as growth factors (reviewed by Florini and Ewton, 1996; Dodson et al., 1996). The majority of muscle fiber nuclei accumulate postnatal, as a result of satellite cell incorporation. It is generally accepted that each muscle fiber nucleus accommodates a finite quantity of volume (Cheek, 1971). Consequently, accumulation of muscle fiber DNA via satellite cells is a prerequisite for muscle fiber hypertrophy.

In young, growing lambs myofibrillar proteins increase disproportionately with DNA, resulting in an increased protein to DNA ratio. (Lorenzen et al., 2000). Accumulation of muscle protein occurs when fractional protein synthesis exceeds degradation. Muscle protein accumulation leads to increases in myofibril number

and length. Myofibrils increase in length due to addition of new sarcomeres, the smallest functional contractile units of muscle, at the tendon ends of muscle fibers (Williams and Goldspink, 1971). Myofibrils also increase in diameter as myofibrillar proteins accumulate. Myofibril enlargement is followed by longitudinal splitting, which results in formation of two myofibrils that can subsequently increase in diameter (Goldspink, 1970). Muscle fiber size is ultimately related to sarcomere number as well as the number and size of myofibrils.

Callipyge Sheep

A Dorset ram, *Solid Gold*, from the Moffat flock in Piedmondt, Oklahoma exhibited a unique muscle hypertrophy phenomenon in 1983 (Cockett et al., 1999). Offspring from this heavily muscled ram soon became popular with commercial and club lamb producers due to their muscularity and trimness. The gene responsible for this muscular condition was mapped to ovine chromosome 18 and named callipyge (CLPG), which is Greek for beautiful buttocks (Cockett et al., 1994). Initial studies showed that CLPG lambs have 32% more muscle mass than non-CLPG lambs. This increase is due to hypertrophy of the longissimus dorsi and muscles of the pelvic limb (Cockett et al., 1994). Callipyge lamb carcasses were also found to be 7% leaner than normal carcasses (Cockett et al., 1994). These findings fueled producers' interest in callipyge sheep and prompted numerous studies confirming the CLPG gene's ability to increase muscle mass and decrease fat (Freking et al., 1998b; Jackson et al., 1997a,b,c; Koohmaraie et al., 1995). Callipyge lambs also consumed less feed, but had the

same average daily gain as normal lambs. Therefore, CLPG lambs have an improved feed utilization efficiency (Jackson et al., 1997a).

Genetic Inheritance of Callipyge Phenotype

Initial studies on the inheritance of the callipyge gene showed segregation of the muscular phenotype to be 49%, or 97 out of 200 lambs (Cockett et al., 1994). This was confirmed by similar matings producing greater than 300 lambs (Freking et al., 1998a,b; Jackson et al., 1997a). Thus, inheritance patterns of the hypertrophied sheep suggested a single autosomal dominant gene was responsible. However, further research demonstrated the muscle hypertrophy phenomenon is expressed only in CN offspring, C denotes the mutant CLPG allele and N denotes the wild-type normal allele (Cockett et al., 1996). The first allele indicates paternal origin (Table 1). This pattern of inheritance is called polar overdominance (Cockett et al., 1996). Polar refers to reciprocal heterozygous genotypes expressing different phenotypes. Overdominance is when both homozygous genotypes produce similar phenotypes (Cockett et al., 1996). The genotypes possible from mating a heterozygous CLPG ewe with a heterozygous CLPG ram are CC, NC, NN and CN, the paternal allele being written first. Of these offspring, only animals of the CN genotype express the muscle hypertrophy phenotype.

Chromosomal position of CLPG was further mapped to a 3.9cM interval on chromosome 18 bounded by CSSM18 and OY3-OY5-BMS1561 flanking markers (Freking et al., 1998a). Although the precise identity of the CLPG

mutation is unknown, candidate genes have been proposed. Bidwell et al. (1999) reported *GTL2* (gene trap locus 2) expression, measured at 56 days of

Table 1. Possible offspring from CN sired matings. Paternal alleles are on the left hand side of the boxes and maternal alleles are written above. The first allele written denotes paternal origin and C denotes the mutant callipyge allele. Asterisk next to genotype denotes phenotypic callipyge and percentages to the right are estimated number of offspring that exhibit the callipyge phenotype.

	N	N	
C	CN*	CN*	50% CLPG
N	NN	NN	

	C	N	
C	CC	CN*	25% CLPG
N	NC	NN	

	N	C	
C	CN*	CC	25% CLPG
N	NN	NC	

	C	C	
C	CC	CC	0% CLPG
N	NC	NC	

age, was elevated in hypertrophied muscles containing at least one CLPG gene. Furthermore, *GTL2*'s chromosomal position makes it an ideal candidate gene for CLPG but *GTL2* lacks an open reading frame, making it a non-coding sequence (Bidwell et al., 1999; Wylie et al., 2000). While the candidate gene identified by Bidwell et al. (1999) did not clarify the biological mechanisms responsible for the CLPG condition due to the non-coding sequence of *GTL2*, it did help researchers solidify future findings on imprinted genes. Imprinted genes on human chromosome 14q32, homologus to mouse distal chromosome 12 and sheep distal chromosome 18, were identified as *GTL2* and *DLK1* (Wylie et al., 2000). The maternally expressed gene, *GTL2*, lacks a significant open reading frame, while the paternally expressed gene, *DLK1*, encodes for a cell-surface transmembrane protein containing epidermal growth factor-like repeats (Wylie et al., 2000). The findings from Bidwell et al. (1999) and Wylie et al., (2000) suggest CLPG lambs result from imprinted genes *GTL2/DLK1*. Furthermore, Miyoshi et al. (2000) contends the maternally imprinted *GTL2* in humans is the same as maternally imprinted *MEG3* in mice. Miyoshi's findings along with Fahrenkrug et al. (2000), who suggested *PREF-1* (preadipocyte factor-1) and *MEG-3* (maternally expressed gene 3) as candidate genes, based on comparative mapping of the ovine CLPG locus, lead to the same conclusion. Depending on the preferred name, the functional candidate genes for CLPG are the maternally expressed, non-coding *MEG3* (*GTL2*) and the paternally expressed, adipogenesis regulator *PREF-1* (*DLK1*, *pG2*, *ZOG*; Fahrenkrug et al., 2000; Wylie et al., 2000). These are candidates due to chromosomal position,

mode of inheritance and negative impact on adipogenesis, as seen in CLPG lambs. As the impact of these genes on muscle growth is not understood, further research is warranted.

Prenatal growth

There have been no studies addressing prenatal development of CLPG lambs. However, it is assumed that prenatal growth of CLPG (CN) lambs is similar to prenatal growth of non-CLPG (NN) lambs. The CLPG gene does not affect birth weights, percentage of dystocia, multiple births or gender distribution (Jackson et al., 1997a). Additionally, no increase in apparent muscle fiber number exists (Koohmaraie et al., 1995). Since muscle fiber hyperplasia occurs prenatally, these data suggest that increased muscle mass in CLPG lambs is due to postnatal muscle hypertrophy.

The CLPG condition in lambs is distinct from the well-studied condition of double muscling in cattle. Recent discoveries have identified the genetic factor controlling double muscling in cattle. In attempts to understand the physiological role of transforming growth factor-β superfamily members, McPherron et al. (1997) "knocked out" the gene for a growth factor and subsequently produced GDF-8 (myostatin) null mice. These mice had a 200-300% increase in muscle mass compared with control mice. McPherron et al. (1997) further established the increase in musculature was associated with an 86% increase in muscle fiber number, as well as hypertrophy. Muscle fiber hyperplasia clearly does not occur in CLPG sheep. It is thought that myostatin (GDF-8) does act as a negative regulator of myoblast proliferation and/or differentiation, since myostatin (GDF-8)

null mice exhibit an increase in muscle fiber number. Myostatin null mice also exhibit 14-50% increases in fiber diameter (McPherron et al., 1997). Thus, the increased muscularity can be attributed to an increase in number of muscle cells, as well as muscle fiber hypertrophy.

Belgian Blue and Piedmontese cattle have long been known for their "double muscled" features. It has only been since McPherron's work in 1997 that GDF-8 was identified as the mutation resulting in double muscled cattle. Belgian Blue and Piedmontese have an 11-bp deletion and a G-A transition, respectively, in the coding region for myostatin. Therefore, it appears these breeds have mutations within the myostatin gene and myostatin is a negative regulator of muscle growth in cattle as well as mice (Grobet et al., 1997; Kambadur et al., 1997). From a production standpoint the CLPG mutation is preferable to mutations in myostatin. Increasing muscle fiber number often increases the size and form of the fetus, thereby impairing ease of parturition in double muscled animals. An increase in muscle fiber number does not occur in CLPG sheep and hence, no difficulties in parturition are observed.

Postnatal Growth

Callipyge (CN) lambs phenotype is indistinguishable from normal lambs until 4-6 weeks of age when the CLPG phenotype becomes apparent (Jackson et al., 1997a,b). Duckett et al. (2000) reported selective muscular hypertrophy of CLPG lambs begins between 7 and 20 kg, which was 2.7 and 14 weeks of age, respectively. Despite discrepancies in onset of muscle hypertrophy, several studies have shown muscles of pelvic limbs and torsos from CLPG lambs are

significantly larger than normal lambs. These studies also found no hypertrophy occurred in muscles of the CLPG thoracic limb (Duckett et al., 2000; Jackson et al., 1997a,b; Koohmaraie et al, 1995). Callipyge lambs have 32%, 42% and 39% heavier longissimus, biceps femoris (BF) and semimembranosus muscles, respectively, but no change in infraspinatus (IS) and supraspinatus muscle weights in comparison to non-CLPG lambs at 6 months of age (Koohmaraie et al, 1995).

