IN VITRO COMPARISON OF SATELLITE CELLS ISOLATED FROM NORMAL AND CALLIPYGE SHEEP EXPOSED TO GROWTH PROMOTING COMPOUNDS

by

Kathleen Ida Neary

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Kathleen Ida Neary

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Jane A. Boles

Approved for the Department of Animal and Range Sciences

Brett E. Olson

Approved for the Division of Graduate Education

Carl A. Fox

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ABSTRACT

Sheep producers are continually faced with the need to increase pounds of retail product sold. One way to increase total retail pounds of lamb available for consumption without increasing sheep numbers or producer costs is through genetic selection for increased size and growth. Another option would be to select sheep with the callipyge mutation or understand what the mutation changes to increase growth. Callipyge is a selective hypertrophic condition exhibited primarily in the hind limbs of affected lambs. The cause of the increased growth at a molecular level has not been thoroughly explored. Other studies have evaluated differences in satellite cells (SC) isolated from high and low growth line animals but none compared normal and callipyge sheep satellite cells using defined media. The objective of the present study was to compare the effect of genotype (normal and callipyge), growth compound (estradiol benzoate, trenbolone acetate, IGF-I, and FGF) and level of growth compound (0, 2.5, 5, 10, 30, and 50 ng/μL) on proliferation of sheep satellite cells. Sheep SC isolated from normal and callipyge sheep were evaluated. Cells were plated and grown in standard growth media for three days and then treated with estradiol benzoate, trenbolone acetate, FGF, and IGF-I in a defined growth media. Proliferation differences between SC types and hormones were evaluated. At intervals of 24 h, SC were fixed, stained, and counted. Data interpretation may be inhibited due to concerns with experimental design and procedures; however, in the present experiment callipyge and normal SC proliferated at similar rates when not exposed to any growth compound. While the difference in number of SC at 24 h and 48 h of growth was significant, there was not a significant interaction between cell genotype and time of growth. A significant interaction between genotype and growth promoting compound was seen. Proliferation of callipyge SC was greater in response to IGF-I than normal SC; callipyge SC proliferated less in response to FGF than did normal SC. Cells deprived of FGF and IGF-I did not exhibit impaired proliferation as expected. Response to IGF-I may explain some differences that are seen in the callipyge phenotype.

CHAPTER 1

INTRODUCTION

Over the last sixty years, the total sheep and lamb crop in the U.S. has been drastically reduced while the demand for retail lamb has remained constant (NASS, 2006). Average price per hundredweight (cwt) of lamb has increased from $79.80 in 2000 to $101.00 in 2004 (NASS, 2006). This increase indicates the continued value of sheep production to U.S. agriculture. In order to meet consumer demand for lamb, producers must increase total pounds of lamb they take to market. The typical method for increasing total pounds of lamb sold is to increase the number of lambs sold. However, due to a decrease in total sheep numbers in the U.S., alternatives that would increase meat production must be investigated.

Two ways to increase total retail pounds of lamb available for consumption without increasing sheep numbers or producer costs are through genetic selection for increased size and growth, and through use of the callipyge mutation. Callipyge is a selective hypertrophic condition exhibited in hind limbs of affected lambs. This condition is due to a single base pair change on chromosome 18 (Freking et al., 2002) and was first discovered in 1983 by a sheep producer (Cockett et al., 1996; Cockett et al., 1994).

Callipyge is not inherited in the typical Mendelian pattern; rather it is passed on in a pattern of polar over dominance (Freking et al., 1998a; Cockett et al., 1996; Cockett et

al., 1994). Simply, only heterozygous lambs that inherit the callipyge allele from the ram and a normal allele from the ewe express the selective hypertrophy phenotype. Jackson et al. (1997) and Cockett et al. (1999) reported that the callipyge phenotype, while expressed in both sexes, is only inherited through the paternal allele. Lambs with any other combination of genes, including lambs with two callipyge alleles, appear to be normal (Cockett et al., 1999; Cockett et al., 1996; Cockett et al., 1994).

The callipyge phenotype is associated with increased muscling in the lamb that occurs four to six weeks after birth (Jackson et al., 1997) and thus causes no dystocia in the ewe. Jackson et al. (1997) reported that lambs expressing the callipyge phenotype had increased muscling on the back as well as extreme muscling in the hind limbs. Affected muscles reside in the pelvic limb (superficial gluteal, tensor facia latae, gluteus medius, gracilis, semitendinosus, adductor, semimembranosus, rectus femoris, and vastus group), torso (longissimus, psoas major, and psoas minor) and thoracic limb (biceps brachii and extensor carpi radialis) (Jackson et al., 1997b). Increased muscling along the back contributed to a larger dorsal groove as well as more pronounced superficial gluteal muscles. Further, lambs expressing callipyge also had a more defined twist, giving the appearance of increased inner leg muscling (Jackson et al., 1997).

Koohmaraie et al. (1995) found that muscle expressing the callipyge phenotype had approximately 30% greater DNA content than normal muscle. One source for increased DNA content in normal muscle is satellite cells, which could play a part in some of the differences seen between normal and callipyge muscle. To date, only Carpenter et al. (2000) have compared satellite cells from normal and callipyge sheep.

Carpenter et al. (2000) compared growth of the two cell types when exposed to growth media containing horse serum and serum collected from sheep expressing normal and callipyge phenotypes and found that there were no proliferative or fusion differences between the two cell types under these conditions. This study used serum from animals in the treatments and thus may have contained uncontrollable or unknown factors, thus skewing the results of the study thus making it difficult to determine the exact mode of action that is causing the increased musculature in the callipyge sheep (Dodson et al., 1990). Knowledge of how satellite cells from callipyge animals are different from normal satellite cells could help develop ways to modify growth of muscle.

Satellite cells from heavyweight turkeys showed a greater proliferative capacity than those from lightweight turkeys (Merly et al., 1998). In addition, Velleman et al. (2000) found that in turkeys selected for high growth rate, there were differences in satellite cell activity between genders as well as bird growth rate (birds selected for rapid growth rate compared to non-selected random control birds). These researchers found overall, satellite cells isolated from lines selected for rapid growth rate proliferated faster when compared to cells isolated from the random control line.

Research on satellite cells isolated from animals and birds with different growth rates in other species is limited. Currently little information is available explaining increased musculature in callipyge sheep and how satellite cells from callipyge and normal sheep respond to normal or elevated level of growth promoting hormones.

Thus, the objective of the present study was to compare the effect of estradiol benzoate, trenbolone acetate, IGF-I, and FGF on proliferation of callipyge and normal

sheep satellite cells. The hypothesis tested was that satellite cells from callipyge and normal sheep would not differ in their proliferation rate nor would their response to growth promoting compounds insulin-like growth factor-I, fibroblast growth factor, estradiol benzoate, and trenbolone acetate be different.

CHAPTER 2

LITERATURE REVIEW

The Domestic Sheep

Origins

Domestic sheep (*Ovis aries*) have played an integral role in human history. Since the earliest domestication of sheep by primitive man approximately 8,000 - 10,000 years ago (Meadows, 2005; Hargreaves and Hutson, 1997; Sheep Production Handbook, 2002; ASIA, 2006), sheep have been used to produce meat, wool, milk and numerous other by-products. While a large number of subspecies exist, until recently the origins of the sheep have not been fully understood (Hiendleder et al., 2002; Hiendleder et al., 1998). Using mitochondrial DNA control region sequences, Hiendleder et al. (2002) investigated classification and origin of domestic sheep. They concluded that of the possible candidates, Eurasian Muflon is the most likely progenitor of existing sheep.

Selection for thousands of years has resulted in over 200 breeds of sheep worldwide (www.ansi.okstate.edu/breeds/sheep/Sheep-w.htm). Selection has also resulted in sheep changing from an animal with a hairy coat giving birth to a single lamb each year to one that is capable of producing over ten pounds of wool and having multiple lambs per year (Sheep Production Handbook, 2002).

Consumer preferences for lamb cuts are affected by the size of cuts and their fatness (Jeremiah et al., 1993). Further, health organizations are recommending

reductions in red meat fat consumption (WHO, 1990) and consumers are demanding leaner cuts (Capps et al., 2002; Woodward and Wheelock, 1990). One implication of these factors is the need of U.S. sheep producers to raise more muscular lambs with less fat. Exploitation of the callipyge phenotype is one tool to achieve this end.

Value

The United States currently ranks seventh overall (Fig. 1) in sheep inventory worldwide, with roughly 7,700,000 head total (FAO, 2006). In Montana, the most common breeds are Rambouillet, Targhee, Columbia, Hampshire, Suffolk, Finn and Polypay (2002 Census of Agriculture, NASS). Currently, Montana ranks seventh in overall sheep production (Fig. 2) with the majority of this production focused in the eastern half of the state (2002 Census of Agriculture, NASS).

Of the seven million sheep in the US, 67,620 sheep operations produced more than three million lambs weighing an average of 134 pounds for slaughter in 2003 (ASIA, 2006). In the first quarter of 2006, the commercial lamb and mutton production totaled 49,000,000 pounds (ERS-USDA, 2006). The 2005 average price was $97.76/cwt, thus, the total value of lamb produced in the first quarter of 2006 was approximately $47,902,400; this equates to roughly $191,609,600 per year.

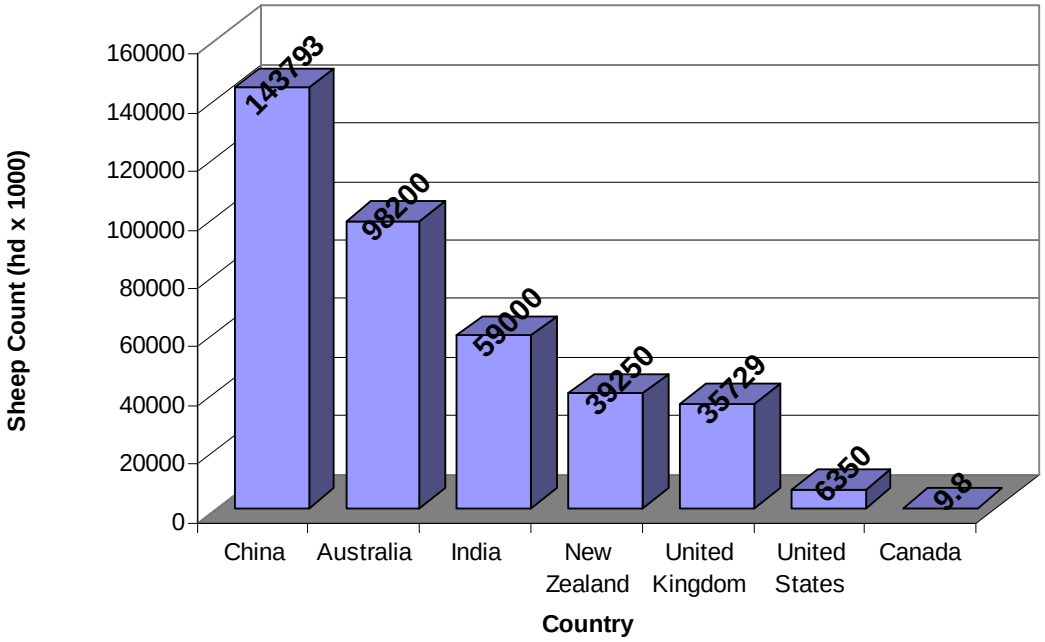

Figure 1: 2004 world sheep inventory by country (ASIA, 2006; FAO, 2006).

