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IMPACT OF ERGOT ALKALOID AND ESTRADIOL 17B ON WHOLE-BODY PROTEIN TURNOVER AND EXPRESSION OF MTOR PATHWAY PROTEINS IN MUSCLE OF CATTLE

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture, Food and Environment
at the University of Kentucky

By

Taylor Dawn Ferguson

Lexington, Kentucky

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Lexington, Kentucky

2020

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ABSTRACT OF THESIS

IMPACT OF ERGOT ALKALOID AND ESTRADIOL 17B ON WHOLE-BODY PROTEIN TURNOVER AND EXPRESSION OF MTOR PATHWAY PROTEINS IN MUSCLE OF CATTLE

Beef cattle consuming endophyte infected tall fescue typically exhibit reduced performance in terms of both decreased dry matter intake (DMI) and growth rates. It has been suggested that lower concentrations of circulating IGF-1 (an important stimulator of the mTOR pathway and ultimately protein synthesis), as observed with consumption of ergot alkaloids, contribute to reduced growth rates. The objective of the current study was to determine if fescue-derived alkaloids decrease muscle protein synthesis though inhibitory action on the mTOR pathway via a direct effect on signal proteins, and if these negative effects can be alleviated by implantation with anabolic agents. Thirty-two Holstein steers were used in a 2x2 factorial design, and treatments consisted of intramuscular administration of bromocriptine (vehicle or 0.1 mg/kg BW) and a subdermal estradiol implant (with or without). Throughout the 35-day experiment, steers were fed a corn silage-based diet, with intake restricted to 1.5 times maintenance energy requirement. Bromocriptine injections were given every three days for 34 days. On days 27 through 32, steers were moved to metabolism stalls for urine collection and whole-body protein turnover was determined using a single pulse dose of [^{15}N] glycine into the jugular vein on day 28. On day 35, muscle samples were collected from the musculus obliquus externus abdominis before (basal state) and 60 mins after (stimulated state) an i.v. glucose challenge (0.25 g glucose/kg). Blood samples were collected at regular intervals before and after glucose infusion for determination of circulating concentrations of glucose and insulin. Bromocriptine reduced insulin and glucose clearance following the glucose challenge, indicating decreased insulin sensitivity and possible disruption of glucose uptake and metabolism in the skeletal muscle. This suggests that fescue-derived alkaloids are detrimental to growing cattle in terms of overall glucose homeostasis and energy metabolism. Conversely, analysis of whole-body protein turnover demonstrated that bromocriptine does not appear to affect protein synthesis or N retention and western immunoblot analysis of skeletal muscle showed that it did not affect abundance of S6K1

or 4E-BP1, so does not appear to inhibit activation of the mTOR pathway or protein synthesis. Implantation improved N retention, decreased protein turnover, and had no effect on protein synthesis, suggesting that steroidal implants promote protein accretion through unchanged rates of synthesis and decreased degradation, even in the presence of bromocriptine, resulting in improved daily gains. Implanted steers likely experienced increased IGF-1 signaling, but downstream activation of mTOR, S6K and 4E-BP1, and thus increased protein synthesis did not occur as expected. Overall, this data suggests that fescue derived alkaloids do not have a negative impact on muscle protein synthetic pathways, independent of DMI.

KEYWORDS: ruminant, bovine, ergot alkaloids, mtor pathway, protein metabolism

Taylor Dawn Ferguson

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10/30/2020

Date

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FREQUENTLY USED ABBREVIATIONS

E-	Endophyte-free
E+	Endophyte-infected
KY-31	Kentucky-31
5-HT	5-hydroxytryptamine (serotonin) receptor
D2	dopamine receptor 2
h	Hour
d	Day
min	Minute
Da	Dalton
N	nitrogen
DM	dry matter
DMI	dry matter intake
ADG	average daily gain
BW	body weight
IVGTT	intravenous glucose tolerance test
AUC	Area Under the Curve
TBA	Trenbolone Acetate
GH	Growth Hormone
IGF-1	Insulin-like Growth Factor-1
BROMO	Bromocriptine
B+I	Bromocriptine and Estradiol Implant
CON	Control
IMP	Estradiol Implant

CHAPTER 1. INTRODUCTION

The most important pasture forage in the United States is the cool-season grass tall fescue (*Festuca arundinacea*) (Hoveland, 2009). Predominantly grown in the southeastern region of the country, tall fescue occupies 12 to 14 million ha and supports millions of cattle and other livestock (Buckner et al., 1979). Following the release of the Kentucky-31 cultivar, tall fescue experienced a meteoric rise in popularity due to its adaptability, persistence under heavy grazing, and extended growing season (Buckner et al., 1979). But even as tall fescue spread, it was gaining a reputation for causing poor animal performance despite appearing to be a nutritionally ideal forage for grazing. The multifaceted syndrome it caused was termed fescue toxicosis. Researchers discovered that grass infected with the endophytic fungi *Epichloë coenophiala*, which produces a variety of toxic ergot alkaloids, was responsible for fescue toxicosis; and that a mutualistic relationship between the fungus and tall fescue gave the plant its exceptional survivability (Bacon et al., 1977; Bacon and Seigel, 1988).