Hypertrophied longissimus, biceps femoris and semimembranosus muscles have greater than 100% more calpastatin activity than non-hypertrophied control muscles. In contrast, infraspinatus and supraspinatus muscles of CLPG sheep, which do not hypertrophy, are similar in calpastatin activity to phenotypically normal lambs (Koohmaraie et al., 1995). These authors suggested increased calpastatin activity increases muscle mass by inhibiting the calcium activated neutral proteases (calpains), which are thought to initiate myofibrillar protein breakdown. If calpastatin's inhibitory effect on calpains does increase muscle mass, hypertrophied muscles of CLPG sheep would have less protein turnover and a greater net protein accretion. Lorenzen et al. (2000) evaluated protein turnover by measuring in vivo protein synthesis and accretion, then determined degradation by difference. Callipyge lamb LD and BF muscle fractional accretion rates were greater than those of non-CLPG lambs (Lorenzen et al., 2000). These authors attributed the increased fractional accretion rate to a reduction in fractional protein synthesis and an even greater reduction in fractional protein degradation. However, this study only looked at one window of

time , 5-11 weeks of age, which is after CLPG lambs have already begun to exhibit their extreme musculature. Therefore, Lorenzen et al. (2000) were unable to conclude that the mechanisms responsible for maintaining muscle hypertrophy are the same as those at the onset of muscle hypertrophy.

The precise role of the calpain system in muscle protein turnover is unclear. For example, Brahman cattle have increased levels of muscle calpastatin, but do not exhibit muscle hypertrophy relative to other cattle (Cole et al., 1963; Whipple et al., 1990). Thus, a causal relationship between muscle calpastatin activity and muscle growth remains to be demonstrated. It seems likely that several factors may contribute to CLPG muscle hypertrophy.

Protein, DNA, and RNA content as well as RNA concentration and RNA:DNA ratio are greater in the longissimus and semitendinosus of CLPG lambs compared with non-CLPG lambs. Protein concentration, DNA concentration and protein:DNA are not altered by the CLPG phenotype (Koohmaraie et al., 1995). In contrast, Carpenter et al. (1996) found protein:DNA ratio to be greater in the semitendinosus, longissimus and gluteus medius muscles but not the supraspinatus muscle of CLPG lambs compared with non-CLPG lambs. Protein:RNA and RNA:DNA ratios in the semitendinosus, longissimus, gluteus medius and supraspinatus were similar between CLPG and non-CLPG (Carpenter et al., 1996). Traditionally RNA:DNA and protein:RNA have been used as indices of transcription and translation, respectively. While this is a crude method to measure transcriptional or translational efficiency it does provide insight into areas where further research needs to be performed.

The above studies suggest the CLPG phenotype does alter the rate of transcription and/or translation.

During postnatal growth, increases in muscle and decreases in fat imply CLPG lambs undergo a repartitioning effect. Considering the increase in feed efficiency previously discussed, a partitioning of more energy to muscle and less to other metabolic functions or biological products occurs in CLPG lambs. Energy repartitioning is also supported by the lighter fleece weights ($P < .01$) and shorter staple lengths ($P < .03$) observed in CLPG ewes (Jackson et al., 1997a).

Callipyge Muscle Fiber Type Characteristics

Muscle fiber phenotypes are influenced by intrinsic properties of myoblasts and extrinsic factors such as innervation. Muscle fiber phenotype alterations result in fast, slow or mixed fast/slow fiber types. Muscle hypertrophy can be obtained by fiber type transitions, since white muscle fibers (type IIb) typically have larger diameters than red (type I) or intermediate fibers (type IIa). Fiber type differences result from production of isoforms of muscle contractile protein (Robbins et al., 1986). The most abundant skeletal muscle contractile protein is myosin and myosin heavy chain isoforms are more abundant than other contractile protein isoforms, making them easier to evaluate. Myosin heavy chain isoform gene expression occurs in the order I↔IIa↔IIx↔IIb (arrows denote possible transitions between different MHC isoforms within a muscle fiber) (Schiaffino and Reggiani, 1994). Conventional actomyosin ATPase typing does not allow distinction between type IIx and IIb muscle fibers (Lefaucheur et al., 1998). A higher percentage of type IIb fibers have been observed in

hypertrophied CLPG lamb muscles. In the longissimus of 24-week-old CLPG lambs, 9% of the muscle fibers are type I (slow, oxidative), 34% are type IIa (fast, oxidative-glycolytic) and 57% are type IIb (fast glycolytic) vs. normal lambs, which have 10%, 46% and 44% of these fiber types, respectively (Koohmaraie et al., 1995). Lorenzen et al., (2000) observed comparable values at 5, 8 and 11 weeks of age with only the type IIb fibers increasing statistically in CLPG lambs. Hypertrophied muscles (gluteus medius and semitendinosus) but not a non-hypertrophied muscle (supraspinatus) had similar increases in the percentage of fast, glycolytic fibers (Carpenter et al., 1996; Koohmaraie et al., 1995). Koohmaraie et al. (1995) also demonstrated type IIa and type IIb muscle fibers of the LD muscle in CLPG undergo a 47% and 45% increase in cross-sectional area, respectively.

The semitendinosus type I, type IIa, type IIb muscle fiber area is increased in the CLPG condition by 3%, 99% and 52%, respectively. Increases in both fiber type distribution and fiber area occur only with type IIb muscle fibers. These characteristics and an increase in type IIa fiber diameter account for the muscle hypertrophy in CLPG lambs (Carpenter et al., 1996; Koohmaraie et al., 1995; Lorenzen et al., 2000).

Potential Mechanisms of Callipyge Gene Action

Although the CLPG gene has not been identified, several experiments have characterized biological mechanisms that may be associated with CLPG gene action. Whisnant et al., (1998) examined blood hormone profiles to determine if these differed between CLPG and normal sheep. Serum

concentration, frequency or pulse amplitude of growth hormone secretion did not differ between CLPG and normal lambs. Likewise, there were no differences in insulin, IGF-I or serum thyroxine concentrations between CLPG and normal lambs. Serum cortisol levels increased similarly with restraint in CLPG and non-CLPG lambs (Whisnant et al., 1998). This information implies that levels of hormones that are generally thought to regulate growth and body composition are similar between CLPG and non-CLPG lambs.

β-adrenergic agonists (βAA) are a class of endogenous or synthetic compounds that elicit numerous physiological responses. The physiological βAA compounds are the catecholamines, epinephrine and norepinephrine. Synthetic forms of βAA, similar to endogenous forms in both pharmacological and chemical properties, include; cimaterol, clenbuterol, L 664,969, ractopamine, and salbutamol. All of the βAA initiate physiological responses by binding to β-adrenergic receptors. β-adrenergic agonists have been used to increase muscle mass and decrease fat in many species (Mersmann, 1998).

Studies in lambs show biceps femoris (BF) muscle mass is increased 18.6%-32.8% with βAA treatment (Koohmaraie et al., 1991; Beermann et al., 1987). Differences in efficacy are dependent upon the agent used and length of administration. Increases in muscle mass are accompanied by increases in protein and RNA content as well as concentration (Koohmaraie et al., 1991; Beermann et al., 1987). Concentration of muscle DNA is decreased, but content is not altered with treatment (Kim et al., 1987; Koohmaraie et al., 1991; Beermann et al., 1987). Muscles of βAA treated lambs have an increased

proportion and diameter of type II muscle fibers (white) (15%-50%) (Kim et al., 1987; Beermann et al., 1987).

The similarities between βAA treated and CLPG lambs led researchers to examine the effects of βAA treatment on CLPG lambs. Administration of the βAA (L-644,969) to CLPG lambs elicited no response (Koohmaraie et al., 1996). These authors suggested CLPG lambs may not respond to βAA because muscle growth rates may already be near maximum. Additionally the CLPG gene may exert its effects through intracellular events similar to βAA treatments (Koohmaraie et al., 1996).

While the biological mechanism affecting the CLPG phenotype has not been found, evidence suggests biological differences occur in homozygous and heterozygous CLPG sheep. Freking et al. (1998) demonstrated that hypertrophied CN lamb muscles had the highest calpastatin activity and shear force values (least tender), CC lambs were intermediate, and NN and NC lambs had the lowest calpastatin activity and shear force. The study performed by Freking et al. (1998) was the first to evaluate all genotypes from CLPG matings. Other studies have quantified characteristics of phenotypic CLPG or non-phenotypic normal lambs. The above information also allows for pooling of NN and NC genotypes when there is no statistical difference. Nevertheless, a biological difference in CC lambs provides evidence for studying skeletal muscle characteristics of CLGP genotypes to better understand CLPG gene action.