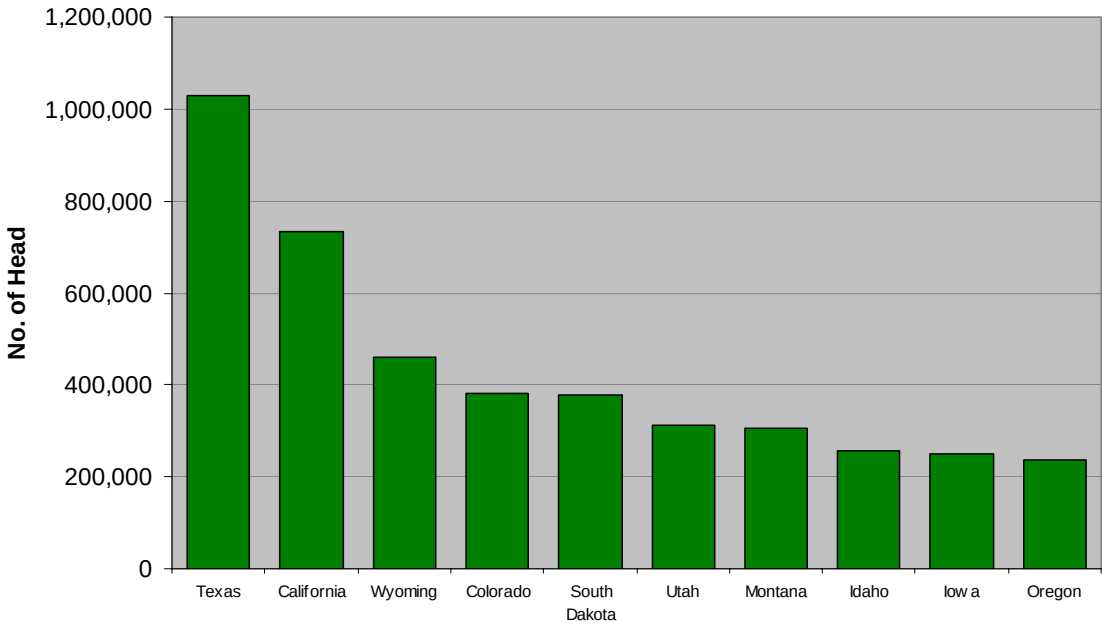

Figure 2: Top ten sheep producing states in the U.S. (NASS, 2006).

Callipyge

Genetics of Callipyge

Callipyge, a selective muscle hypertrophic condition occurring predominantly in the hind limbs of affected lambs, is caused by a single base pair change on chromosome 18 (Freking et al., 2002). This change is an A-to-G switch (or a single nucleotide polymorphism) that lies approximately 32,775 base pairs (~33 kb) upstream of the *GTL2* gene on chromosome 18 (Cockett et al., 2005; Smit et al., 2003; Freking et al., 1998a). Inheritance of the callipyge phenotype does not follow the typical Mendelian pattern. Instead, it is inherited in the pattern of polar overdominance (Freking et al., 1998a; Cockett et al., 1996; Cockett et al., 1994). Simply, only heterozygous lambs that inherit the callipyge gene from the ram and the normal gene from the ewe express the selective hypertrophy phenotype. Jackson et al. (1997) and Cockett et al. (1999) reported that the callipyge phenotype, while expressed in both sexes, is only inherited as a paternal allele. Lambs with any other combination of genes, including lambs with two callipyge alleles, appear to be normal (Cockett et al., 1999; Cockett et al., 1996; Cockett et al., 1994) (Table 1).

Table 1: Allelic inheritance of the Callipyge phenotype (Freking and Leymaster, 2006).

Genotype	Phenotype
$C^{mat}C^{pat}$	Normal
$C^{mat}N^{pat}$	Normal
$N^{mat}N^{pat}$	Normal
$N^{mat}C^{pat}$	Callipyge

Callipyge was first identified in 1983 by a sheep producer who noticed a ram lamb exhibiting extreme muscling. The animal had only a small amount of subcutaneous fat and remarkable hind quarter muscling (Cockett et al., 1996; Cockett et al., 1994). This Dorset ram lamb, Solid Gold, most likely had the mutation very early in embryonic development (Cockett et al., 2005; Smit et al., 2003).

Recently, the callipyge phenotype has been mapped between Maternally Expressed Gene 3 (*MEG3*) and Delta, Drosophila, Homolog-Like 1 (*DLK1*) genes, or the *GTL2* region (Smit et al, 2003; Freking et al., 2002; Fahrenkrug et al., 2000). Delta, Drosophila, Homolog-Like 1 is a protein that functions in regulation of cellular growth and differentiation, neuroblast differentiation and adipogenesis (Murphy et al., 2005). When *DLK1* expression is removed (i.e. by knockout), mice exhibit severely retarded growth and rapidly develop obesity. Mice are also unable to maintain normal lipid metabolite levels. These factors illustrate a role of *DLK1* in adipogenesis and growth (Murphy et al., 2005; Moon et al., 2002). These findings were reinforced by Smas and Sul (1993), who showed that *DLK1* is highly expressed in preadipocytes. This expression inhibits differentiation of preadipocytes. Once differentiation begins *DLK1* expression is suppressed. Delta, Drosophila, Homolog-Like 1 is also inherited in a paternal fashion similar to callipyge (Murphy et al., 2005; Cockett et al., 2001). Due to its close proximity to callipyge in combination with individual roles of each gene, *DLK1* may have an impact on function and activity of the callipyge gene (Murphy et al., 2005; Fahrenkrug et al., 2000; Fahrenkrug et al., 1999).

Murphy et al. (2005) found that the callipyge phenotype was highly correlated with *DLK1* expression during lamb development. This correlation led them to conclude that the effect of callipyge is to interfere with postnatal down regulation of *DLK1* expression. Thus, Murphy and colleagues (2005) were first to show that the callipyge phenotype coincides, both spatially and temporally, with another anomalous gene expression (*DLK1*). These researchers however, did not explain why the difference in muscle development happens only after birth in affected sheep.

Identification and Characteristics

Solid Gold sired many lambs, passing the mutation to roughly 10% of his descendants through which the mutation was traced (Smit et al., 2003). This indicates Solid Gold to be a germline mosaic animal (Smit et al., 2003). In general, germline mosaic animals have the typical two alleles plus an additional mutated allele (L. Alexander, personal communication). As Solid Gold began to develop embryonically, some sex cells were formed that contained the A allele (~90%). Then, the mutation occurred and the sex cells containing the G allele were formed (~10%). These G-containing sex cells produce mutated gametes that are the source of the callipyge allele. The mutation also occurred early enough in development to not only affect gametes produced by Solid Gold, but also to cause Solid Gold to express the callipyge phenotype. All animals that express the callipyge phenotype are descendants of this ram (Cockett et al., 2005).

Jackson et al. (1997) reported that lambs expressing the callipyge phenotype had increased muscling on the back as well as extreme muscling in the hind limbs. Affected

muscles reside in the pelvic limb (superficial gluteal, tensor facia latae, gluteus medius, gracilis, semitendinosus, adductor, semimembranosus, rectus femoris, and vastus group), torso (longissimus, psoas major, and psoas minor) and thoracic limb (biceps brachii and extensor carpi radialis) (Jackson et al., 1997b). Increased muscling along the back contributed to a larger dorsal groove and more pronounced superficial gluteal muscles. Further, lambs expressing callipyge also had a more defined twist, giving the appearance of increased inner leg muscling (Jackson et al., 1997).

Jackson et al. (1997) also found that ewes delivering callipyge lambs had no greater frequency of dystocia than ewes delivering normal lambs and all lambs appeared normal at birth. Expression of the callipyge phenotype begins at approximately four to six weeks of age; general lamb shape is consistent with that of normal lambs until this time. Being a carrier of the callipyge allele may affect the animal in other ways as it matures. Ewes possessing the paternal callipyge allele exhibited lower ovulation rates (~0.25 ovum fewer) and lower live weights at breeding time (~2.5 kg less) than did ewes with both normal alleles (Freking and Leymaster, 2006). However, the decrease in ovulation rate did not affect the number of lambs born to ewes that had the paternal callipyge allele (Freking and Leymaster, 2006). In addition, inheritance of the paternal callipyge allele did not significantly affect conception rates, fertility, maternal ability, or lifespan and longevity of the ewe (Freking and Leymaster, 2006). With the current information available, there are no apparent detrimental effects to ewe productivity when used in a callipyge lamb production system.

Production, Commercial Yield, and Consumer Issues

Callipyge expression can be advantageous to the sheep producer in several ways. First, lambs expressing the callipyge phenotype more efficiently convert feed to gain than normal sheep (Cockett et al., 2005). From this, the producer has lower production costs, decreased grazing pressure on available pastures, and higher retail product yield. Total muscle yield from callipyge lambs is equal to or greater than 50% more than from normal lambs (Cockett et al., 2005; Duckett et al., 2000; Freking et al, 1998b; Koohmaraie et al., 1995). Callipyge lambs also have higher dressing percentages and leg scores, larger loin eye areas, and are leaner (Cockett et al., 2005; Cockett et al., 2001; Freking et al., 1998b). Protein accretion in callipyge lambs is 12.5 grams per day compared to just 10.2 grams per day in normal lambs (Freking et al., 1998b). Overall, callipyge lambs produced carcasses that were 4.1 kg heavier with 4.3% less fat than carcasses produced by normal lambs (Freking et al., 1998b).

Meat from callipyge lambs has been shown to be considerably less tender than meat from normal lambs (Cockett et al., 2005; Freking et al., 1999). Warner-Bratzler shear force values from lambs expressing the callipyge phenotype were higher than the values from normal lambs (Duckett et al., 2000; Freking et al., 1999; Shackelford et al., 1997; Koohmaraie et al., 1995). Thus, consumer satisfaction may be reduced due to reduced tenderness of callipyge meat. This may be dealt with by selecting for tenderness, using post-mortem tenderization methods, or altering the cooking method used; for example, roasting produces more tender meat than broiling or grilling (Shackelford et al., 1997). More recently, Kerth et al. (2003) evaluated consumer acceptability of retail cuts

from callipyge sheep and found that consumers rated fewer callipyge loin and shoulder cuts acceptable than cuts from normal lambs. However, acceptability of leg chops was not affected by phenotype.

A second option for dealing with decreased tenderness is utilizing a postmortem tenderization system when the sheep are harvested. Clare et al. (1997) explored using calcium chloride injections 24 h postmortem and found that using calcium chloride injections increased tenderness of retail cuts as well as increasing consumer acceptability of retail cuts by 25%. While these treatments may assist in improving tenderness issues, they are costly and almost always extremely difficult to implement with current lamb marketing systems (Freking et al., 1999). Electrical stimulation of carcasses has been explored and been found to improve tenderness of loin chops by up to 34% (Kerth et al., 1999).

Freking et al. (1999) proposed long term selection for tenderness within the callipyge phenotype as an alternative to carcass and post-mortem treatments. The phenotypic correlation between lean accretion rate (or the callipyge phenotype expression) and shear force value (tenderness) is estimated to be 0.18 (Freking et al., 1999). However, 0.18 is a relatively low correlation and would take a great deal of input in order to make effective selection for tenderness. While this alternative has not been investigated, it presents possibilities for improving customer satisfaction.

Muscle Composition

Composition of muscle fibers from normal and callipyge sheep may help explain why the two grow differently. Affected muscles reside in the pelvic limb, torso and

thoracic limb (Jackson et al., 1997b). Carpenter and colleagues (1996) found callipyge lambs had a higher percentage of fast twitch glycolytic fibers and lower percentages of slow twitch oxidative and fast twitch oxidative glycolytic fibers. Diameter of fast-twitch fibers was also greater in callipyge lambs than normal lambs and less for slow twitch fibers (Carpenter et al., 1996; Cockett et al., 2001). Koohmaraie et al. (1995) found similar results and that callipyge muscle is associated with a higher DNA content than normal muscle (~30% higher). One source for the increased DNA content in muscle is satellite cell fusion into existing muscle, which may also play a part in some of the differences between normal and callipyge muscle.

Satellite Cells

Characterization and Origins

Muscle satellite cells were discovered in 1961 (Mauro, 1961). Despite being studied extensively, their characterization has remained similar to the original description. Mauro (1961) described them as being "wedged" between plasma and basement membranes of the muscle fiber. When viewing a satellite cell, it projects inward, moving myofibrils to the periphery (Mauro, 1961). Furthermore, satellite cells appear tubular with three or four "feet" attached to the extracellular matrix. In comparison to normal muscle cells, satellite cells are unique not only because of location but also due to exhibition of an increased nucleus to cytoplasm ratio, decreased organelle content, and a smaller nucleus size (Chargè and Rudnicki, 2004; Valente et al., 2002).

Researchers have speculated on the origin of satellite cells and have suggested four possibilities for their origin. Possibilities include:

> "1) descendants of specific lineages of myoblasts that retain their structural and functional identity throughout the lifespan of the animal…; 2) remnant of embryonic or fetal myoblasts…; 3) generic stem cells that were trapped in association with specific myofiber types and subsequently adapted to, and assumed properties of, juxtaposed myofiber… or 4) flexible myogenic stem cells that are capable of adapting to the myofibrillar environment in which they reside, while retaining some capability of changing, depending on environmental cues (extrinsic factors) impinging on them…" (Dodson et al., 1996)

Function

No matter their origin, the basic function of satellite cells is to donate DNA and cellular contents to adjacent muscle fibers for repair, regeneration, and hypertrophy (Mathison et al., 1989; Valente et al., 2002). Donation of nuclei from satellite cells to myoblasts accounts for as much as 98% of new nuclei in muscle (Dodson et al., 1987), and is the primary pathway of new muscle growth and repair. After puberty, satellite cells remain quiescent until a stimulus, like an injury, induces release of growth factors that signal satellite cells to reenter the cell cycle (Dhawan and Rando, 2005; Kokta et al., 2004). Activation of satellite cells in callipyge animals would be necessary to explain increased DNA and growth in muscles.