Fescue toxicosis is seen in cattle grazing fescue infected with high levels of endophyte, and the symptoms are generally categorized into three syndromes: fescue foot, fat necrosis, and summer slump. The most notable symptoms of affected animals include: elevated respiration rate and gangrene of the extremities, failure to shed the winter haircoat, heat intolerance, and reductions in weight gains, feed intake, reproductive efficiency, and milk production (Hoveland, 2009). This disease is the largest animal health related production cost in the grazing industry, and when considering its impact on all livestock species, the combined annual loss to the industry from fescue toxicosis likely exceeds $1 billion (Strickland et al., 2011).

Ergot alkaloids liberated from forage in the gastrointestinal tract are considered the causal agents of the animal disorders associated with tall fescue consumption. Their diverse biological effects are directly related to the structural similarities between ergot alkaloids and the biogenic amine neurotransmitters norepinephrine, dopamine, and serotonin (Berde and Stürmer, 1978). This allows alkaloids to bind to multiple receptors associated with these neurotransmitters, which are located throughout the body, and prevent them from performing correctly. For example, interference with serotonin and adrenergic receptors has been shown to cause vasoconstriction of bronchial, subcutaneous and peripheral blood vessels; this is believed to be the cause of the respiratory and circulatory issues that are common in affected animals (Oliver, 1997; Dyer, 1993). A classic indication of fescue toxicosis is the sharp reduction in serum prolactin, which is caused by activation of dopamine receptors by ergot alkaloids (Schillo et al., 1988).

Since cattle exposed to ergot alkaloids have such pronounced losses in weight gain, there have been many attempts to improve their performance. Use of steroidal implants in stocker cattle is one approach that has been shown to increase ADG in animals grazing infected pasture (Brazle and Coffey, 1991; Beconi et al., 1995; Aiken et al., 2006; Carter et al., 2010). Davenport et al. (1993) demonstrated that cattle grazing endophyte-infected pastures not only had lower rates of gain compared to controls, but also exhibited a slight decrease in IGF-1 responsiveness to estradiol-17β. IGF-1 is an important stimulator of the mTOR pathway, which in turn is responsible for protein synthesis and muscle gain in growing animals (Yoon, 2017). Ergot alkaloids and structurally similar chemicals (indoles) have been shown to inhibit the growth hormone

axis (Flückiger et al., 1978) and the mTOR pathway (Ahmad et al., 2013), respectively, in other species. Research investigating the effects of ergot alkaloids on net protein synthesis and the mTOR pathway in cattle is severely lacking. The objective of the current study was to determine if fescue-derived alkaloids decrease muscle protein synthesis though inhibitory action on the mTOR pathway via a direct effect on signal proteins, and if these negative effects can be alleviated by implantation with anabolic agents.

CHAPTER 2. LITERATURE REVIEW

Introduction

One of the most wide-spread and heavily utilized forages in the United States is the cool-season grass, tall fescue (*Festuca arundinacea*). To some extent, all sectors of the livestock and equine industries rely on tall fescue for grazing, but beef cow-calf operations became especially dependent on the grass after its widespread introduction to the country during the mid-20th century. The species appeared to be well-suited for livestock grazing because of its hardiness, nutritive qualities, and ease of cultivation, however, consumption of tall fescue quickly became associated with poor animal health and performance. The multifaceted syndrome, dubbed tall fescue toxicosis, can be costly to producers and has garnered attention from scientists for many years. Once it was recognized that the disease was caused by ergot alkaloids, which are present in most tall fescue through its relationship with the endophytic fungus *Epichloë coenophiala*, research began to focus on how alkaloid consumption altered body homeostasis in order to fully understand the underlying mechanisms of fescue toxicosis. With regard to their influence on essential metabolic pathways, such as protein expression, insulin signaling, and lipid metabolism, there is still much to be discovered and their effects are virtually unknown at the present time.

History of Tall Fescue and Fescue Toxicosis

Introduction and Selection in the United States

Even though tall fescue is the most important pasture grass in the United States, its introduction into the country is shrouded in speculation. The story likely begins with another native of Europe and close relative, meadow fescue, which was first introduced

into cultivation around 1820, but has long been recognized for its value as a forage plant (Smith, 1899). Almost all the meadow fescue seed planted in the US until the late 1880s was imported from England, tall fescue was likely a contaminant in that seed (Vinall, 1909). These accidental additions to meadow fescue pastures were quickly recognized for their ability to thrive even in conditions where meadow fescue began to wane. By 1900 it was proving its worth for pasture and mowing in grass trials in Kentucky (Garman, 1900) and Virginia (Kennedy, 1900); and was praised for its superior growth, height, competitive ability, and drought tolerance.