Characteristics of Meat from Callipyge Lambs

The hypertrophied condition of CLPG lambs adds lean tissue to the carcasses, but increased levels of muscle calpastatin have adverse effects on carcass quality. Callipyge lamb loin chops are significantly tougher than normal lamb chops and do not undergo typical tenderization during postmortem storage. Warner-Bratzler shear force values are 44.8%, 112.2% and 144.7% greater for CLPG lamb loin chops than control lamb chops on days 1, 7, and 21, respectively (Koohmaraie et al., 1995). It is important to note that even after 21 days of aging CLPG shear force values were still greater than shear force values for day 1 normal lambs (Koohmaraie et al., 1995). Myofibril fragmentation index values are higher for normal lambs, indicating there is less postmortem proteolysis of myofibrillar proteins in CLPG lambs. Western blot analysis confirmed a lack of postmortem degradation of myofibrillar proteins troponin-T, desmin, vinculin, nebulin and titin from CLPG lambs (Koohmaraie et al., 1995).

Elevated levels of collagen and increased collagen crosslinks also decrease meat tenderness. However, collagen content and hydroxylysylpyridinoline crosslink concentration are lower in CLPG lambs than non-CLPG lambs (Field et al., 1996). Collectively, these data demonstrate that decreased tenderness in CLPG lambs results from less postmortem proteolysis.

MATERIALS AND METHODS

Sample Collection

Muscle samples were obtained after slaughter of market weight (approximately 22-week-old), phenotypically CLPG (CN, n=6) and non-CLPG (NN, NC, CC, n=3 per genotype) ram lambs. Lamb genotyping was performed as described by Freking et al., (1998) and genotype data was provided Dr. Freking (USDA-ARS, Clay Center, NE). Muscle samples were collected in the abattoir at Roman L. Hruska U.S. Meat Animal Research Center (USDA-ARS, Clay Center, NE). Skeletal muscle samples were collected within 15 minutes after exsanguination. Longissimus dorsi, infraspinatus, and biceps femoris samples were removed from the right side of each lamb, diced and quick-frozen in liquid nitrogen, then stored at -80°C. Samples were kept on dry ice during transportation to the Muscle Biology Laboratory at Michigan State University, where they were stored at -80°C.

Carcass measurements and protein and nucleic acid measurements were collected from all genotypes and muscles. Myofibrillar degradation analysis, myosin heavy chain isoform and sarcoplasmic protein separation was performed with LD tissue of NN, NC, CC and CN lamb samples.

Carcass Measurements

Lamb carcass weights were recorded on an in-line rail scale immediately after slaughter and evisceration. To offset muscle weight removed during sample collection 0.9 kg was added to each carcass weight. Ribeye area (REA) was determined as the cross-sectional area of the longissimus dorsi. Backfat

was defined as the subcutaneous fat thickness measured at a point half the lateral distance of the longissimus muscle from the vertical process of the thoracic vertebra. Both measurements were taken adjacent to the 12th rib interface after the longissimus muscle was exposed by a cut perpendicular to the vertebral column between the 12th and 13th rib. Tracer paper was applied to the cut face and the outside perimeter of the loin muscle was traced onto the paper, along with the outside perimeter of subcutaneous fat. Ribeye area was taken from the right side of lamb carcasses. Traced REA's and backfat thickness were measured using a standard lamb REA grid and backfat ruler. Ribeye area and backfat thickness measures were averaged from independent measures taken by three evaluators.

After a 24 hour chill of the carcass, the longissimus dorsi (LD), biceps femoris (BF) and infraspinatus (IS) were dissected from the left side of the carcass and weighed. The LD was removed by making an incision perpendicular to the long axis of the muscle and adjacent to the cranial edge of the ilium. A knife was run along the vertical processes of the lumbar and thoracic vertebrae to remove the medial side of the LD from the spine. This was followed by an incision running along the transverse processes of the lumbar vertebrae, ribs, and ending at the cervical vertebrae to detach the LD from its insertion. The LD muscles were removed from carcasses, and then separated from attached superficial muscles such as the spinalis or multifidus dorsi.

The BF was removed from the left side of the carcass with the proximal detachment being achieved with an incision on the caudal edge of the ilium and sacral tuber. Distal detachment was at the distal end of the femur.

Infraspinatus removal was performed by palpation of the shoulder to locate the spine of the scapula. Once the spine of the scapula was located, an incision was made on the ventral/caudal edge of the spine, partially detaching the IS from the scapula. The IS was pulled from the infraspinous fossa. The proximal end of the IS was detached from scapular cartilage and the distal end from the lateral tuberosity of the humerus.

All muscles were closely trimmed of any other muscles and excess connective tissue or fat, then weighed. The same individual performed all trimming and weighing of muscles.

Protein and Nucleic Acids

Protein concentrations were determined by the biuret method (Gornall et al., 1949). The DNA concentrations were determined according to Labarca and Paigen (1980) using Hoechst 33258 reagent. Concentrations of RNA were determined by the method of Munro and Fleck (1969). Detailed procedures for quantifying protein and nucleic acid concentrations is in the appendix.

Sample Preparation

A list of all solutions used in the following methods is included in the appendix. Half gram LD muscle samples were placed in 10 volumes of homogenization buffer containing 75 mM KCL, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM ethylenediamine tetra acetic acid (EDTA), 50 mM NaF, 2 mM

phenylmethylsulfonyl flouride (PMSF), 6 mg/L leupeptin and homogenized by Polytron® (Brinkmann, Westbury, NY) for 10 seconds at speed 6 using a 1.5 cm generator. Sodium fluoride, PMSF and leupeptin were dissolved in stocks and added to homogenization buffer the day of extraction. Samples were homogenized a second time for 10 seconds at speed 6 to ensure a homogeneous mixture. Samples were centrifuged at 10,000 x*g* for 15 minutes at 4°C to separate myofibrillar proteins from sarcoplasmic proteins. The supernatant containing sarcoplasmic proteins was poured off and stored at -80°C. Myofibrillar proteins were suspended in homogenization buffer, vortexed, and centrifuged as indicated above. The supernatant fluid was discarded. This wash procedure was performed two more times and the final pellet was resuspended in 10 mL homogenization buffer without sodium flouride, PMSF or leupeptin and stored at -80°C. A 0.5 mL aliquot of sarcoplasmic and myofibrillar protein preparations was removed prior to freezing and mixed 1:1 v/v with 2x Laemmli buffer (0.125 M Tris, 4% SDS, 20% glycerol) without β-mercapto-ethanol (MCE), heated at 90°C for 5 minutes and stored at -20°C.

Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL) for myosin heavy chain (MHC) studies and by biuret (Gornall et al., 1949) for degradation, as well as sarcoplasmic protein studies. A serial dilution of bovine serum albumin was used to create a standard curve from 10 mg/ml to 0 mg/ ml. The biuret assay was used on non-denatured myofibrillar protein samples to increase solubility.

Myosin Heavy Chain Isoform Separation

Myofibrillar proteins from the LD muscle of three lambs per genotype were suspended in 1x Laemmli buffer plus glycerol (0.0625 M Tris, 2% SDS, 5% MCE, 40% glycerol, pH 6.8). Proteins (0.5 or 1μg/lane) were loaded onto discontinuous 8% gels (50:1 ratio of acrylamide to N,N'-methylene-bis-acrylamide) with 4% stacking gels (50:1). Gels (20 cm x 20 cm x 0.75 mm) were run at 275 volts for 24 hours at 4°C using a BIO-RAD Protean II xi cell (BIO-RAD, Hercules, CA) with a water-cooled chamber. A two-buffer system was used. The upper running buffer consisted of 0.1 M Tris (base), 150 mM glycine, and 0.1% SDS, and lower running buffer contained 50 mM Tris (base), 75 mM glycine, and 0.05% SDS (Talmadge and Roy, 1993). Myosin heavy chain (MHC) isoforms separated by SDS-polyacrylamide gel electrophoresis (PAGE) and were silver stained (BIO-RAD, Hercules, CA). Electronic images were acquired and analyses were performed with a BIO-RAD Gel Doc imaging system and Quantity One, respectively (BIO-RAD, Hercules, CA). Designation of myosin heavy chain isoforms was based on the relative migration of type IIa, IIx, IIb and I, as described for rat myosin (Talmadge and Roy, 1993).

Myosin Heavy Chain Western Blots

Myosin heavy chain gels were electrophoretically transferred to polyvinylidene fluoride (PVDF, Immobilon™-P, Millipore Corporation, Bedford, MA) overnight at 4°C. Transfer buffer (25 mM Tris, 0.192 Glycine, 0.1% SDS, pH 8.3) was mixed gently with a stir bar during transfer. From cathode to anode, a sandwich consisting of clamping cassette, one fiber pad, two pieces of Whatman

filter paper, gel, PVDF membrane, two pieces of Whatman filter paper, one fiber pad and the anode side of transfer clamping cassette was assembled prior transfer. Proteins were transferred from the gels to membrane at 70 mA constant current. After removal from the transfer apparatus, membranes were rinsed with double distilled (ddI) water, placed in 10 ml of 1% non-fat dry milk (NFDM) blocking solution and incubated for 1 hour to minimize non-specific antibody binding. All blocking, washing and incubations occurred on a red rocker at speed 7 in Falcon® tissue culture plate tops. Primary mouse anti-myosin monoclonal antibody (Na4) was diluted 1:5000 in 1% NFDM. Blocking solution was poured off membranes and diluted Na4 Ab was poured onto membranes and incubated for 1.5 hours at room temperature. Membranes were washed 3 times for 5 minutes each time with 10 mL 1% NFDM. After washes, 20 mL of 1% NFDM with anti-mouse IgG alkaline phosphatase (AP) conjugated 2° Ab (1:10,000 concentration) were dispensed onto membrane and incubated for 30 minutes. Membranes were washed 3 times for 5 minutes each time with 10 mL 1% NFDM followed by 2 washes with 10 mL Tris-buffered saline containing 0.1% Tween-20. Membranes were exposed to BIO-RAD AP substrate working solution (BIO-RAD, Hercules, CA) until desired band intensity appeared, but no longer than 1 minute. Membranes were rinsed several times with ddI water to remove residual substrate and halt the enzymatic activity. Membranes were dried on paper towel before image acquisition.