Response to Aging & Disease

Studies show density and activity of satellite cells decrease as animals age (Hawke and Garry, 2001; Seale and Rudnicki, 2000). Growth normally occurs rapidly in a very short period of time and then plateaus. After the plateau, muscle growth is reduced, corresponding to a decrease in satellite cell activity (Hawke and Garry, 2001).

In a conventional animal production system, satellite cells are most active during the steepest portion of the growth curve. When growth is occurring rapidly, satellite cells are continually contributing their DNA and cellular contents to adjacent muscle cells (Hawke and Garry, 2001; Moss and Leblond, 1971).

In response to aging and disease, functions of satellite cells vary. During aging, length of the telomere of each chromosome is reduced with each successive cell replication. These decreases in length reduce the ability of the cell to proliferate (Hawke and Garry, 2001). Hawke and Garry (2001) showed that, in humans, satellite cells can replicate as many as 60 times in neonatal muscle and as few as 20 to 30 times in older muscle. Interestingly, replications in pre-teen (nine year old) muscle and in senile (> 60 years old) muscle were similar (Hawke and Garry, 2001). The difference due to ageing comes as satellite cells fuse to form myotubes. As an individual ages, satellite cells form thinner and more fragile tubes, thereby reducing their ability to effectively repopulate the myofiber. Thus, a lesser amount of repair occurs in the muscle after injury or extensive stress due to fragility of myotubes formed from "older" satellite cells (Hawke and Garry, 2001).

Impaired response of satellite cells is not due solely to limited ability to regenerate; environment plays a large role as well. As the basal lamina of the aged thickens, increased fibrosis in skeletal muscle reduces density of capillaries so blood and nutrient flow to satellite cells decrease (Hawke and Garry, 2001). These findings reinforce those by Schultz and Lipton (1982), who found that the average number of new

cells was inversely proportional to donor age; therefore, proliferation potential of satellite cells decreases with age.

For normal satellite cell operation, there must be appropriate immune system response to remove or degrade injured or necrotic muscle tissue. Once injured or necrotic tissue is removed, satellite cells may begin regeneration. Satellite cell proliferation also increases and differentiation may be mediated by soluble factors released by macrophages (Cantini et al., 1994). In aged or diseased muscle, lower blood flow causes insufficient immune response, decreasing the macrophage's ability to degrade injured tissue. This decrease limits the ability of satellite cells to regenerate the muscle (Hawke and Garry, 2001).

In situations such as muscle denervation, atrophy, immobilization, or degeneration, the ability of satellite cells to donate their DNA and cellular contents is reduced. The lack of signaling between satellite cells and nerves after denervation causes an initial increase in satellite cell proliferation followed by a dramatic drop (as much as 8%) in cell numbers (Jejurikar and Kuzon, 2003). In the case of a reduction in muscle mass due to prolonged immobilization, malnutrition, or injury, called sarcopenia, a decline in the number of satellite cells in the muscle also occurs. However, when muscle is allowed to recover, the satellite cell population also recovers (Jejurikar and Kuzon, 2003). These findings are reinforced by Fauconneau and Paboeuf (2000) who showed satellite cells extracted from fish exposed to a starvation period had decreased satellite cell activity, but when the fish were allowed to refeed the satellite cell population showed almost complete recovery. Similarly, Halevy et al. (2000) found satellite cell populations

isolated from fed and starved chicks responded similarly when nutrition was restored. When IGF-I was introduced during recovery, satellite cells showed greater ability to assist in muscle regeneration and a significant increase in the proliferative potential (Jejurikar and Kuzon, 2003). Recovery may be further facilitated by exposure to adult muscle extracts. Bischoff (1990) showed satellite cells exposed to an extract of crushed adult muscle increased proliferation relative to those not exposed to the extract.

Regeneration

For satellite cells to perform throughout the life of an animal, the pool of available cells must be regenerated. Inactive satellite cell numbers remain constant throughout many cycles of use (Seale and Rudnicki, 2000; Asakura et al., 2002). Without regeneration, restoration of injured muscle is not possible; nor could any hypertrophy of existing muscle occur. Zammit et al. (2004) present three theories regarding satellite cell regeneration:

1) Satellite cells are a heterogeneous population. Some cells of this population are designated for use in repair and differentiate rapidly when needed. Others are designated for population self-renewal and differentiate more slowly. 2) The population is homogenous; all cells are simultaneously active. If needed for regeneration, they adopt opposite paths, with some providing new nuclei and some preserving the existing population. 3) Satellite cells are continually created and used as needed. Existing cells move to the area for regeneration or growth; replacements come from a stem cell located either inside or outside the muscle tissue. These theories are supported by Seale and

Rudnicki (2000), who describe similar regeneration methods including generation of daughter and self cells and origin via stem cells.

Further investigation of the third process reveals additional supportive evidence. Asakura et al. (2002) found, in mice, muscle-derived stem cells undergo preferential differentiation and are a distinct cell population, closely associated and coexisting with muscle satellite cells. Although different from satellite cells, muscle-derived stem cells are capable of transformation to satellite cells when injected into regenerating muscle, making them available for repair and growth. Together, muscle-derived stem cells and satellite cells are steps on the regeneration ladder and are available in the adult skeletal muscle equilibrium.

Activation and termination of satellite cell activity may be affected by numerous things. In newly hatched chicks, a one-day starvation period activated satellite cells (Halevy et al., 2001). However, a starvation period closer to hatch time made it more likely the chick would make no compensatory gain. Knowledge of this finding is important in regulation of the growth of newborn animals. Being able to induce the cell cycle may encourage additional gain per animal. Contrary to this, Mozdziak and colleagues (2002) found that early supplementation for gain did not occur through the satellite cell pathway in post hatch chicks.

Treatment by Growth Compounds

Satellite cell populations have been subjected to a variety of both endogenous and exogenous growth compounds. Hawke and Garry (2001) reviewed effects of various growth factors on satellite cell activity. They found fibroblast growth factor (FGF) and

hepatocyte growth factor (HGF) increased proliferation and decreased differentiation of satellite cells in culture. In addition, Sheehan and Allen (1999) found that of the nine members of the FGF family, FGF1, FGF2, FGF3 and FGF6 are found in or associated with muscle tissue. These four FGFs may stimulate rat satellite cell proliferation *in vitro*. Finally, Sheehan and Allen (1999) found that HGF, used in conjunction with FGF, stimulates the expression of FGF receptor 1. The above illustrates the role of HGF as an activator of rat satellite cells as well as an increaser of FGF responsiveness in satellite cells. Additionally, Doumit et al. (1993) showed that the same effect occurs in porcine satellite cells, with a marked increase in the number of cells when cultures were treated with FGF when compared to cells with no treatment.

In cultured cells, removal of FGF causes the cells to arrest in the G1 phase of the cell cycle and move into terminal differentiation (Wray-Cahen et al., 1998). It has also been shown that the re-addition of FGF to the media after terminal differentiation begins is not affective in reversing the process (Wray-Cahen et al., 1998). This is most likely due to the fact that FGF receptors on the cells are lost during the terminal differentiation process (Wray-Cahen et al., 1998; Olwin and Hauscha, 1988). It has also been shown that FGF in the media of cultured cells is critical to activation of quiescent satellite cells (Johnson and Allen, 1995). In the animal, FGF is stored in the extracellular matrix of muscle and plays a role in regulation of muscle hypertrophy (Wray-Cahen et al., 1998).

Another widely explored growth factor is growth hormone (GH). Growth hormone has been shown to increase growth rate, feed to gain ratio, and lean muscle percentage (Scanes, 2003) in animals. However, Dodson et al. (1996) reported that GH

had no direct effect on satellite cells in the concentration range of 10^{-8} to 10^{-7}M. Within their studies only testosterone directly affected satellite cells isolated from beef and swine (10^{-7} to 10^{-6}M) (Dodson et al., 1996).

In the animal, GH effects are mediated through insulin-like growth factor-I (IGF-I) and is thought to be the primary regulator of IGF-I expression (Sanders and Harvey, 2004; Halevy et al., 1996). Insulin-like growth factor-I and insulin-like growth factor-II (IGF-II) have been associated with release of growth hormone in growing animals. Increasing levels of circulating GH result in increased levels of IGF-1 circulating which is part of the feedback mechanism that controls GH release (Sanders and Harvey, 2004). Insulin-like growth factor-I released from the liver in response to elevated levels of circulating GH results in increased muscle and bone growth.

In the body, IGF-I functions to regulate muscle growth, development and maintenance through autocrine and paracrine actions (Wray-Cahen et al., 1998; Kokta et al., 2004). The release of IGF-I from adjacent cells can be stimulated by exercise or muscle overload, resulting in increased satellite cell proliferation (Hawke and Garry, 2001). In muscle, autocrine or paracrine action is mediated through IGF binding proteins (IGFPBs) that are secreted by myoblasts (Wray-Cahen et al., 1998). In both cattle and pigs, IGF-I levels are low during prenatal development and increase as the animal ages (Wray-Cahen et al., 1998).

Insulin-like growth factor-I and IGF-II are present in (Minshall et al., 1990) and increase proliferation and differentiation of isolated satellite cells in culture (Oksbjerg et al., 2004; Hawke and Garry, 2001). These findings are supported by Hodik et al. (1997),

who showed *in vitro* IGF-I treatment induced DNA synthesis in chicken satellite cells. In addition, IGF-I and IGF-II stimulated proliferation in turkey satellite cells, but not differentiation of satellite cells (McFarland et al., 1993). However, FGF must be present in order for this increased proliferation to occur (McFarland et al., 1993). Insulin-like growth factor-I may also mediate effects of androgens on satellite cells. Chen et al., (2005) and Kokta et al. (2004) suggested IGF-I signaling may be responsible for timing and regulation of satellite cell proliferation and differentiation.

Transforming growth factor-beta (TGF-β), generally thought of as a decreasor of muscle proliferation and differentiation, has been shown to both increase and decrease muscle proliferation and differentiation (Hawke and Garry, 2001). Dodson et al. (1996) stated that TGF-β is generally regarded as a decreasor of both proliferation and differentiation of satellite cells. However when serum was removed from pig satellite cell treatment media in culture, TGF-β increased proliferation. Hathaway et al., 1994 showed variation in response of satellite cells when exposed to TGF-β. They found that TGF-β suppressed proliferation of satellite cells isolated from five day old lambs, but did not suppress proliferation in satellite cells isolated from 30- or 150-day old lambs. Little of the available research compared the effect of growth promoting hormones (i.e. FGF, GH, IGF-I, and IGF-II) on satellite cells isolated from animals with different genetic potential for growth such as callipyge sheep. Alteration of the responses of satellite cells to growth compounds may explain some of the increased muscle growth seen in callipyge sheep.

Extrinsically Administered Growth Promoting Compounds

Two hormones often administered to an animal to increase growth, especially in cattle, are estradiol benzoate and trenbolone acetate. Commonly combined in implant form, estradiol benzoate and trenbolone acetate have been shown to increase muscle growth and growth efficiency, but decrease tenderness, marbling, and quality grade (Herschler et al., 1995). The effect of androgens, such as testosterone and trenbolone acetate, on muscle satellite cells has also been extensively studied.

Chen et al. (2005) reviewed the effects of androgens on muscle satellite cells and stated that testosterone enhanced proliferation of primary rat myoblasts, but had no effect on proliferation and reduced differentiation of pig satellite cells. Dodson et al. (1996) reported that testosterone only affected satellite cells isolated from beef and swine in the range of 10^{-7} to 10^{-6} M. Androgen-treated satellite cells also appeared to have increased responsiveness to IGF-I than control cells. Initial studies suggested that testosterone had no effect on protein accretion because it did not directly cause muscle cells in vitro to stimulate protein accumulation (Allen et al., 1984; Dodson et al., 1996). Furthermore, administration of testosterone had no effect on beef embryonic myoblasts (Gospodarowicz et al., 1976) and porcine satellite cells (Doumit et al., 1996). Testosterone has been shown to decrease the responsiveness of satellite cells to growth factors like basic FGF, and IGF-I (Doumit et al., 1996). Conversely, testosterone has been shown to up-regulate the androgen receptors in pig (Merkel et al., 1994) and beef (Kamanga-Sollo et al., 2004) muscle.