Sarcoplasmic Protein Separation

Ten micrograms of LD muscle protein per lane were loaded on a discontinuous 10% (37.5:1 ratio of acrylamide to N,N'-methylene-bis-acrylamide) separating gel poured up to 18 mm from the top of the inner plate on a Bio-Rad mini protean II (BIO-RAD, Hercules, CA). Gels were allowed to set-up for 1 hour before a 4% (37.5:1) stacker was poured. Samples were electrophoresed at a constant voltage of 180 until the dye front ran off (40-50 minutes). Gels were stained in Coomassie blue R-250 and destained (1-hour wash in destain 1, 3-hour wash in destain 2) before being dried between sheets of cellophane and scanned using a Bio-Rad fluor-s image analysis system. Electronic images were acquired and analyses were performed with a BIO-RAD Gel Doc imaging system and Quantity One, respectively (BIO-RAD, Hercules, CA).

m-Calpain Mediated *In vitro* Proteolysis

Myofibrillar proteins from three NN and three CN lambs were purified according to procedure of Goll et al., (1974). All samples were visually appraised by light microscopy to ensure homogeneous and uniform myofibrils.

Six milligrams of protein suspended in 50 mM Tris (pH 6.7-7.0) were dispensed in a 2 mL microcentrifuge tube. Volume was adjusted to 2 mL with 50 mM Tris and 165 mM NaCl, then centrifuged at 10,000 x g for 10 minutes. Supernatant fluid was poured off and the pellet was resuspended in 2 mL of digestion buffer (50 mM Tris, 165 mM NaCl, 10 mM MCE, 5 mM $CaCl_2$) by vortexing and inversion. Microcentrifuge tubes were placed on a mechanical inverter at 10 rpm for incubation with m-calpain. An initial 250 µl sample (time 0)

was drawn and added 1:1 v/v to 2x Laemmli buffer and heated to 95°C for 5 minutes. One unit of protease activity per 12 mg myofibrillar protein was then added to the digestion buffer. At 5, 15, 30, 60, 120, 240, and 480 minutes after m-calpain addition, samples were prepared as described for the initial sample. A second *in vitro* digestion was performed using 0.5 unit of active protease (m-calpain) per milligram myofibrillar protein for 120 minutes. One unit of m-calpain activity was defined as the amount of enzyme that catalyzed an increase of 1.0 absorbance unit at 278nm in 60 minutes at 25°C. All m-calpain was graciously provided by Dr. M. Koohmaraie from his laboratory at the Roman L. Hruska U.S. Meat Animal Research Center (ARS, USDA,Clay Center, NE).

Separation of myofibrillar proteins was conducted using SDS-PAGE (10% gel with 4% stacker 37.5:1 ratio of acrylamide to N,N'-methylene-bis-acrylamide) at 180 volts for 55 minutes. Proteins were electrophoretically transferred to PVDF at 150 mA for 2 hours using a BIO-RAD mini protean II transfer unit, with ice blocks, placed on a stir plate (BIO-RAD, Hercules, CA). After removal from and disassembly of the transfer apparatus, a mark in pencil was made on the membrane between the BIO-RAD broad range gel electrophoresis molecular weight marker (BIO-RAD, Hercules, CA) lane and the sample lane. Two more marks were made on the membrane at the 65 kiloDalton (kD) marker to aid in removal of proteins greater than 66 kD. The molecular weight marker lane and proteins greater than 66 kD were removed from the rest of the membrane and stained in amido black (Figure 3). Proteins greater than 66 kD were used to verify equal protein load amongst lanes. The far right lane was removed and

used as a negative control. The remaining membrane and negative control lane were placed in 10 ml of 1% bovine serum albumin (BSA) blocking solution and incubated for 1 hour to minimize non-specific antibody binding.

All blocking, washing and incubations occurred on a red rocker at speed 7 in Falcon® tissue culture plate tops. Primary mouse anti-desmin antibody was produced by the D76 hybridoma cell line and harvested on day 12 of culture. Supernatant fluid was harvested then stored in aliquots in a -20°C freezer (growth and harvesting of cells is described in appendix). Primary antibody (1° Ab) was thawed at 37°C prior to use. After blocking for at least 1 hour, 1% BSA blocking solution was poured off and 10 mL of primary mouse anti-desmin hybridoma supernatant fluid was applied to membranes, covered and incubated for over 4 hours at room temperature. Primary mouse anti-desmin antibody was removed and stored for second use (1° Ab can be used twice with no loss in intensity). The negative control lane remained in blocking solution during 1° Ab incubation. Membranes, including negative control, were washed three times for 5 minutes each time with 10 mL 1% BSA. After washes, 10 mL of 1% BSA was dispensed onto the membrane and 20 µl anti-mouse IgG biotin conjugated secondary antibody (2° Ab, 1:500 concentration) were added. Incubation plates were covered and incubated for 45 minutes at room temperature. Membranes were washed 3 times for 5 minutes each time with 10 mL 1% BSA, then incubated with Extra-Avidin conjugated AP (Sigma, 1:1000 dilution in 1% BSA) for 35 minutes. Membranes were washed three times for 5 minutes each time with 10 mL 1% BSA followed by 2 washes with 10 mL 0.1% TTBS. Membranes

were exposed to BIO-RAD AP substrate working solution (BIO-RAD, Hercules, CA) to visualize Ab binding to desmin and desmin degradation products. Membranes were rinsed several times with ddI water to remove residual substrate and stop the enzymatic reaction. Membranes were allowed to air dry on a paper towel before visual appraisal.

Statistical Analysis

All data containing a numeric value of measure were analyzed using the General Linear Model procedure of SAS (SAS Inst. Inc., Cary, NC) with the Tukey-Kramer test for pair-wise comparisons of mean differences. Sample sets NN and NC were pooled as non-CLPG lambs before being compared to CN and CC genotypes.

RESULTS

Effects of lamb genotype on carcass traits and skeletal muscle characteristics were examined. Three lamb genotypes (CN, CC, NC) contain at least one mutant CLPG allele, but only the CN genotype exhibits the phenotypic muscular hypertrophy of the CLPG condition. Freking et al. (1998) demonstrated that hypertrophied CN lamb muscles had the highest calpastatin activity and shear force values (least tender), CC lambs were intermediate, and NN and NC lambs had the lowest calpastatin activity and shear force. Although lambs with the CC genotype do not exhibit muscular hypertrophy, they are distinct from lambs of NN and NC genotypes regarding some charateristics. Therefore, CN (referred to as CLPG) and CC genotypes were compared to the pooled NN and NC genotypes (referred to as non-CLPG) in the current study.

Carcass Measurements and Muscle Weights

Carcass traits and dissected muscle weights expressed as a percent of carcass weight are listed in Table 2 for all lamb genotypes. Carcass weight and backfat thickness were similar among genotypes (Table 2). Callipyge lambs had higher leg scores and larger REAs than the average of non-CLPG. Longissimus dorsi and BF muscles were 26% and 22% heavier, respectively in CLPG lambs compared with non-CLPG (Tables 3 and 4). Additionally, LD and BF muscles comprised a higher percentage of carcass weight in CN lambs than non-CLPG lambs (Table 2). Infraspinatus muscle weights were similar among genotypes (Table 5) and the IS made up a comparable proportion of carcass weight in all genotypes (Tables 2).

Table 2. Carcass characteristics of ram lambs of NN, NC, CN and CC genotypes. The mutant callipyge allele is represented by C and N represents the wild-type normal allele. The paternal allele is listed first. The only animals expressing the phenotypic callipyge muscle hypertrophy are genetically annotated as CN, while NN, NC, and CC genotypes are phenotypic normal.

Trait	NN	NC	CN	CC	Pooled SEM
Carcass Weight, kg	27.7	26.2	27.6	21.8	1.1
12th Rib Backfat, mm	4.2	4.2	4.2	3.8	0.4
Leg Score, 1-15 scale 15 = Most Desirable	11.0	10.7	14.8[a]	11.3	0.3
REA, cm^2	15.1	13.5	20.4[a]	14.0	0.8
Longissimus dorsi, % Carcass Weight	2.4	2.6	3.2[a]	2.7	0.1
Biceps femoris, % Carcass Weight	1.4	1.5	1.8[a]	1.4	0.03
Infraspinatus, % Carcass Weight	0.6	0.7	0.6	0.6	0.1

a = different from pool of NN and NC ($P<0.05$).