Often, trenbolone is used in growth implants to mimic the effects of testosterone. Trenbolone, like testosterone, had no effect on proliferation of rat satellite cells (Chen et al., 2005). Similar results have been seen when treating satellite cells and myoblasts with estrogen (Kahlert et al., 1997). In addition, Johnson et al. (1998) showed satellite cells isolated from steers implanted with trenbolone acetate and estradiol benzoate had greater proliferation, fusion, and differentiation rates than did satellite cells isolated from non-implanted steers. These findings are reinforced by Thompson et al. (1989) who showed that satellite cells isolated from trenbolone treated rats showed greater proliferative capacity and sensitivity to IGF-I and FGF than those isolated from non-treated control rats. In contrast, Thompson et al. (1989) found that the treatment of satellite cells with trenbolone did not affect the proliferation rate. While treatment of satellite cells with trenbolone has no effect on their proliferative ability (Johnson et al., 1998; Thompson et al., 1989), trenbolone used as an implant in the animal may stimulate satellite cell activity and therefore skeletal muscle growth by increasing sensitivity to IGF-I and FGF (Thompson et al., 1989).

Carpenter et al. (2000) suggested lambs expressing callipyge genotype are not different due to hormone patterns, but due to another unknown factor. In animals, hormone levels vary by species. In sheep, estradiol can be found at roughly 1.20 pg/mL of serum (Evans et al., 1994). Insulin-like growth factor I, the main control for GH in satellite cells, is found at 268 ng/mL (Whisnant et al., 1998). Fibroblast growth factor, a promoter of satellite cell fusion, is found from 10-200 ng/mL (Allen et al., 1984).

Finally, trenbolone can be measured at 0.05 +/- 0.19 pg/mL (Henricks et al., 1997). The above compounds comprise the basis for satellite cell treatment in this study.

Action of Satellite Cells Isolated from Animals with Different Growth Potentials

The only known comparison of satellite cells from normal and callipyge sheep was done by Carpenter et al. (2000). They compared growth of two cell types when exposed to growth media containing horse serum and serum collected from sheep expressing normal and callipyge phenotypes. There were no proliferative or fusion differences between the two cell types under these conditions (Carpenter et al., 2000). This study used serum from animals in the treatments and thus may have contained uncontrollable factors, thus limiting the comparability to the present defined media study.

Satellite cells from heavyweight and lightweight strains of turkey were found to differ in proliferative capacity with cells from heavyweight turkeys having greater proliferation than cells from lightweight turkeys (Merly et al., 1998). On the contrary, no difference in appearance of muscle markers (desmin and the myosin heavy chain) was shown, confirming the authors' finding that selection for growth rate does not affect muscle fiber maturation in turkeys (Merly et al., 1998).

In turkeys selected for high growth rate, differences between genders as well as differences in growth rate have been observed (Velleman et al., 2000). Velleman et al. (2000) showed that overall satellite cells isolated from males categorized as rapid growth rate selected line (F-line) proliferated faster than cells isolated from the random control line. Also, differentiation was faster in cells from F-line male cells than in cells from random control male and F-line female cells. These findings are reinforced by Yun et al.

(1997) who found that faster growing satellite cell clones were more responsive to treatment by fibroblast growth factor and insulin-like growth factor-I than were slower growing satellite cell clones. Taken together, these results suggest proliferation differences attributable to both growth rate and gender effects (Velleman et al., 2000; Yun et al., 1997).

Rouger et al. (2004) found that highly proliferative satellite cells tended to fuse only with myotubes or myofibers. On the contrary, less proliferative satellite cells tended to fuse only with each other (Rouger et al., 2004). This illustrates the ability of satellite cells with different proliferation (or growth) rates to differ, thereby suggesting that the different growth potentials of normal and callipyge sheep may have different satellite cell activity.

Statement of Problem

There is currently little information explaining increased musculature of callipyge sheep relative to normal sheep. As satellite cells have been implicated in muscle growth, differences between these two genotypes satellite cell proliferation will be investigated. In addition, as exogenous administration of growth promoting compounds is standard practice in feeding of ruminant livestock, in vitro response of satellite cells from callipyge and normal sheep to increased levels of four of these compounds will also be evaluated.

Therefore, the objective of the present study was to compare the effect of genotype, growth compound (estradiol benzoate, trenbolone acetate, IGF-I, and FGF) and

level of growth compound on proliferation of sheep satellite cells. The hypothesis tested was that satellite cells from callipyge and normal sheep would not differ in their proliferation rate nor would their response to the growth promoting compounds insulin-like growth factor-I, fibroblast growth factor, estradiol benzoate, and trenbolone acetate be different.

CHAPTER 3

MATERIALS AND METHODS

Cell Lines

Callipyge and normal sheep satellite cells (SSC) were donated by Dr. Michael Dodson from the Animal Science Department at Washington State University. Satellite cells were isolated from the semimembranosus muscle of sheep expressing the callipyge (n = 4) and normal (n = 4) genotype, expanded in culture, stored in liquid nitrogen (Dodson et al. 1986), and used in the present study.

Cell Isolation

Cells were isolated from four normal and four callipyge sheep from the Dubois, ID USDA U.S. Sheep Experiment Station (USSES) before the start of the experiment following the procedures of Dodson et al. (1986) and Burton et al. (2000). A brief summation of this procedure follows. First, the semimembranosus muscle was aseptically isolated from the animal at harvest. Next, muscle was treated with antimycotics and antibiotics and connective tissue removed. Remaining muscle was weighed, ground through a sterile grinder, and reweighed. Minced muscle was aliquoted into 50 mL centrifuge tubes, treated with pronase (~ 1 mg/mL), incubated in a 37^{o}C static water bath, and agitated every ten minutes. After incubation, the cell suspension was centrifuged, supernatant was removed and the cells were re-suspended in media. The suspension was then washed three times by centrifugation and supernatant saved each

time and pooled. After final centrifugation, the supernatant was poured off and cells re-suspended in fresh media, plated in the desired dish layered with pig skin gelatin, and grown.

Cells were transported from Washington State University Muscle Biology Laboratory to the Fort Keogh Livestock and Range Research Laboratory in liquid nitrogen. Upon arrival, cells were moved to a larger liquid nitrogen storage tank and kept there until needed. For experimentation, passage of SSC ranged from passage one to four. In relation to treatment of cells, passage number for each treatment was random. Prior to use, evaluation involved comparing isolated cells visually to those described in Dodson et al. (1990), Molnar and Dodson (1993), and Bischoff (1986) and found to be normal, viable satellite cells (Fig. 3).

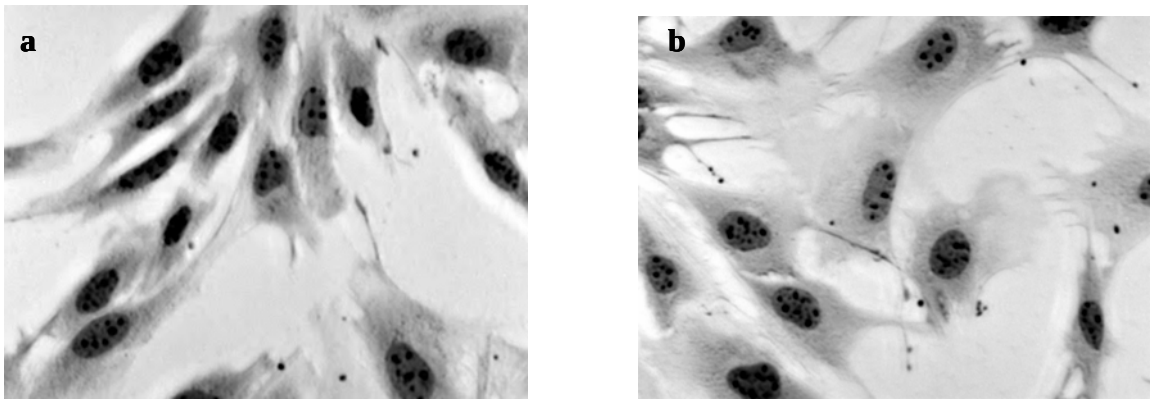

Figure 3: Healthy normal (a) and callipyge (b) satellite cells 24 h post-plating.

Media and reagents

Cells were grown in Dulbecco's modified Eagle's medium (DMEM [Invitrogen, 12800-017, 0.044 M $NaHCO_3$ [Fisher Biotech, BP328-500], pH=7.08). The medium was sterilized by filtration through a 0.22 μm pore filter (NUNC, Millex-GP50) into sterile glass bottles and stored at 4°C. Dulbecco's modified Eagle's medium was supplemented with 10% (vol/vol) horse serum (HS, Invitrogen, 26140-079), 1% (vol/vol) penicillin–streptomycin (Invitrogen, 15140-122), and 0.5% (vol/vol) of gentamicin (Invitrogen, 15710-064). Cells were grown in DMEM, supplemented with defined media components (Table 2), and supplemented with various hormone concentrations after three days of growth.

All defined media compounds were brought to the desired concentration in sterile phosphate buffered saline (PBS, 0.2738 M NaCl [Sigma-Aldrich, S-5866], 0.54 mM KCl [Sigma-Aldrich, P-5405], 16 mM Na_2HPO_4 [Sigma-Aldrich, S-3264], 3 mM KH_2PO_4 [Sigma-Aldrich, P5655], pH=7.08]) and added to DMEM and stirred gently to assure proper distribution.

The PBS/EDTA (ethylenediamine tetraacetic acid, 0.0015 M [Sigma-Aldrich, E-0399) solution was prepared, steam-sterilized and used to free cells from the culture dish. A 50 ml aliquot of sterile trypsin (Invitrogen, 15090-046) was added to 450 ml of sterile PBS/EDTA and stored at 4°C.

Ten percent pig skin gelatin (PSG) (Sigma-Aldrich, G-1890) was prepared and steam-sterilized then stored at 4°C.

Giemsa stain (Sigma-Aldrich, GS1L-1L) working solution was purchased for use.

Table 2. Concentration of components in defined growth media.

Component	Stock Conc.	Working Conc.	Catalog No.
Fibroblast Growth Factor	2 µg/mL	20 ng/mL	F-3133
Fibronectin	200 µg/mL	2 µg/mL	F-4759
Bovine Serum Albumin	100 mg/mL	500 µg/mL	A-4919
Fetuin	50 mg/mL	500 µg/mL	F-3385
Insulin	10^{-6} M	10^{-9} M	I-1822
Dexamethasone	10^{-4} M	10^{-7} M	D-2915
Transferrin	2 mg/mL	5 µg/mL	T-2036
L-Glutamine	200 mM	2 mM	G-8540
Selenium	5 µg/mL	3.8 ng/mL	S-5261
Vitamin C	10 mg/mL	10 µg/mL	A-4403
Biotin	200 µg/mL	200 ng/mL	B-4639
Linoleic Acid	50 mg/mL	10 µg/mL	L-1012
Calcium Chloride	1 M	0.5 mM	C-5670
Peptone	300 mg/mL	600 mg/L	P-5905
Sodium Pyruvate	110 mg/mL	110 µg/mL	P-5280
Hepes	1.5 M	10 mM	H-4043
Myoinositol	1 mg/mL	1 µg/mL	I-7508
Penicillin/Streptomycin	10,000 units/mL	10 ml/L	*Added separately*
Gentamicin	10 mg/mL	5 ml/L	*Added separately*

**** Catalog numbers are for Sigma-Aldrich Company, St. Louis, MO.*

Cell Culture

Sheep satellite cells were thawed in a 37°C static water bath and suspended in DMEM + 10% HS. Each sample was centrifuged (2700 rpm for 3 min; 1500 x g) using a Beckman Coulter Allegra25R centrifuge (swing bucket rotor) (Beckman Coulter, Inc.), and re-suspended in DMEM + 10% HS. Callipyge SSC and normal SSC were plated in PSG-coated 80 cm^2 flasks (NUNC, 178891). All cultures were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2) and 95% air for 24 h. Dulbecco's

modified Eagle's medium + 10% HS was removed and fresh media added daily. When the cells were approximately 75% confluent, they were detached from the culture dishes using 1X trypsin/0.0015 M EDTA, centrifuged at 2700 rpm (1500 x g) for 3 min and resuspended in DMEM + 10% HS. Sheep satellite cells were counted by hand on a hemacytometer (Hausser Scientific, 500) under an Accuscope PH3030 (Great Scopes) inverted research grade microscope equipped with a 40X lens with 10X oculars. Cells were then re-plated on PSG coated 24-well plates (NUNC, 35-3047). Normal and callipyge SSC were plated at 20,000 cells per well. Cells were grown in DMEM + 10% HS for a period of 72 h. At 24 h and 48 h, DMEM + 10% HS was removed and fresh media + 10% HS added. At 72 h, the DMEM + 10% HS was removed; cells were rinsed three times with PBS; defined media plus hormone replaced the DMEM + 10% HS. Plates were stained at 0 h, 24 h, 48 h, 72 h and 96 h of hormone addition and placed in a cool, dark place until counted. Defined media plus hormone was removed from the remaining plates and fresh medium was added daily. There were two identical plates per day of treatment.

Hormone Treatment

Each animal's satellite cells were subjected to estradiol benzoate (Sigma-Aldrich, R-187895), insulin-like growth factor-I (IGF-I) (Sigma-Aldrich, I-3769), fibroblast growth factor (FGF) (Sigma-Aldrich, F-3133), and trenbolone acetate (Sigma-Aldrich, T-3925) at the levels described in the diagram below (Fig. 4). Each column of four wells

contained a duplicate of cell types giving two duplicates of the treatment on each plate (Fig. 4).

Estradiol was dissolved to 1 mg/mL in 100% ethanol, and then diluted in warmed PBS to 0.1 mg/mL. Trenbolone was dissolved in 1mL dexamethasone (DMSO) to a concentration of 1 mg/mL, and then slowly dissolved in warmed PBS to 0.1 mg/mL. FGF and IGF were dissolved in warmed PBS to 0.1 mg/mL. For the FGF treatment, no FGF was added to the defined media; the only FGF available to the cells was that which was added to the media in the experimental levels previously described. All hormones were added to the media to the required experimental concentration per well (Fig. 4).

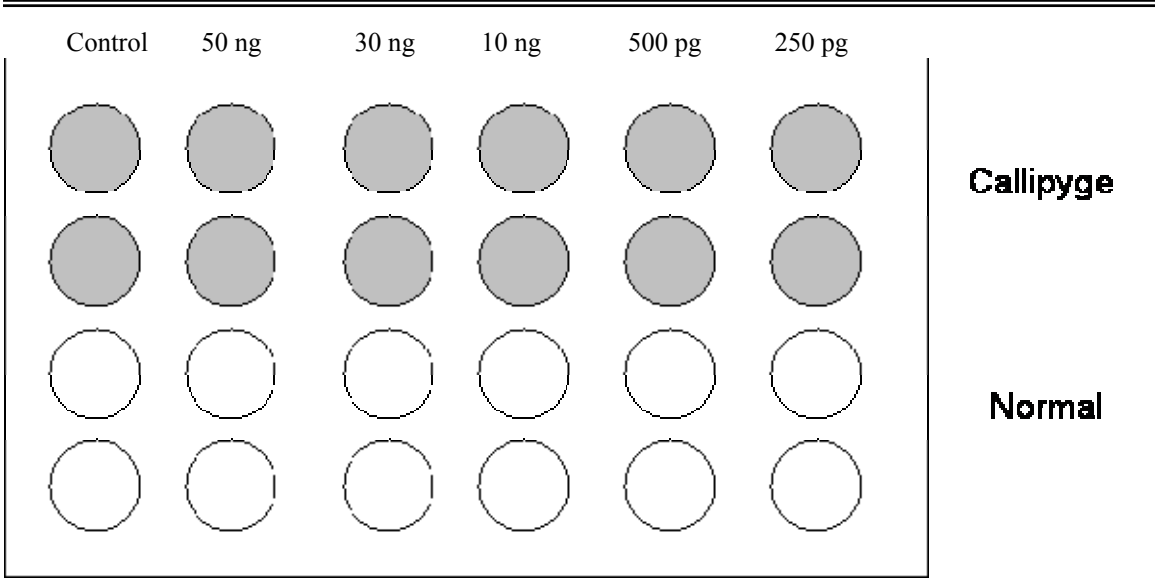

Figure 4: Culture plate (24-well plate) indicating cell type and hormone treatment level in each well.

Cell Staining

After incubation for 0 h, 24 h, 48 h, 72 h and 96 h with hormone, cells were stained using methanol and Giemsa stain (Sigma-Aldrich, GS1L-1L) (Molnar and Dodson, 1993). Growth media was removed from the plates; plates were rinsed twice with PBS. Methanol (200 uL) was added and allowed to stand for 35 minutes to fix cells. Methanol was then poured off and Giemsa stain (200 uL) was added and allowed to stand for a minimum of 1 h. After staining the cells were rinsed with distilled water, air dried and counted.

Analysis & Counting

Plating efficiency was evaluated with an Accuscope PH3030 (Great Scopes) inverted research grade microscope. Counting was done using Accuscope PH3030 (Great Scopes) and IMT-100 (Olympus) inverted, research-grade microscopes equipped with 40X lenses with 10X oculars. Each microscope field was measured to determine diameter and 10 fields in each well were counted at random; the average count in the 10 fields was used to calculate total number of nuclei. Number of total nuclei, number of fused nuclei, defined as three or more nuclei in one tube, and average number of nuclei per well were recorded and used for statistical analysis. Data were recorded as nuclei per square mm.

Statistical Analysis

The design of the study was a split plot in time with the original plot being the genotype (callipyge and normal) with the first split as growth promoting compound treatments (IGF-I, FGF, estradiol benzoate, and trenbolone acetate) and the second split as concentration of growth promoting compound (0, 2.5, 5, 10, 30, and 50 ng/μL) as well as inclusion of time of treatment (0, 24, and 48 h). There were four animals of each genotype (eight total animals) with six hormone treatments and five days on the hormone treatment. On each plate a callipyge and normal animal was tested in duplicate with all six hormone treatment levels. Two duplicates of each plate were done. The dependent variable was number of cells per mm^2. A preliminary analysis was conducted using PROC GLM of SAS (v9.1; SAS Institute, Cary, NC) to calculate residuals using a linear mixed model that included genotype of the animal (callipyge and normal), growth promoting compound (fibroblast growth factor, insulin-like growth factor-I, estradiol benzoate, and trenbolone acetate), concentration of growth promoting compound (0, 2.5, 5, 10, 30, and 50 ng/μL), and time after treatment with growth promoting compound (0, 24, and 48 h) as fixed classification variables. Random classification effects were animal within genotype and the interaction of animal within genotype and growth promoting compound. Residuals were standardized and the distribution of the residuals was evaluated for the presence of outliers and normality using PROC UNIVARIATE (v9.1; SAS Institute, Cary, NC). No additional data editing was done, as the distribution of the data was nearly normal. This is illustrated in the skewness and kurtosis values from the UNIVARIATE analysis, which were 0.399 and 1.268, respectively. Data containing a

normal distribution have an ideal skewness and kurtosis of zero. The values of 0.399 and 1.268 are quite close to zero, therefore reinforcing the normal distribution. The normal distribution of data is further illustrated in figure 5, a box and whisker plot with estimates from the UNIVARIATE analysis.

Subsequently, mixed-model analyses of variance were conducted using restricted maximum likelihood as implemented in PROC MIXED of SAS (v9.1; SAS Institute, Cary, NC). The first analysis considered genotype of animal, growth promoting compound, concentration of growth promoting compound, and time after treatment with growth promoting compound as fixed classification variables. Random classification effects were animal within genotype and the interaction of animal within genotype and growth promoting compound. Number of cells plated on d -3 was included in the model as a linear covariate.

The initial model (Table 3) contained all possible interactions among the fixed effects and it was reduced in a stepwise fashion by eliminating effects that did not approach significance ($P > 0.30$) and were not components of higher order effects to arrive at a final model (Table 4). Unless otherwise specified, stated differences are set to be significant at the 0.10 level of probability.

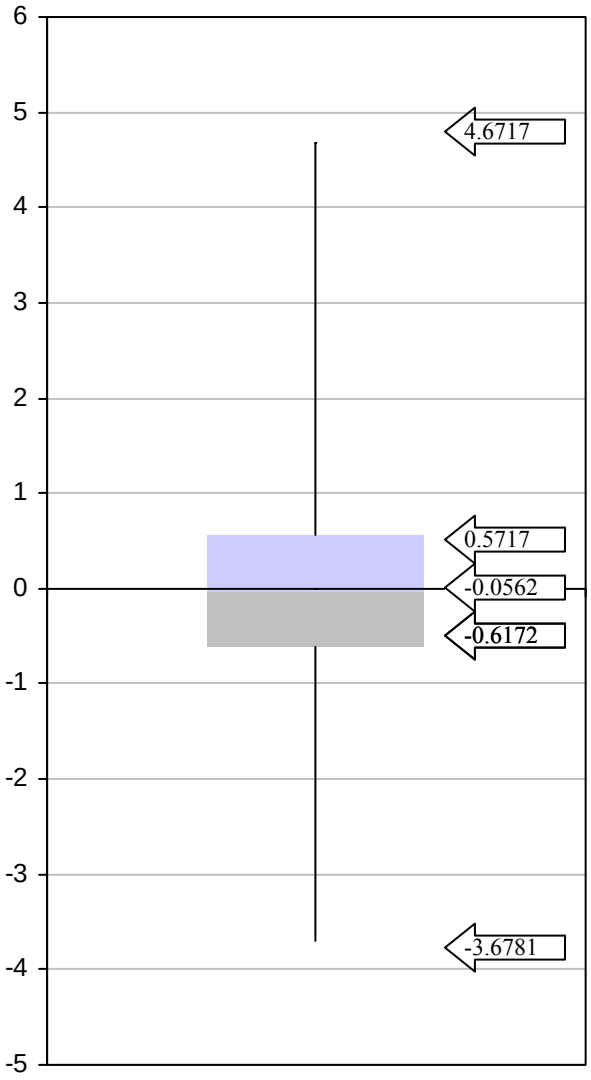

Figure 5: Data distribution as illustrated in a Box and Whisker plot.

Table 3: Initial ANOVA model.

Cov Parm	Estimate
A*T1	10195
A*T1*T2	8819.89
Residual	9484.21

Effect	Num DF	Den DF	F Value	Pr > F
T1	1	6	0.14	0.7207
T2	3	14	2.67	0.0879
T1*T2	3	14	0.16	0.9212
T3	5	1532	12.17	<0.0001
T1*T3	5	1532	0.20	0.9641
T2*T3	15	1532	1.68	0.0487
T1*T2*T3	15	1532	0.51	0.9374
T4	2	1532	910.91	<0.0001
T1*T4	2	1532	39.52	<0.0001
T2*T4	6	1532	14.37	<0.0001
T1*T2*T4	6	1532	11.04	<0.0001
T3*T4	10	1532	1.09	0.3632
T1*T3*T4	10	1532	0.34	0.9704
T2*T3*T4	30	1532	0.30	0.9999
T1*T2*T3*T4	30	1532	0.40	0.9986
Pe	1	1532	5.41	0.0202

** T1= genotype of animal; T2 = growth promoting compound; T3 = concentration of growth promoting compound; T4 = time after treatment; pe = plating efficiency*

Table 4: Final ANOVA model.

Cov Parm	Estimate
A*T1	10123
A*T1*T2	8899.26
Residual	9144.09

Effect	Num DF	Den DF	F Value	Pr > F
T1	1	6	0.14	0.7207
T2	3	14	2.67	0.0879
T1*T2	3	14	0.16	0.9212
T3	5	1532	12.17	<0.0001
T2*T3	15	1532	1.68	0.0487
T4	2	1532	910.91	<0.0001
T1*T4	2	1532	39.52	<0.0001
T2*T4	6	1532	14.37	<0.0001
T1*T2*T4	6	1532	11.04	<0.0001
pe	1	1532	5.41	0.0202

** T1= genotype of animal; T2 = growth promoting compound; T3 = concentration of growth promoting compound; T4 = time after treatment; pe = plating efficiency*

CHAPTER 4

RESULTS & DISCUSSION

Statistical Analysis

Higher order interaction of genotype by growth compound level by time (T1xT3xT4), growth compound by level by time (T2xT3xT4), genotype by growth compound by level (T1xT2xT3) and the four way interaction of genotype, growth compound, level and time (T1xT2xT3xT4) were all not significant (Table 5). Thus, these interactions along with genotype by level (T1xT3) and level by time (T3xT4) were deleted from the initial model to arrive at a final model (Table 6). Subsequently, a simplification to the model was explored wherein the drug response was expressed as a continuous variate rather than as specific classifications. This simplification increased residual variance from 9,144 (Table 6) to 9,312 (Table 7). Thus, there was little loss of precision when the dose-response relationship was taken to be linear as opposed to a more complex form.