Quantification of Skeletal Muscle Nucleic Acids and Protein

Muscle weight, protein, DNA and RNA concentrations are shown for the LD, BF and IS muscles in Tables 3, 4 & 5, respectively. Concentration of protein was not statistically different for LD and IS muscle groups amongst genotypes, but BF protein concentration was lower in CLPG and genotypic CC lambs compared with non-CLPG lambs. Concentrations of DNA and RNA for the three muscles were similar amongst genotypes (Tables 3, 4 & 5). However, LD muscles of CLPG lambs had more total DNA than those of non-CLPG lambs (Table 3). Longissimus and BF muscles of CLPG lambs also had more RNA than those of non-CLPG lambs. No difference in nucleic acid content of IS

muscles were observed among genotypes. Thus, increased nucleic acid content is attributable to hypertrophy of muscles in CLPG lambs.

The RNA:DNA ratio in LD muscles of non-CLPG lambs is 20% less than the mean RNA:DNA ratio of the CN or CC genotypes (Table 3). Protein:RNA ratios of the LD were higher for non-CLPG lambs than CLPG or genotypic CC lambs. Protein:DNA ratio for all three muscles as well as protein:RNA and RNA:DNA ratios for the BF and IS muscle were similar among genotypes (Tables 3, 4 and 5).

Table 3. Longissimus dorsi muscle weight, protein and nucleic acid measurements of ram lambs of from NN, NC, CN and CC genotypes. The mutant callipyge allele is represented by C and N represents the wild-type normal allele. The paternal allele is listed first. The only animals expressing the phenotypic callipyge muscle hypertrophy are genetically annotated as CN, while NN, NC, and CC genotypes are phenotypic normal.

Longissimus Dorsi Trait	NN	NC	CN	CC	Pooled SEM
Wet tissue weight, g	654.7	659.2	892.1[a]	592.7	29.8
Protein, mg/g	222.7	257.9	199.6	215.3	7.8
Protein, g	145.8	168.5	180.8	128.9	9.9
DNA, ug/g	177.2	210.5	187.7	200.6	4.7
DNA, mg	116.0	138.2	167.6[a]	119.5	6.6
RNA ug/g	333.7	481.1	469.7	531.7	21.2
RNA, mg	218.1	316.2	414.8[a]	317.2	17.3
Protein:DNA	1259.7	1237.0	1062.5	1067.0	35.0
Protein:RNA	683.6	553.2	429.7[a]	404.3[a]	27.5
RNA:DNA	1.8	2.2	2.5[a]	2.6[a]	0.1

a = different from pool of NN and NC ($P<0.05$).

Table 4. Biceps femoris muscle weight, protein and nucleic acid measurements of ram lambs of from NN, NC, CN and CC genotypes. The mutant callipyge allele is represented by C and N represents the wild-type normal allele. The paternal allele is listed first. The only animals expressing the characteristic phenotypic callipyge muscle hypertrophy are genetically annotated as CN, while NN, NC, and CC genotypes are phenotypic normal.

Biceps Femoris Trait	NN	NC	CN	CC	Pooled SEM
Wet tissue weight, g	378.0	393.1	494.4[a]	308.4	16.9
Protein, mg/g	274.6	283.6	232.7[a]	224.1[a]	6.7
Protein, g	104.3	111.2	116.1	69.0	5.7
DNA, ug/g	198.7	223.8	207.7	211.3	5.4
DNA, mg	75.0	87.7	102.4	65.1	3.7
RNA ug/g	432.7	512.2	514.0	535.5	17.9
RNA, mg	162.6	200.7	252.8[a]	164.4	8.7
Protein:DNA	1381.5	1265.1	1131.5	1065.3	35.9
Protein:RNA	645.8	558.2	455.9	433.2	25.8
RNA:DNA	2.1	2.2	2.4	2.5	0.1

a = different from pool of NN and NC ($P<0.05$).

Table 5. Infraspinatus muscle weight, protein and nucleic acid measurements of ram lambs of from NN, NC, CN and CC genotypes. The mutant callipyge allele is represented by C and N represents the wild-type normal allele. The paternal allele is listed first. The only animals expressing the phenotypic callipyge muscle hypertrophy are genetically annotated as CN, while NN, NC, and CC genotypes are phenotypic normal.

Infraspinatus Trait	NN	NC	CN	CC	Pooled SEM
Wet tissue weight, g	172.4	169.3	161.0	139.1	5.8
Protein, mg/g	222.6	226.1	228.2	237.8	2.7
Protein, g	38.3	38.3	36.9	33.0	1.5
DNA, ug/g	219.7	228.6	211.8	218.1	3.7
DNA, mg	37.8	38.6	34.3	30.1	1.4
RNA ug/g	538.2	544.8	504.4	537.3	28.2
RNA, mg	92.3	92.3	80.6	72.7	4.1
Protein:DNA	1014.9	991.8	1078.8	1095.0	17.7
Protein:RNA	418.3	420.4	460.5	483.8	22.1
RNA:DNA	2.4	2.3	2.3	2.4	0.1

Measures of Muscle Fiber Type Transition

Gel electrophoresis of muscle proteins from lambs of all genotypes provided a quantitative measure for all four myosin heavy chain isoforms and several sarcoplasmic proteins. Myosin heavy chain isoforms type I, IIb, IIx and IIa were separated using SDS-PAGE (Figure 1). All bands were confirmed to be myosin isoforms using a monoclonal Ab (Na4) that recognizes all forms of sarcomeric myosin. However, specific myosin isoforms were not identified by Western blotting, since available isoform specific antibodies either did not recognize lamb myosin or cross-reacted with multiple myosin isoforms (results not shown). Designation of myosin heavy chain isoforms was based on the relative migration of type IIa, IIx, IIb and I, as described for rat myosin (Talmadge and Roy, 1993). Type I, type IIa and IIb myosin heavy chain isoforms did not change in either CN or CC genotypes in comparison to non-CLPG lambs (Table 6). In contrast, type IIx of CLPG lambs increased 25% in comparison with the mean of non-CLPG lambs (Table 6).

Sarcoplasmic proteins were separated by SDS-PAGE and several bands were assigned putative identities (Figure 2). No differences among genotypes were detected in the proportion of specific protein bands (Table 7).

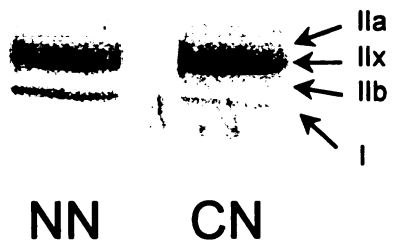

Figure 1. Analysis of myosin heavy chain isoforms (MHC). Separation of MHC from phenotypic non-callipyge (NN) lambs (left lane) and phenotypic callipyge (CN) lambs (right lane) was accomplished by SDS-PAGE. Myosin heavy chain isoforms were visualized by silver staining as described in methods. Designation of myosin heavy chain isoforms was based on the relative migration of type IIa, IIx, IIb and I, as described for rat myosin (Talmadge and Roy, 1993).

Table 6. Percentage of myosin heavy chain isoform in the longissimus muscle of ram lambs of from NN, NC, CN, or CC lambs. C represents the mutant callipyge allele and N represents the wild-type normal allele. The paternal allele is listed first. The only animals expressing the phenotypic callipyge muscle hypertrophy are genetically annotated as CN, while NN, NC, and CC genotypes are phenotypic normal.

Isoform	NN	NC	CN	CC	Pooled SEM
% Type IIa	39.9	37.4	35.0	35.5	1.3
% Type IIx	42.9	38.5	54.3[a]	46.6	0.5
% Type IIb	5.2	5.5	5.5	5.3	0.9
% Type I	12.0	18.6	5.2	12.5	1.3

a = different from pool of NN and NC ($P<0.05$).

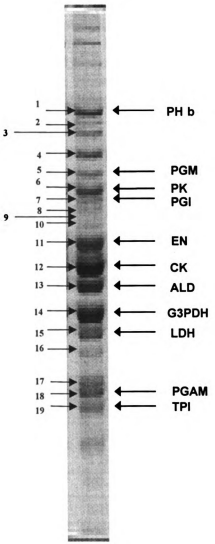

Figure 2. Representative lane showing separation of sarcoplasmic proteins. Left arrows indicate bands detected, right arrows indicate potential proteins as identified by McCormick et al. (1988) in swine. Proteins were separated by SDS-PAGE and visualized by Coomassie blue staining as described in materials and methods.

Table 7. Percentage of sarcoplasmic protein band within lane from NN, NC, CN or CC ram lambs was quantified according to Materials and Methods. The mutant callipyge allele is represented by C and N represents the wild-type normal allele. The paternal allele is listed first. The only animals expressing the characteristic phenotypic callipyge muscle hypertrophy are genetically annotated as CN, while NN, NC, and CC genotypes are phenotypic normal. Protein bands were identified as described in Figure 2.