Comparison of means derived from the model described above indicated substantial differences of number of cells per mm^2 associated with genotype by growth promoting compound at 0 h. Based on the observations from these preliminary analyses, the final model used for the analysis of these data included genotype of animal, growth promoting compound, and time after treatment as fixed classification variables. Random classification effects were animal within genotype and the interaction of animal within genotype and growth promoting compound were included in the model as linear

covariates. Single degree of freedom linear functions were constructed to partition interaction effects that approached significance ($P \leq 0.10$) and aide in their interpretation.

Table 5: ANOVA with concentration of growth promoting compound as a linear effect.

Cov Parm	Estimate
A*T1	10101
A*T1*T2	8910.51
Residual	9312.07

Effect	Num DF	Den DF	F Value	Pr > F
T1	1	6	0.14	0.7212
T2	3	14	2.08	0.1485
T1*T2	3	14	0.16	0.9184
T3	1	1648	24.92	<0.0001
T2*T3	3	1648	3.42	0.0168
T4	2	1648	946.65	<0.0001
T1*T4	2	1648	40.76	<0.0001
T2*T4	6	1648	14.78	<0.0001
T1*T2*T4	6	1648	11.57	<0.0001
pe	1	1648	5.32	0.0212

** T1= genotype of animal; T2 = growth promoting compound; T3 = concentration of growth promoting compound; T4 = time after treatment; pe = plating efficiency*

Response of Satellite Cells to Growth Promoting Compound Treatment

Cells that were stained at 72 h and 96 h of growth were not counted or included in the analysis because they were too numerous to count. In order to make counting of later time period cells feasible, a smaller number of cells per well (i.e. ~5,000 – 10,000 cells per well) need to be plated as well as allowing only 24 hours of growth prior to hormone treatment. Increased density of cells resulted in the cells being too numerous to count and may have cell to cell effects that complicate the interpretation of data.

Main effects were seen but some higher order interactions show differences that occur with the cells. The main effects showed genotype (T1) had no effect on proliferation of satellite cells in culture (Fig. 6). However, a trend was observed for individual growth promoting compounds used (P= 0.14) to increase growth rate of satellite cells. As expected, time of growth (T4) resulted in a significant increase in cell numbers.

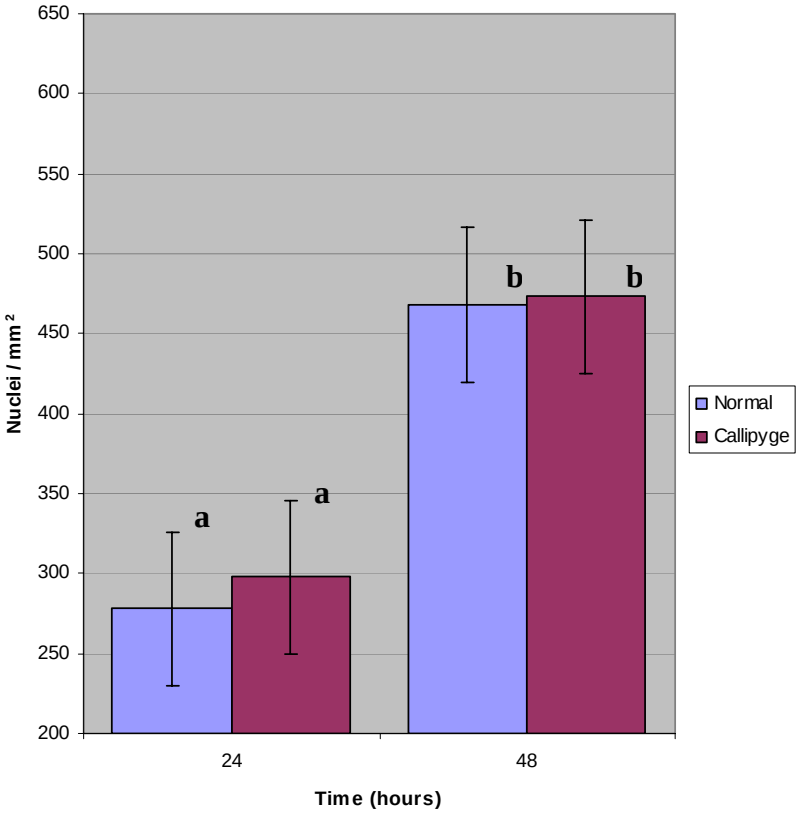

Figure 6: Effect of time and genotype on the proliferation of satellite cells. Bars with common letters do not differ significantly (P ≥ 0.9184).

The finding that callipyge and normal sheep satellite cells grow similarly is in agreement with Carpenter et al. (2000), who found that normal and callipyge sheep

satellite cells had similar population doubling times regardless of satellite cell type or serum type. Carpenter et al. (2000) also found that the percentage of cells fusing was not different between normal and callipyge satellite cell types when serum from callipyge or normal sheep was added.

Growth Promoting Compound

While not statistically significant (P = 0.1485), growth promoting compound shows a trend toward significance. This could be due to the lack of response by cells treated with estradiol benzoate and trenbolone acetate. If we removed these two compounds from the analysis and reran it using only FGF and IGF-I, there is a good possibility that the growth promoting compound would be significant.

Many researchers have shown that treatment of satellite cells with FGF (Michal, et al., 2002; Hawke and Garry, 2001; Sheehan and Allen, 1999; Johnson and Allen, 1995; Doumit et al. 1993; Green and Allen, 1991) and IGF-I (Oksbjerg et al., 2004; Hawke and Garry, 2001; Hodik et al., 1997; Dodson et al., 1996; McFarland et al., 1993; Mathison et al., 1989) stimulates proliferation.

Genotype of Animal x Growth Promoting Compound x Time after Treatment

There was a greater than 2-fold difference in number of cells per mm^2 associated with genotype by growth promoting compound at 0 h (Fig. 7). Therefore, analysis of covariance was used to account for these differences by including number of cells at time 0 as a covariate in the model.

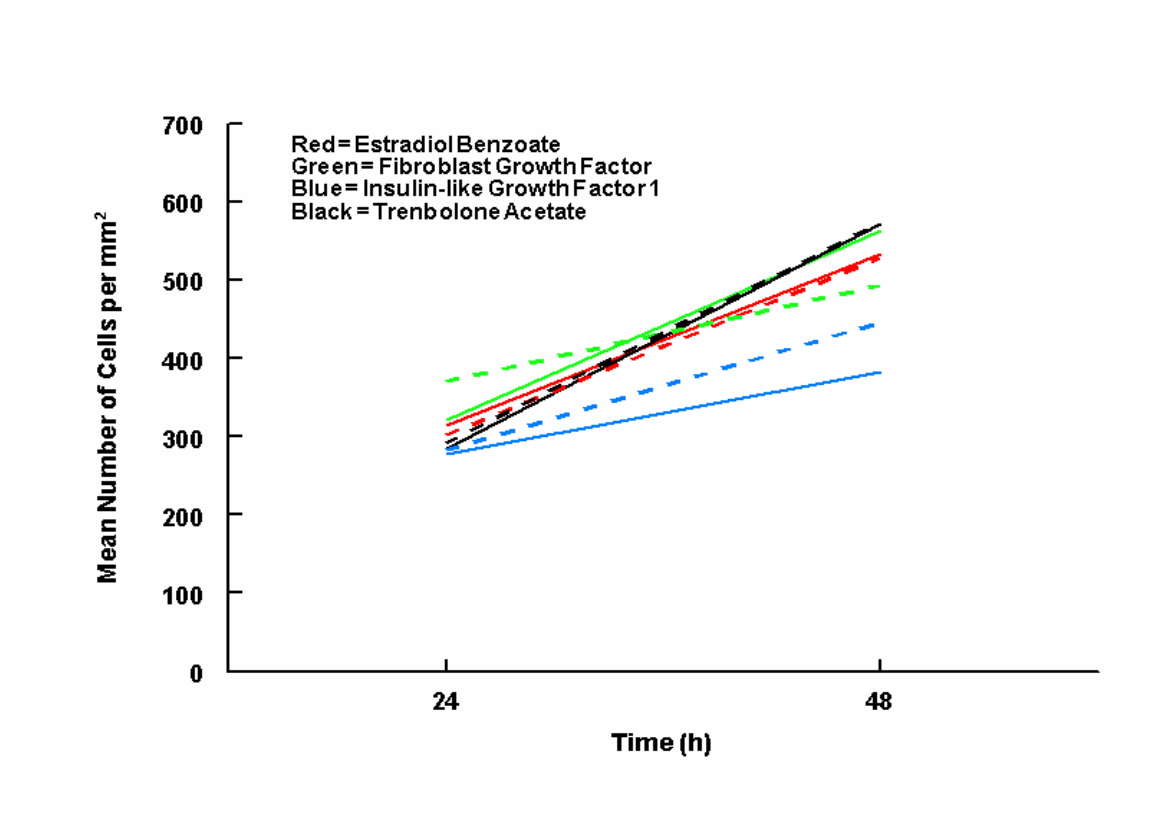

Figure 7: Response of satellite cells isolated from normal and callipyge sheep after standardization at time zero. Solid lines represent the response from normal satellite cells. Dashed lines represent the response from callipyge satellite cells.

The significant interaction between genotype, growth promoting compound and time (T1 x T2 x T4) shows that estradiol and trenbolone had no effect on proliferation of satellite cells over time. For both estradiol benzoate and trenbolone acetate, change in the number of cells from 24 h to 48 h was virtually identical for both callipyge and normal satellite cells (Fig. 7). While estrogens do affect muscle growth *in vivo*, estrogens have not been found to directly influence muscle satellite cell growth, nor have they been found to activate skeletal myoblast proliferation (Kahlert et al., 1997). The lack of

response to estradiol benzoate could be accounted for when looking at the form of growth compound that was used. Because estradiol benzoate is implanted into animals for growth promotion the goal was to determine if the compounds themselves affected satellite cells as opposed to the metabolized form estradiol-17β. With no response reported, estradiol apparently has no direct affect on satellite cell proliferation. When estradiol benzoate is administered, it is first metabolized into estradiol-17β (Fig. 11) by the liver (Bearden et al., 2004; Senger, 1999; Norman and Litwack, 1997). Estradiol-17β's effects are mediated through GH, causing the animal to become more efficient by increasing rate of protein accretion and decreasing amount of protein degradation (Scanes et al., 2003; Scheffler et al., 2003; Perry et al., 1991). This change in efficiency is what increases muscle size and mass (Bearden et al., 2004; Senger, 1999; Norman and Litwack, 1997). The compound used in growth implants apparently has no direct affect on satellite cell proliferation (Fig. 7) and supports research that suggests that estradiol must be metabolized into a usable form of the growth promoting compound to affect growth.

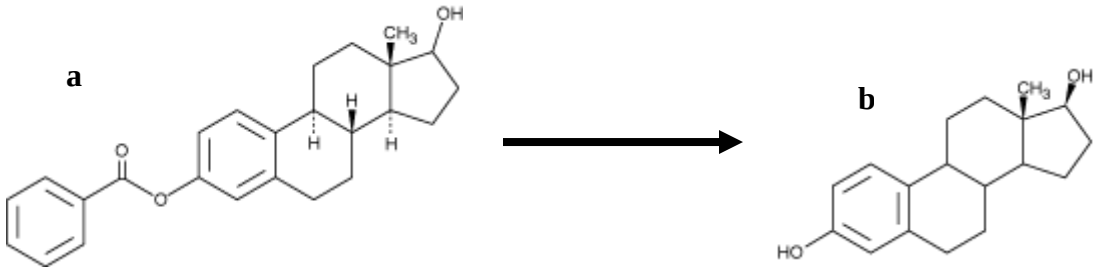

Figure 8: Estradiol benzoate (a) and estradiol-17β (b) (From: www.sigma-aldrich.com; www.caymanchem.com; Bearden et al., 2004; Senger, 1999; Norman and Litwack, 1997).

Trenbolone acetate is a form of testosterone that is used in growth implants and from its lack of affect on isolated satellite cells suggests that it also needs to be metabolized by the liver before the effect is seen. Trenbolone acetate is metabolized into testosterone, which is metabolized into estradiol-17β (Fig. 9) before being used by muscle (Bearden et al., 2004; Senger, 1999; Norman and Litwack, 1997). Similarly to estradiol benzoate, I wanted to determine if the compounds themselves affected satellite cells as opposed to the metabolized form that can affect the cells. However, the non-metabolized (implant) form did not affect proliferation of satellite cells (Fig. 7) so the effect of trenbolone acetate in implants is most probably through metabolized compounds.

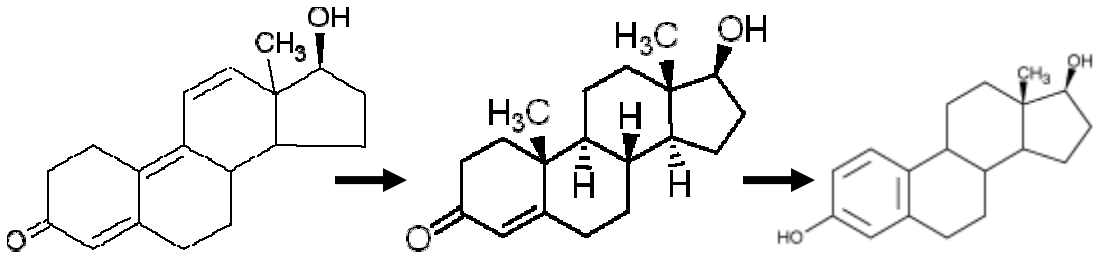

Figure 9: Trenbolone (a), testosterone (b), and estradiol-17β (c) (From: www.sigma-aldrich.com; Bearden et al., 2004; Senger, 1999; Norman and Litwack, 1997).

Little work on trenbolone acetate treatment of satellite cells has been published and available research on testosterone and satellite cells varies. Generally it has been reported that satellite cells are not directly affected by testosterone. Chen et al. (2005) reported that testosterone enhanced proliferation of primary rat myoblasts, but not satellite cells. Chen et al. (2005) and Doumit et al. (1996) found that testosterone had no effect on proliferation of porcine satellite cells. Similarly, Gospodarowicz et al. (1976)

reported testosterone had no effect on beef satellite cells. Conversely, Dodson et al. (1996) reported that testosterone affects on beef and swine satellite cells was dose dependent and cells were only affected in the range of 10^{-7} to 10^{-6} M. While unmetabolized hormone administration prevented any treatment to satellite cells, the chance of an actual effect occurring was slim. While treatment of satellite cells with trenbolone has no effect on their proliferative ability (Johnson et al., 1998; Thompson et al., 1989), trenbolone used as an implant in the animal may stimulate satellite cell activity and therefore skeletal muscle growth by increasing sensitivity to IGF-I and FGF (Thompson et al., 1989).

Insulin-like growth factor-I and FGF affected cells isolated from callipyge animals differently than cells from normal animals. Number of cells increased more rapidly in callipyge than normal in response to IGF-I. Conversely, number of cells increased more rapidly in normal than in callipyge cells in response to FGF (Fig. 7). There is a statistically significant difference between the normal cells and the callipyge cells at each time period; the normal cells have a more drastic increase from 24 h to 48 h (~ 43% more nuclei/mm^2; Fig. 7) than do the callipyge cells (~ 25% more nuclei/mm^2; Fig. 7). This information suggests that callipyge cells do not respond to FGF as much, but when IGF-I is present there is an increase in proliferation.

These findings are in disagreement with Yun et al. (1997) who found that slower growing satellite cell clones isolated from a single bird were less responsive to treatment by fibroblast growth factor than were faster growing clones. Additionally, many researchers have shown that treatment by FGF stimulates proliferation of muscle satellite

cells (Michal, et al., 2002; Hawke and Garry, 2001; Sheehan and Allen, 1999; Johnson and Allen, 1995; Doumit et al. 1993; Green and Allen, 1991), which did not happen to callipyge in this study. An alternative cause of the difference is increased cell density resulted in cell to cell interactions that altered the response to added growth compounds.

While response of satellite cells was different between 24 h and 48 h, there was no increasing difference of proliferation of satellite cells as increasing levels of IGF-I was added, which is contradictory to the result found by several other researchers. These researchers (Oksbjerg et al., 2004; Hawke and Garry, 2001; Hodik et al., 1997; McFarland et al., 1993) reported that IGF-I significantly increased proliferation of satellite cells. Moreover, Mathison et al. (1989) found that IGF-I binding in satellite cell cultures was different between high and low growth lines of Targhee sheep. The findings reported here are similar to this, as normal and callipyge sheep satellite cells did respond differently to treatment by IGF-I. Callipyge satellite cells grew faster than normal initially then leveled off. Normal satellite cells began growing more slowly and ended up growing faster than the callipyge satellite cells. It may be necessary to extend the treatment period to see any additional difference between the two genotypes in response to increasing levels of IGF-I or reduce the number of cells plated to reduce any affect of cell density on the proliferation of the cells.

Insulin-like growth factor-I functions in the body to regulate muscle growth, development and maintenance (Wray-Cahen et al., 1998). It functions primarily during postnatal growth and development (Dodson et al., 1996). Insulin-like growth factor-I has been reported to increase proliferation and differentiation of satellite cells in culture

(Oksbjerg et al., 2004; Hawke and Garry, 2001; Hodik et al., 1997; Dodson et al., 1996; McFarland et al., 1993; Mathison et al., 1989).

Growth Promoting Compound x Concentration of Growth Promoting Compound

The interaction of growth promoting compound and level of compound between 0, 2.5, 5, 10, 30, and 50 ng/μL was significantly different (P = 0.0168) (Table 5). The curves for estradiol and trenbolone show no increase in response to increasing levels of the compound (Fig. 10) as shown by the slope of the line. Insulin-like growth factor-I and FGF, however, show increasing cell numbers in response to increased levels and then show a possible flattening of the curve suggesting no increased response at higher levels.

The possibility that the dip at the 2.5 ng/μL level is due to human error is very high. Errors in dilutions are magnified as the concentration of the solution is reduced. When the value for 2.5 ng/μL is removed from the graph, the resemblance to the upper portion of a typical dose response curves is increased (Fig. 11; Fig. 12).

The zero treatment level in this study contained no FGF. With the absence of FGF in the defined media it was expected that the rapid increasing portion of the dose response curve would be seen. However, this was not the case and the resulting curves more resemble the leveling off of the response as opposed to the exponential portion of a dose response curve.

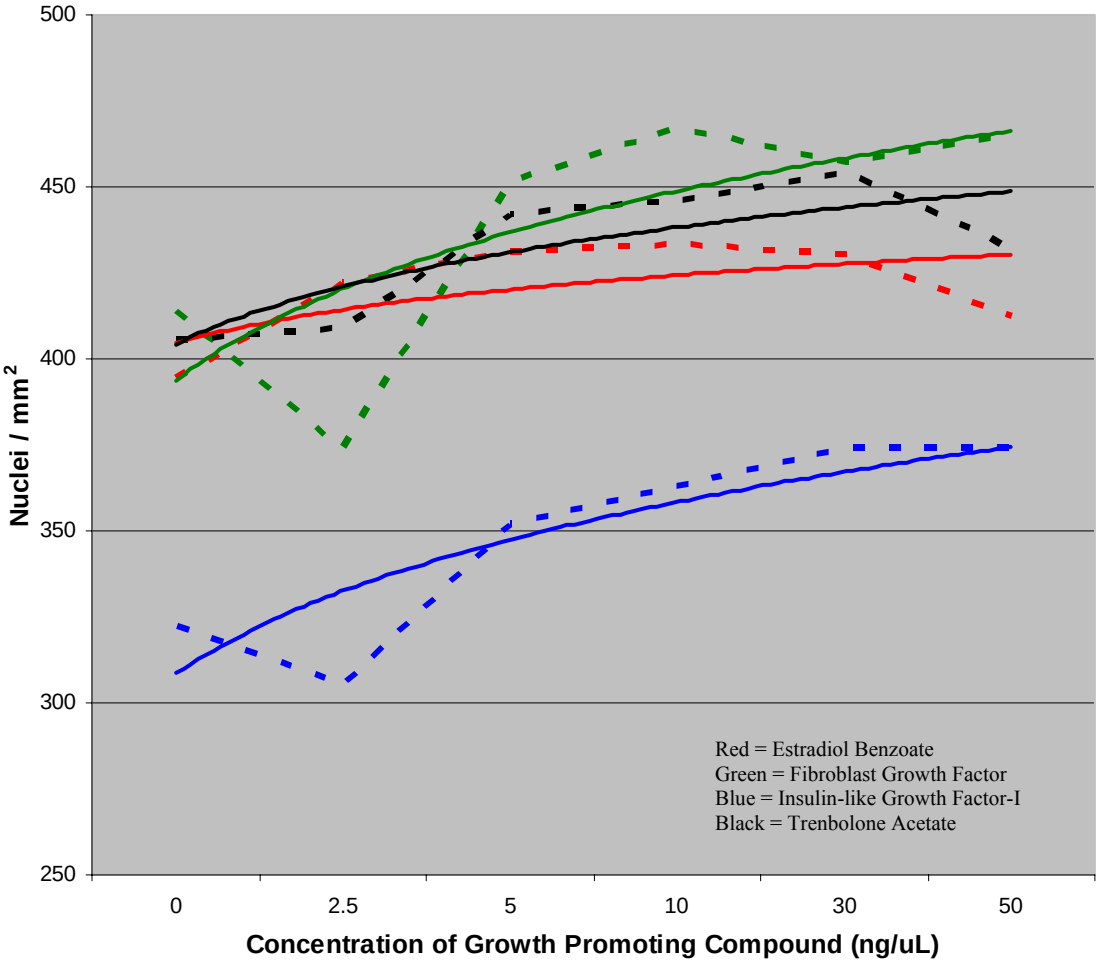

Figure 10: Normal and callipyge satellite cell response when exposed to different levels of growth promoting compounds. Dotted lines indicate actual cell counts; solid lines indicate power fit curves. *FGF: $y=393.65x^{0.0945}$; IGF-I: $y=308.44x^{0.1082}$; Trenbolone acetate: $y=404.33x^{0.0581}$; Estradiol Benzoate: $y=404.51x^{0.0345}$*

In other studies it has been reported that the lack of FGF caused satellite cells to stop growing and move into terminal differentiation (Wray-Cahen et al., 1998). During this process, FGF receptors were lost and re-administration of FGF did not affect cell growth. It was expected that the zero level cells' proliferation would slow or they would show fusion as time of growth increased. However, in this experiment, response at 0

ng/well was no different than response at 50 ng/well for both normal and callipyge satellite cells.

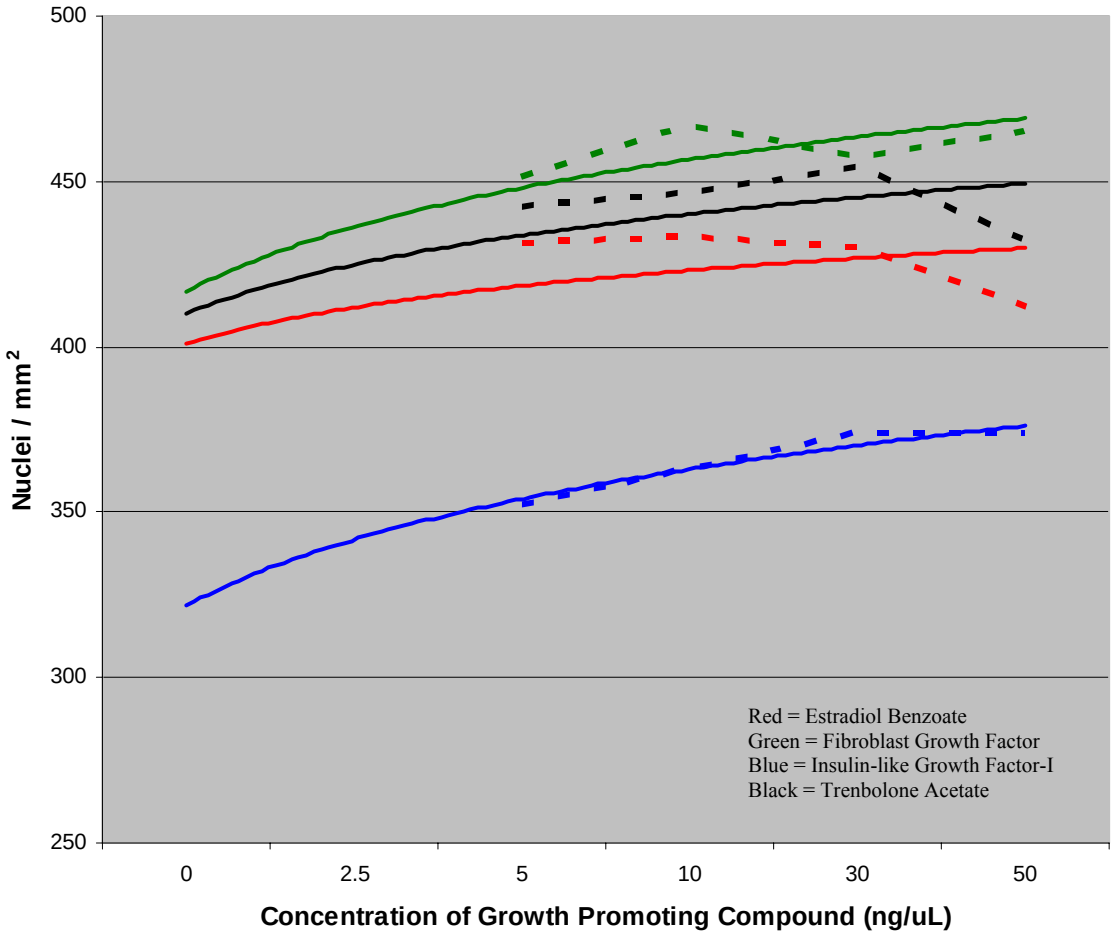

Figure 11: Normal and callipyge satellite cell response when exposed to different levels of growth promoting compounds without the 2.5 ng/µL data point. Dotted lines indicated actual cell counts; solid lines indicate power fit curves. *FGF: $y=416.57x^{0.0663}$; IGF-I: $y=321.62x^{0.0874}$; Trenbolone acetate: $y=410.01x^{0.0512}$; Estradiol Benzoate: $y=400.91x^{0.039}$*