Sarcoplasmic Protein Band	NN	NC	CN	CC	Pooled SEM
1	5.3	5.0	5.4	5.4	0.07
2	0.6	0.7	0.5	0.6	0.20
3	3.3	2.6	2.9	2.5	0.02
4	6.2	5.9	5.6	5.7	0.19
5	2.4	2.2	2.5	2.3	0.04
6	5.0	4.7	5.5	5.3	0.11
7	1.6	1.1	1.9	1.2	0.27
8	1.2	1.2	1.3	0.8[a]	0.11
9	0.3	0.3	0.2	0.2	0.33
10	0.8	0.6	0.8	1.4	0.15
11	6.7	6.8	6.9	6.6	0.05
12	0.2	1.0	0.9	0.6	0.02
13	11.8	15.4	11.7	14.2	0.17
14	7.6	8.6	8.0	9.2	0.32
15	11.9	12.8	11.9	13.4	0.14
16	4.2	4.5	4.1	4.6	0.79
17	3.5	3.6	3.3	2.3	0.28
18	4.8	3.7	4.0	2.4	0.38
19	4.2	2.9	4.0	4.0	0.12

a = different from pool of NN and NC ($P<0.05$).

m-Calpain Mediated *In Vitro* Proteolysis

The disappearance of the myofibrillar protein desmin on Western blots was used to determine degradation by m-calpain. Desmin is a 55 kD intermediate filament protein, which is known to be degraded by calpains. A ratio of 1:12 (unit m-calpain to mg protein) was determined by titration to slowly degrade several myofibrillar proteins (results not shown). These conditions were used to determine the degradation rate of desmin from NN and CN lamb myofibrils. Desmin from either genotype did not completely degrade within 480 minutes (results not shown). However, there appeared to be less intact desmin remaining from NN myofibrils compared with CN myofibrils after 480 minutes of digestion. Several bands representing desmin degradation products between 40 and 50 kD were darker after digestion of myofibrils from CN lambs compared with myofibrils of NN lambs. Desmin or desmin degradation products did not appear in the negative control lane containing no 1° Ab. This indicates that bands on desmin western blots were specific to 1° Ab binding to its antigen (results not shown).

To achieve complete degradation of intact desmin a 1:2 ratio (unit active calpain to mg protein) was used in an *in vitro* digestion of samples for 120 minutes. Intact desmin (55 kD) was not distinctly visible after a 120 minute digest of either NN or CN lamb myofibrils (Figure 3). Intact desmin degraded more rapidly in myofibrils of NN lambs compared with myofibrils of CN lambs. Furthermore, Desmin degradation products of 48-50 kD appeared transiently until 30 minutes of digestion, but were more abundant in myofibrils of CN lambs

compared to NN lambs (Figure 3). Additionally, desmin degradation products of ~38-40 kD were readily apparent after 15 minutes of CN myofibril incubation with m-calpain, and persisted throughout the 120 minute digest. These ~38-40 kD products were barely detectable in digests of NN myofibrils (Figure 3).

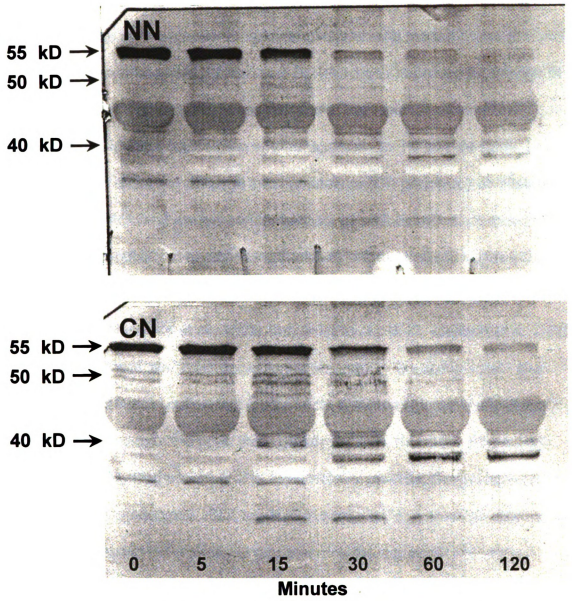

Figure 3. Western blot of desmin after m-calpain induced proteolysis of myofibrils (1:2 unit m-calpain to μg protein). Top blot shows NN myofibrils, while bottom blot shows CN myofibrils after digestion for 0 to 120 minutes. Molecular weight marker and proteins larger than 65 kD (not shown) were removed and stained with amido black to verify size of proteins and equal protein loads among lanes. Bands visible on membrane are specific immunoreactive desmin or desmin degradation products. The arrows notate desmin and desmin degradation products. The 55 kD band is intact desmin, while the bands between 50 and 40 kD are degradation products. Phenotypic normal is annotated NN and CN is phenotypic callipyge.

DISCUSSION

Callipyge lambs are leaner, heavier muscled, and more efficient at converting feed to body weight than non-CLPG lambs. Improved knowledge of muscle development in CLPG lambs will lead to strategies in more efficient production of trim, muscular sheep or other species. In the current study, I determined the effects of callipyge lamb phenotype (CN genotype) on skeletal muscle DNA, RNA, and protein concentrations, as well as indices of muscle fiber type, and susceptibility of myofibrillar proteins to calpain-induced proteolysis.

Previous studies have identified CLPG lambs based solely upon phenotype (Koohmaraie et al., 1995; Carpenter et al., 1996; Jackson et al., 1997; Taylor and Koohmaraie, 1998; Lorenzen et al., 2000). The study herein and Freking et al. (1998b) confirm that lambs of the CN genotype have significantly greater leg scores than the other three genotypes (NN, NC, CC). Phenotypic selection based on leg scores seems to accurately separate CN genotypes from NN, NC and CC genotypes. Still, separation based on leg score can leave up to 66% of the lambs in the non-CLPG group carring the mutant CLPG allele. Freking et al. (1998b) suggested evaluation of all four CLPG genotypes is needed to provide complete information on this mutation. In support of the need to genotype these authors demonstrated hypertrophied CN lamb muscles had the highest calpastatin activity and shear force values (least tender), CC lambs were intermediate, and NN and NC lambs had the lowest calpastatin activity and shear force. This implies that while CN lambs have noticable increases in muscle hypertrophy, other CLPG genotypes may not outwardly show phenotypic

differences, but can exhibit biological characteristics compared to genotypically normal lambs. My study reinforces the need to characterize CLGP genotypes to better understand CLPG gene action. Increased dressing percentage (percentage of animal remaining after skinning, head removal and evisceration) in CLPG lambs can be attributed to heavier carcass weight, lighter internal organ weights or a combination of both (Jackson et al., 1997b, Koohmaraie et al., 1995). There is a 30% increase in carcass weight due to increased muscle weights of the pelvic limb and torso of CLPG lambs, compared with non-CLPG lambs (Jackson et al., 1997c). Therefore, an increase in carcass weight is attributable to individual muscle weights. In my study a similar increase occurred in muscles of the torso (longissimus dorsi) and pelvic limb (biceps femoris), with no change in a muscle of the thoracic limb (infraspinatus). The ability of CLPG lambs to increase muscle mass in specific muscle groups or regions is very perplexing and is yet to be explained. Nevertheless, the increased dressing percentage an muscle mass of the loin and leg make CLPG carcasses extremely desirable to the packer.

The LD and BF muscles of CLPG lambs have been shown to increase an average of 33% and 49% in weight, respectively, compared with non-CLPG lambs (Jackson et al., 1997c, Koohmaraie et al., 1995). Genotypic CN lambs also had heavier LD and BF muscle weights in my study (Table 2). Although average carcass weights among genotypes used in this study were not statistically different, CC lambs had 20% lighter carcass weights than the average of the other three genotypes (Table 2). Therefore muscle weights were

also expressed as a percentage of carcass weight to decrease variation among genotypes. The percentage of total carcass weight of LD and BF muscles was larger for CN lambs than those pooled from NN and NC lambs. The IS muscle, which is not hypertrophied in CLPG lambs, was a similar percentage of carcass weight for all genotypes (Table 2). The observation that muscles of the leg and loin, but not the shoulder, undergo hypertrophy in CN lambs relative to other genotypes is consistent with other studies comparing phenotypic callipyge and non-callipyge lambs (Koohmaraie et al., 1995; Jackson et al., 1997c). Ehile the reason for differential effects of the CLPG gene on these muscles is unclear. My study demonstrated that the muscle hypertrophy is associated with increased muscle RNA content.

Protein, DNA and RNA concentrations were measured in the LD, BF and IS muscles (Tables 3, 4 & 5) to gain a better understanding of muscle characteristics influenced by the CLPG gene. Muscle DNA and protein concentrations were generally not statistically different among genotypes (Tables 3, 4 & 5). These results are in agreement with previous studies on phenotypic callipyge lambs (Koohmaraie et al., 1995; Lorenzen et al., 2000). The RNA concentrations among genotypes were not statistically different (analysis not presented). Nevertheless, my study did find RNA concentrations, on average, to be lower in the LD and BF muscles of NN lambs than the average of the three genotypes that contained at least one CLPG allele. RNA concentrations in LD and BF muscles of these latter genotypes were similar. It is currently unclear why this trend is observed in LD and BF muscles of phenotypic normal lambs

that carry a CLPG allele, but not in non-hypertrophied IS muscle of any lambs carrying a CLPG allele. Koohmaraie et al., (1995) also demonstrated an increase in RNA concentration in phenotypic CLPG lambs compared with control lambs. In contrast, Carpenter et al. (1996) found no difference in RNA concentration among phenotypic CLPG and non-CLPG lambs. The protein, DNA and RNA concentrations of the IS muscle were similar across all four genotypes in my study. These data are consistent with previous characterization of non-hypertrophied muscles in CLPG lambs (Carpenter et al., 1996).