The reason for this anomaly is unknown, but additional evaluation of this treatment would be much more informative to confirm or invalidate this finding. In the case of the FGF-treated cells, the null hypothesis that the callipyge and normal satellite cells would respond differently was supported. This could be due to the residual FGF in

individual components of the defined media. Additionally, the lack of proliferation inhibition could be due to autocrine and/or paracrine effects between cells. In order to effectively address the FGF issue, the present study needs to be repeated

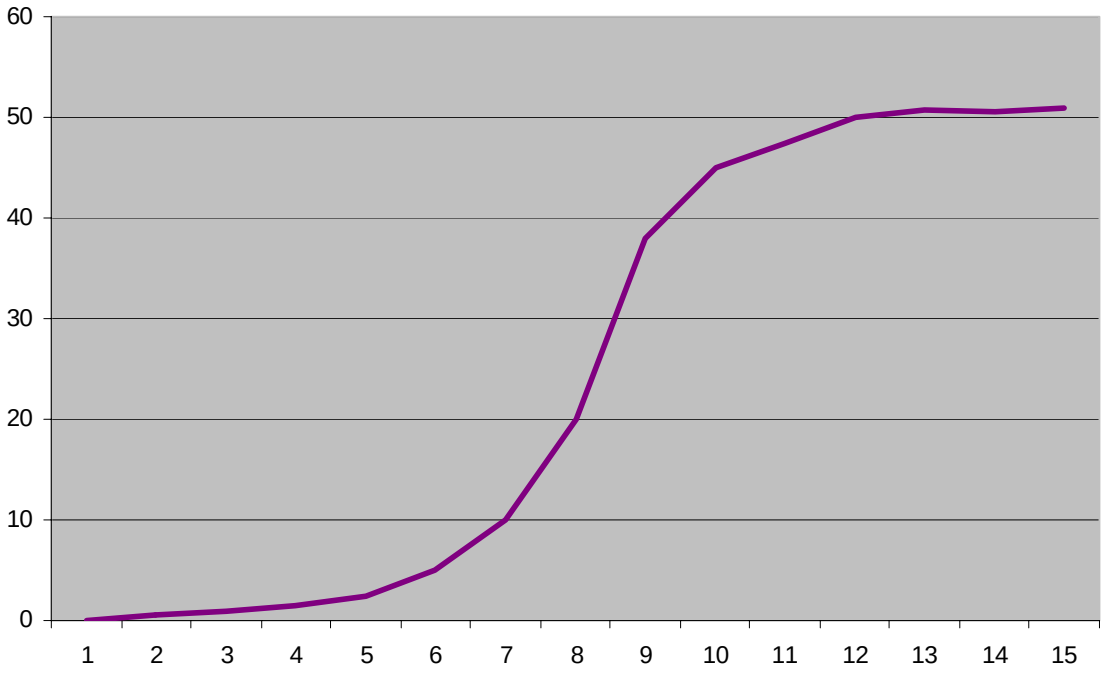

Figure 12: Typical dose response curve.

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Challenges to Interpretation of Data

In hindsight, there are several flaws in this experiment. To begin, there were not sufficient independent replications to effectively evaluate each data point. While I clearly do not have enough independent replications to estimate variance at each observational point, I did test homogeneity of variance and establish the appropriateness

of using the pooled error variance for comparisons. Secondly, replications of individual animals were not independent. By creating and incubating replications simultaneously, I did set up a situation where replications were not independent. Any environmental factors (incubator problems, media misformulation, human error, etc.) that would have affected one replication of an animal would also affect the second replication. Even though each animal was done at different times, if I were to do the experiment over again, I would do it so that each replication was completed before the next commenced. Additionally, checking plating efficiency to ensure accuracy would be done prior to proceeding. Thirdly, our analysis was not one typically seen in cell culture experiments. In general, the analysis used is the PROC GLM function of SAS (v9.1; SAS Institute, Cary, NC) (Mozdziak et al., 2002; Carpenter et al., 2000; Velleman et al., 2000; Sheehan and Allen, 1999; Merly et al., 1998; Doumit et al., 1996; Hathaway et al., 1994; Doumit et al., 1993; Mathison et al., 1989) with a completely random design (Michal et al., 2002; Dodson et al., 1990). Granted, the atypical analysis is indicated by the design of the experiment and lack of sufficient replication; however, this analysis of the data is more powerful than alternatives given the design. Finally, the treatments we used were not entirely applicable to the sheep industry because estradiol and trenbolone are rarely used as growth implants in sheep, nor were they effective in their action. A better understanding of the mode of action of these growth promoting compounds at the outset would have led to fewer and different treatments than I used.

In addition to those mentioned above, there were additional drawbacks in my experiment. First, I would consider this a very good “introductory” experiment. Using

this broad set of data an evaluation of which hormone was the most informative would be appropriate. With the focus on one hormone, less clutter would be forced into a single experiment. For example, these data may tell me that the most informative part of the dose/response curve was between 12 and 24 hours of growth or beyond 48 hours. Therefore, ideal design would be an experiment that had many times (0 h, 6 h, 9 h, 12 h, 15 h, etc.) of staining/counting. I would try a few smaller experiments to find an "ideal" hormone range to be working in and focus there rather than having such a large concentration difference. Maybe the hormone I am interested in shows the most promise from 0 to 2.5 ng/μL – this could be broken down into smaller increments to see where the actual effect is happening. Treatment would commence sooner so that there is less chance that the cells would be starting at a different level. Also, plating cells and allowing them to grow prior to addition of growth compounds could lead to erroneous interpretation of data because of cell to cell interaction because of increased cell density.

I should have been able to replicate studies that showed FGF and IGF-I would cause a decrease in proliferation or movement into terminal differentiation in the satellite cells (including cell death when deprived of FGF or IGF-I) regardless of genotype (Oksbjerg et al., 2004; Michal, et al., 2002; Hawke and Garry, 2001; Sheehan and Allen, 1999; Hodik et al., 1997; Johnson and Allen, 1995; Doumit et al. 1993; McFarland et al., 1993; Green and Allen, 1991). No inhibition of proliferation could be attributable to a small amount of residual FGF in the media component. Perhaps I wasn't able to rinse the cells thoroughly enough to remove all of the FGF thereby allowing them to keep proliferating even though the defined media did not contain any FGF. Removal of IGF

should also have been detrimental to the cells. However, the defined media did contain insulin which will occupy the same binding sites as IGF-I on the satellite cells and activate cell components at a lower level than IGF-I and keep the cells proliferating in the absence of IGF-I. Additionally, the unexpected result at the zero level of treatment for IGF-I and FGF could be due to the autocrine and paracrine interactions between the proliferating cells. Since the density was so high initially, autocrine and paracrine effects would counteract any lack of treatment in the defined media.

Lack of effect by growth promoting compounds may be due to concentration of hormone used. The range of growth promoting compound used was 6.5×10^{-6} to 3.3×10^{-7} M for IGF-I, 3.05×10^{-6} to 1.5×10^{-7} M for FGF, 1.3×10^{-4} to 6.6×10^{-6} M for estradiol benzoate, and 1.8×10^{-4} to 9.2×10^{-6} M for trenbolone acetate. The literature suggests concentrations between 10^{-8} to 10^{-7} (Dodson et al., 1996) and I used 10^{-4} to 10^{-7}, which may have eliminated any chance of a response by the cells to treatments. Dodson et al. (1996) suggest a range of 10^{-7} to 10^{-6} M for testosterone, which should be comparable to what one would expect when using trenbolone acetate. Most of my range was well above 10^{-6} M. Additionally, the ranges of 10^{-10} to 10^{-7} M for IGF-I and 10^{-11} to 10^{-10} M for FGF are suggested (Dodson et al., 1996). Again, the concentrations that I used were well above that suggested by the literature. The levels of growth promoting compound used had no effect on the proliferation of the cells; therefore either the compound had no effect because it was not in a metabolized form or the levels were too high to see a typical dose response curve. On the contrary, the zero level of treatment

was not too large. Lack of response to this level of treatment could be due to autocrine and paracrine effects shown at such a high plating density of cells.

When looking at proliferation, a good experimental system would allow cells to undergo numerous cell divisions before termination of the experiment, which the present experiment did not do. Further, an exact cell numbers need to be known prior to the addition of any growth promoting compounds to evaluate increased proliferation. I did count the cells at time zero, or the time which the growth promoting compound treatments were added but this count was used as a covariate to adjust the proliferation of the cells to the same point. So, if 20,000 cells were initially plated and allowed to attach for 24 h, the cultures washed, and the experimental treatments added then a proliferation test system would cover 20,000 cells (100 nuclei/mm^2) to at least 80,000 – 100,000 cells per well (400-500 nuclei/mm^2). Since cell division is not 100 percent, there may be only 2-3 cell cycles to see if the treatments worked. Alternatively, if 2,000 cells per well (10 nuclei/mm^2) were initially plated and the experiment started at that number, greater than 6 cell divisions would be seen over the span of the proliferation experiment. Plating fewer cells per well initially would allow greater accuracy and confidence in cell counts as well as interpretation of results.

Further complications were apparent due to addition of 10% serum for 3 days before adding growth promoting compound treatments. While there was a cell count at time zero, or the time at which growth promoting compound treatment was added, that count was not integrated into the data analysis; rather, the count was used as a covariate. Further, sheep satellite cells sometimes can explode in numbers for whatever reason,

making cell proliferation counts impossible when cells were plated at high numbers and left in serum for some time. Finally, too many cells in a proliferative experiment negate the effects of many growth factors, since the cells themselves produce growth factors and the autocrine and paracrine effects take over. In the present experiment, the cell-produced factors might have overwhelmed the ones that were added as treatments which could lead to erroneous conclusions about the results.

Use of a clonal line of satellite cells may have proven to be more informative than use of primary isolates. Blanton et al. (1999) demonstrated that clonal myoblasts have the same cell size distribution and cytoplasmic/nuclear ratio as primary cell isolates. Additionally, use of clones would have been useful because of decreased risk of contamination by fibroblasts and elimination of paracrine effects (Doumit et al., 1993; Burton et al., 2000). However, the primary isolates are more informative when looking at the muscle environment because they are more representative of the muscle as a whole.

In order to clarify differences between normal and callipyge satellite cells as well as differences in the muscle hypertrophy in the animals, the previously expressed concerns would all need to be addressed. In addition, more extensive experimentation using additional growth promoting compounds (FGF, IGF-I and IGF-II, hepatocyte growth factor, etc. as reviewed by Hawke and Garry, 2001) as well as muscle growth and satellite cell markers (desmin, MyoD, myf5, etc. as reviewed by Hawke and Garry, 2001) would prove informative in filling in additional pieces of the puzzle.

Summary

Taking all of the previously expressed concerns into account, few conclusions about the data can be made with confidence. However, under the conditions used in the present experiment, genotype had no effect on the proliferation of the satellite cells in culture. Individual growth promoting compounds used tended (P= 0.1485) to affect the growth rate of satellite cells. As expected, time cells were allowed to proliferate resulted in a significant increase in cell numbers. Cells from different genotypes responded differently to growth compounds used. Estradiol and trenbolone had no effect on the proliferation of cells but callipyge cells responded differently to IGF and FGF than did normal cells. Callipyge cells showed increased growth rate in response to IGF-I suggesting that part of the difference in muscle growth seen in callipyge sheep could be due to increased response of the satellite cells to IGF-I not due to differences in the proliferation rate of the satellite cells from different genotypes.

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