Koohmaraie et al. (1995) demonstrated that protein:RNA ratios decreased and RNA:DNA ratios increased in hypertrophied muscles of CLPG lambs. I also found the RNA:DNA ratio to be higher in LD muscle of CN and CC lambs compared with NN and NC lambs. This ratio supports the trend of increased RNA concentration and suggests that there is either less RNA degraded or an increase in transcription in CLPG lambs. Protein:RNA ratios of LD and BF muscles tended to be lower for lambs with a mutant CLPG allele than NN lambs for my study. Previous studies have suggested that CLPG muscle hypertrophy is associated with a decline in muscle protein degradation (Koohmaraie et al., 1995; Carpenter et al., 1996; Lorenzen et al., 2000). If this proves to be true, then decreased protein:RNA ratio observed herein suggests that translational efficiency in CLPG lamb muscle may be low relative to non-CLPG lamb muscle. These possibilities warrant further evaluation of protein and nucleic acid concentrations of more genotypic CLPG and non-CLPG lambs. If the current

findings are confirmed, future studies on RNA stability, gene transcription and translational efficiency in these lamb genotypes may be informative.

Muscle fiber phenotype alterations result in type I (slow), type IIb (fast) or type IIa (fast/slow) fiber types. Ultimately, fiber type differences result from production of muscle contractile proteins of differing isoforms (Robbins et al., 1986). My study was the first to measure myosin heavy chain isoforms associated with muscle fiber types of the LD muscle from CLPG lamb genotypes. There was a 25% increase in type IIx of CLPG lambs compared with the mean of non-CLPG lambs. The type IIx myosin heavy chain isoform is considered to be associated with fast-twitch muscles and is a transitional isoform between type IIa and IIb. While other fiber types analyzed did not show statistical differences, the increased proportion of type IIx MHC isoform was coincident with numerical decreases in both type I and IIa. This information is consistent with the general idea that MHC gene expression occurs in the order I↔IIa↔IIx↔IIb (arrows denote possible transitions between different MHC isoforms within a muscle fiber) (Schiaffino and Reggiani, 1994). Conventional actomyosin ATPase typing does not allow distinction between type IIx and IIb muscle fibers (Lefaucheur et al., 1998). Still, type IIb muscle fibers of CLPG LD muscles increase in size and distribution (Carpenter et al., 1996, Koohmaraie et al., 1995, Lorenzen et al., 2000). My data indicate the increased percent and size of type IIb muscle fibers in the LD of CLPG lambs is due primarily to an increase in proportion of type IIx MHC isoform. Increased percent and size of type IIb muscle fibers, along with an increase in diameter of type IIa muscle fibers in the LD of CLPG lambs, accounts

for the muscle hypertrophy (Carpenter et al., 1996, Koohmaraie et al., 1995).

Type IIb muscle fibers have also been shown to have a greater capacity for glycolytic metabolism and a decreased capacity for oxidative metabolism than type I muscle fibers. I expected these changes to be reflected as differences in the proportions of sarcoplasmic proteins, which include glycolytic enzymes. However, no significant differences among genotypes were apparent.

Jackson et al. (1997a, b) observed that feed intake (kg/d) in CLPG lambs was decreased an average of 14% whereas feed conversion (lb of gain/lb of feed) of the same group increased 10% over their non-CLPG contemporary group. An increase in percentage of type IIb muscle fibers in CLPG lambs may contribute to improved production efficiency of these sheep (Lorenzen et al., 2000). Type IIb muscle fibers have lower protein turnover rates than type I muscle fibers, making them potentially more efficient (Garlick et al., 1989). Decreased protein turnover via elevated calpastatin activity was speculated by Koohmaraie et al. (1995) to decrease proteolysis of myofibrillar proteins and subsequently increases muscle mass in CLPG lambs. However, calpastatin activity is also elevated in CC lambs (Freking et al., 1998) and Bos Indicus cattle (Whipple et al., 1990), which do not exhibit muscle hypertrophy. Therefore, I believe additional factors, such as translational rate and/or phosphorylation of myofibrillar proteins contribute to either decreased myofibrillar proteolysis and increased efficiency of CLPG lambs.

A reduced susceptibility of CLPG proteins to proteolysis could help explain the decrease in protein turnover observed in CLPG muscle tissue (Lorenzen et

al., 2000) and the reduced rate and extent of postmortem proteolysis seen in CLPG meat (Koohmaraie et al., 1995). My study focused on susceptibility of CN and NN myofibrillar proteins to m-calpain-mediated *in vitro* proteolysis. The disappearance of the myofibrillar protein desmin on Western blots was used to determine degradation by m-calpain. Desmin is a 55 kD intermediate filament protein, which is known to be degraded by calpains. Desmin from both CN and NN lambs was susceptible to calpain-induced degradation. Differences in rate of calpain-mediated proteolysis were evident between CN and NN lamb muscle proteins. Desmin degradation products between 38 to 40 kD, persisted longer during digestion of CN myofibrils compared with NN myofibrils. It is my belief that these degradation products are more readily digested in NN lamb myofibrils compared with CN lamb myofibrils and subsequently undetectable by my detection method. Results from my experiment suggest desmin and other myofibrillar proteins of CN lambs are more resistant to degradation than those of NN lambs. Although the reason for differential sensitivity of myofibrillar proteins to calpain cleavage has not been determined, this may partially explain the observation that muscle protein fractional breakdown rate is reduced in CLPG lambs, resulting in muscle hypertrophy (Lorenzen et al., 2000). Subsequently, this information supports the conclusion that CLPG lambs have reduced postmortem proteolysis.

Collectively, results from my experiment demonstrate that LD and BF muscles are heavier in CN lambs than other genotypes. No differences were detected in characteristics of IS muscles among genotypes. In LD muscles of

CN and CC genotypes the RNA:DNA ratios are higher than in LD muscles of non-CLPG lambs. This suggests CN and CC lambs either have a decrease in RNA degradation or a more efficient transcription process. Greater muscle mass in CN lambs is associated with an increase in the proportion of type IIx myosin heavy chain isoform. A decrease in susceptibility of myofibrillar proteins to degradation may also contribute to CN lamb muscle hypertrophy, reduced fractional breakdown of muscle protein, and increased efficiency of lean growth.

APPENDIX

Homogenization buffer

75 mM KCL
10 mM KH_2PO_4
2 mM $MgCl_2$
2 mM EDTA

*Sodium Fluoride, Phenylmethylsulfonyl flouride (PMSF) and Leupeptin were dissolved in stocks and added to homogenization buffer the day of protein extraction.

50 mM NaF
2 mM PMSF
6 mg/L Leupeptin

Digestion Buffer

50 mM Tris
165 mM NaCl
10 mM β-mercaptoethanol (MCE)
5 mM $CaCl_2$
pH = 6.7 - 7.0

Myosin Heavy Chain Isoforms Lower Running Buffer

	1 L	2 L	4 L
50 mM Tris Base	6.05 g	12.1 g	24.2 g
75 mM Glycine	5.63 g	11.26 g	22.52 g
0.05% SDS	0.50 g	1.0 g	2.0 g
ddH_20	fill to 1 L	fill to 2 L	fill to 4 L

*pH adjustment is not necessary.

Myosin Heavy Chain Isoforms Upper Running Buffer

	1 L	2 L	4 L
0.1M Tris Base	12.11 g	24.22 g	48.44 g
150 mM Glycine	11.26 g	22.52 g	45.04 g
0.1% SDS	1.0 g	2.0 g	4.0 g
ddH_20	fill to 1 L	fill to 2 L	fill to 4 L

*pH adjustment is not necessary.

Standard Running Buffer, pH 8.3

	1 L	2 L	4 L
0.025 M Tris (F.W. 121.1)	3.0 g	6.0 g	12.1 g
0.192 M Glycine (F.W. 75.07)	14.4 g	28.8 g	57.6 g
0.1% SDS	1.0 g	2.0 g	4.0 g
ddH_20	fill to 1 L	fill to 2 L	fill to 4 L

*This solution can be made directly in a large jug. Check the pH and store at room temperature. However, it is usually a better idea to make the solution in a beaker and then pour it into a jug after everything has dissolved.

Standard Transfer Buffer

	1 L	2 L	4 L
Glycine	14.42 g	28.83 g	57.66 g
Tris Base	3.03 g	6.06 g	12.12 g
15% Methanol	150 mL	300 mL	600 mL
ddH_20	fill to 1 L	fill to 2 L	fill to 4 L

*pH 8.1-8.3, adjustment is not necessary. This solution may be reused 4-5 times with filtering (#1 Whatman) between each use. Dispose in Methanol Hazardous Waste container.

1% Blocking Solution Bovine Serum Albumin (BSA)

ddH_20	360 mL
10XTris Buffer Solution	40 mL
0.05% Tween 20	0.2 mL
1% BSA (Sigma A2153)	4.0 g

TTBS (0.05% Tween 20), pH 7.4

Add 250 µL of Tween 20 to 500 mL TBS

2X Laemmli buffer minus MCE, pH 6.8

0.125 M Tris
4 % SDS
20% Glycerol
*Cut concentrations of Tris and SDS in half for 1X

Use the 2X Treatment Buffer minus MCE from above to soluble proteins prior to BCA Protein Assay. Add MCE and 4 mg/mL Bromophenol Blue in the proportions shown below.

	10 mL	30 mL	50 mL
2X Treatment Buffer Minus MCE	9.0 mL	27.0 mL	45.0 mL
MCE	1.0 mL	3.0 mL	5.0 mL
4 mg/mL Bromophenol Blue	200 µL	600 µL	1.0 mL

*Make in hood due to smell and toxicity of MCE. Make fresh daily (or use aliquots that have been frozen). Any solution not used may be aliquoted and frozen for future use. Disposal is in the Hazardous Waste container. If the dye is not dark enough or the samples are not dense enough, add 50% more Bromophenol Blue.

Coomassie Blue Stain R_{250}

(0.025% Coomassie Blue R_{250}, 40% Methanol, 7% Acetic Acid)

	1 L	2 L	4 L
Coomassie Blue R_{250}	0.25 g	0.5 g	1.0 g
Methanol	400 mL	800 mL	1600 mL

Acetic Acid	70 mL	140 mL	280 mL
ddH_20	fill to 1 L	fill to 2 L	fill to 4 L

*Dissolve Coomassie Blue in Methanol only. Stir Methanol and Coomassie Blue until it dissolves then add ddH_20 and Acetic Acid. Filter solution with a Whatman #1 filter.

Destaining Solution I (40% Methanol, 7% Acetic Acid)

	1 L	2 L	4 L
Methanol	400 mL	800 mL	1600 mL
Acetic Acid	70 mL	140 mL	280 mL
ddH_20	fill to 1 L	fill to 2 L	fill to 4 L

*Mix in hood and store at room temperature.

Destaining Solution II (5% Methanol, 7% Acetic Acid)

	1 L	2 L	4 L
Methanol	50 mL	100 mL	200 mL
Acetic Acid	70 mL	140 mL	280 mL
ddH_20	fill to 1 L	fill to 2 L	fill to 4 L

*Mix in hood and store at room temperature.

TISSUE PROTEIN/DNA/RNA DETERMINATION

HOMOGENIZATION (polytron homogenizer)

1. Place 0.5 to 1.0 gram of muscle a 50 ml conical centrifuge tube (orange cap). Add 25 x volume by weight of cold extraction buffer.

EXTRACTION BUFFER	**1 liter**
Tris (10 mM)	1.21 g
EDTA (5 mM)	1.86 g

pH 8.0

2. Homogenize sample for 2 min at setting 4 (30 sec on, 30 sec off 4 times; keep sample tube in beaker containing ice slush)

3. Take aliquots of homogenate for RNA, DNA, and protein determinations.
   250 µL for protein x 3
   100 µL for DNA x 3
   3 mL for RNA x 3

DNA DETERMINATION

1. In 3 ml methacrylate cuvets (pre-selected for uniformity), add 100 µl of homogenate and 2900 µl of DNA assay buffer.

2. DNA standard curve: Calf thymus DNA stock = 200 µg/ml
   Prepare standards by serial dilution.

µg DNA/ml	DNA stock	Extraction buffer (Tris, EDTA)
100	500 µl	500 µl mix, remove 500 µl, dilute 1:1
50		"
25		"
12.5		"
6.25		"
3.125		"
0		Extraction buffer only

   Place 100 µl of each standard in duplicate cuvets.

3. In a dimly lit room, add 3 ml of Hoechst 33258 Reagent (diluted to 1µg/ml in DNA Assay Buffer) to all cuvets. Incubate in the dark for 15 min. Record time that Hoechst reagent is added to standards and samples.

4. Turn on DYNAQUANT fluorometer and allow 15 min warm-up. **FOLLOW THIS SEQUENCE:** SETUP, PROMPT OFF, UNITS ng/ml, ESC, place 0 standard in fluorometer, READ, ZERO, place 25 µg/ml standard in fluorometer, CALIBRATE, 2500 ng/ml (this actually represents 2500 ng in the cuvet), ENTER.

   Read all standards (duplicate) and samples (triplicate) in sequence that Hoescht Reagent was added. Record time that standards and samples are read peiodically (each 9-12 tubes). Time from addition of Hoechst reagent to sample read should be kept constant (± 3 min).

5. Use the standard curve to determine the concentration of the samples. Divide by 1000 to get the µg DNA per cuvet, and divide that value by .004 g fresh tissue/cuvet to get µg DNA/g muscle. (or multiply by 250 dilution factor)

DNA REAGENTS

DNA Assay buffer	1 liter
0.05 M NaH_2PO_4 * HOH (FW 138.0)	6.90 g
2 M NaCl (FW 58.45)	116.90 g
2 mM EDTA*Na (FW 372.2)	0.744 g

pH to 7.4

Hoechst 33258 reagent

1 mg/ml stock solution in ddi water
Store in the dark at 4 C
Dilute fresh 1:1000 in DNA Assay buffer (H33258 = 1 µg/ml)

DNA Stock Solution

Calf thymus DNA 2.0 mg

1. Dissolve 2.0 mg of calf thymus DNA in 8 ml Extraction Buffer in original DNA bottle. Allow 2.5 to 3 hr at room temp.

2. To find the purity of the solution, dilute it 1:10 and read its absorbance at 280nm and 260 nm.

Divide A_{260} by A_{280} to get the purity. It should be around 1.8

3. To find the concentration, multiply the A_{260} reading by 10. It is known that at A_{260}, 50µg/ml DNA will read 1.0, therefore divide 50 by 1 and multiply by your A260 reading.

4. Dilute DNA stock solution to 200 µg/ml. Freeze in aliquots at -80° C.

PROTEIN DETERMINATION

1. Place 250 µl aliquots (in triplicate) of homogenate in 16 x 100 mm borosilicate glass tubes. Add 750 µl 1 N NaOH to each.

   Prepare duplicate BSA standards in 25% extraction buffer, 75% 1 N NaOH @ 8, 4, 2, 1, 0.5 and 0 mg/mL. Use 1 ml of standard for readings.

2. Parafilm tubes, vortex briefly and let incubate at 37 C for 3 to 4 hours. Turbid samples should become clear.

3. Add 4 ml of biuret reagent to each tube, parafilm and vortex. Place in dark for 30 min at room temp.

4. Remove 200 µL from each standard or sample, dispense in microtiter plate and read at 540 nm. Determine protein concentration of samples based on standard curve.

BIURET REAGENT

Dissolve the following in about 500 ml of D.D. H_2O in a 2 liter volumetric·

$CuSO_4 \cdot 5\ H_2O$	1.5 g
Sodium potassium tartrate	6.0 g

Add with constant stirring 300 ml of fresh carbonate free 10% NaOH.

Make up to 1 liter and store in a dark polyethylene bottle. Discard if black or red precipitate appears.

RNA DETERMINATION

1. Place 1 ml of homogenate in 1.5 ml micro-centrifuge tubes and add 330 µl of 1.2 N perchloric acid (PCA). Allow to sit on ice for 10 min.

2. Centrifuge at 10,000xg at 0-4°C for 5 min. (F-20/micro rotor = 8,900 rpm.) Decant and discard supernatant.

3. Add 700 µl cold .2 N PCA. Break up pellet with a glass rod and vortex. Centrifuge as above. Discard supernatant. Repeat 2X's using 700 µl of .2 N PCA.

4. Add 1.0 ml of .3 N KOH (room temp). Break pellet and vortex. Heat at 37° C for 60 min. Place in incubated shaker after heating in water bath. (DNA determination may be done during the incubation.)

5. Add 330 µl of cold 2.4 N PCA and allow tubes to sit in an ice bath for 10 min. Centrifuge at 10,000xg at 0-4°C for 5 min. (F-20/micro rotor = 8,900 rpm.) Decant (retain supernatant.)

6. Wash precipitate with 500 µl of cold .2N PCA. Break pellet and vortex. Centrifuge at 10,000xg at 0-4°C for 5 min (F-20/micro rotor = 8,900 rpm.) and mix the supernatant with the supernatant from step 5. Repeat.

7. RNA Measurement- Measure O.D. of RNA hydrosylate at 260 nm and 232 nm.

   Calculation: µgRNA= (39.1xO.D. 260) - (15.5 x O.D. 232)
   X2.3 (volume of supernatant)
   x 25

RNA Reagents

1.2 N PCA	20.49 ml of 70% PCA bring up to 200 ml with dd water.
0.2 N PCA	1 part 1.2 N PCA + 5 parts dd water
0.3 N KOH	KOH-4.21grams/250 ml dd water

Growth and harvest of hybridoma cell lineage, D76

1. Hybridoma cells were grown in 75 cm^2 tissue culture flasks (#3376, Corning Incorporated, Corning NY) in Dulbecco's Modified Eagle Medium

   Dulbecco's Modified Eagle Medium
   25 mM HEPES (#23700-040, Gibco BRL, Grand Island NY)
   10% fetal bovine serum
   0.1 mM Non-Essential Amino Acids (#11140-050, Gibco BRL)
   1 mM sodium pyruvate (S-8636, Sigma Chemical Co., St. Louis MO)
   2 mM L-glutamine (G-5763, Sigma Chemical Co.)
   0.5% antibiotic/antimycotic.

2. Frozen cell suspensions were thawed in a warm water bath and re-suspended in 10 ml of media to dilute freezing medium and centrifuged at 300 xg for 2 minutes. The cell pellet was then re-suspended and plated at $2.5x10^6$ cels/75 cm^2 flask in 20 ml of medium (125,000 cells/ml).

3. Cells were supplied with new medium every 48 hours at a ratio of 1:4 (existing cell suspension to fresh media) and cell suspensions were transferred into new flasks when necessary. Feeding was continued until the desired volume of supernatant was attained. Cultures were then left undisturbed for 12 days.

4. Cultures were harvested, pooled and centrifuged at 2000 xg for 15 minutes to pellet cells. The clarified supernatant was then collected and stored in aliquots at -20°C until use.

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