The popularity of tall fescue was not fully realized until the release of two cultivars in the 1940s, Alta and Kentucky 31 (KY-31). Starting in 1918, the ecotype Alta was selected for winter hardiness, persistence, and ability to remain green during the dry summers of western Oregon; and then released cooperatively by the Oregon Agricultural Experiment Station and the USDA (Cowan, 1956). Alta was planted throughout the Pacific Northwest and intermountain regions of the western United States. The ecotype, KY-31 was first collected in 1931 from a mountain pasture in Menifee County, Kentucky. The population had been under natural selection on the site when the property was purchased in 1875 and local farmers held it in high regard for pasture and erosion control (Fergus and Buckner, 1972). The Kentucky Agricultural Experiment Station conducted lengthy tests of the cultivar, and it was released in 1942. KY-31 was noted for its dependability, adaptability to a wide range of soils, and ability to provide grazing over much of the year (Buckner et al., 1979). With the release of KY-31, the popularity of tall fescue soon eclipsed that of other cool season perennial grasses, mostly because of its greater persistence in pastures compared to other species. These qualities can be

attributed to its course, deep root system which develops a thick sod; allowing it to grow on sites with moist, heavy soils, that are often waterlogged or flooded, making tall fescue particularly valuable for conservation purposes (Buckner et al., 1979). The previously barren and brown winter landscape of the southern United States was transformed during the 1940s and 1950s by the widespread planting of tall fescue for forage, soil conservation, roadside cover and turf (Hoveland, 2009).

During the 30 years following the release of Alta and KY-31, tall fescue cultivation rapidly spread in the United States, until it was the predominant cool-season perennial grass. In 1940 it grew on approximately 16,000 ha, over 1.6 million ha in 1956, and by 1973 it occupied an estimated 12 to 14 million ha; the species also spread from its original range in the transition zone and is now grown from Florida to Canada in a wide range of climatic conditions (Cowan, 1956; Buckner at al., 1979).

Tall Fescue Toxicity

With the widespread use of tall fescue for pasture came the rise of a conglomerate of health issues that were discovered to be a direct result of livestock consuming the grass. By the 1950s, fescue had gained a reputation for causing problems in animals that resulted in poor performance (Pratt and Haynes, 1950). This puzzled animal scientists since well-managed fescue was a high-quality forage when it came to crude protein, digestible dry matter, amino acid and mineral content, and should result in good animal performance (Bush and Buckner, 1973). The first health issue to be reported and associated with fescue pastures was the syndrome fescue foot. In the summer months, animals consuming large amounts of tall fescue struggle to dissipate heat, resulting in excessive panting and spending extended time in shade or in ponds; while in the winter,

animals cannot maintain peripheral body temperature, which can result in frost bite and subsequent loss of ears, tail switches, and hooves (Jacobson et al., 1963). This was first reported in Australia by Cunningham (1949) and then in New Zealand by Pulsford (1950), who said it had been known by stockman for many years. In the United States the first report came from Colorado in 1952; it had been recognized for about 30 years, but was confused with other conditions like foot rot, frozen feet, or ergot poisoning (Goodman, 1952).

The second syndrome, known as fat necrosis, occurs where high rates of nitrogen fertilizer are applied to tall fescue pasture; mainly from poultry litter or other manure. Hard fat accumulates in the abdominal cavity of cattle, which do not show symptoms until vital body processes are affected, most frequently digestive upset occurs when the necrotic tissue constricts the intestines (Bush et al., 1979). Fat necrosis may also interfere with the functions of the kidney and heart, as well as cause difficult births if the birth canal is surrounded (Edgson, 1952; Williams et al., 1969) This problem was first clinically diagnosed by Williams et al. (1969) in the United States in 1967.

The third syndrome, first described by Robbins in 1973 is commonly called summer slump, because it is exhibited especially during the summer, when temperatures are highest (Robbins, 1983). Symptoms are varied and include nervousness, elevated body temperature, intolerance to heat, failure to shed winter coat, depressed feed intake, poor weight gains and milk production, excess salivation, and reduced conception rates (Hoveland et al., 1983). The major economic losses to the United States beef industry associated with consumption of endophyte infected tall fescue can mostly be attributed to this disorder (Sleper and West, 1996). Poor performance has also been observed in other

livestock. Dairy cattle grazed on tall fescue have lower milk production than on other grasses (Seath et al., 1956) and mares have higher foal mortality rates and agalactia (Putnam et al., 1990).

Even though financial losses attributed to tall fescue are great, producers often do not recognize the problem, given that the signs can go undetected until they become severe, this makes fescue toxicosis the largest animal health related production cost in the grazing industry (Strickland et al., 2011). A survey in 1993 of state extension specialists where tall fescue is most common, showed that losses from reduced conception rates and weaning weights in beef cattle total $609 million annually (Hoveland, 1993). Beef stocker grazing on tall fescue is also unprofitable because of low daily gains, so it is rarely done, forcing producers to graze cattle on more expensive annual pastures. Losses to the horse industry from fescue toxicosis are exceedingly difficult to estimate, but nearly all the states reported reproduction issues. Compared to those of cattle, increased foal mortality may not result in as many deaths, but considering the price of horses, especially in the thoroughbred industry, these losses still hold a high monetary value on an individual basis. When regarded in today's dollars and considering the impact on the equine and small ruminant industries, the combined annual loss to the livestock industry from fescue toxicosis likely exceeds $1 billion (Strickland et al., 2011). The cost of this disease drove researchers to uncover the root of these syndromes.

Determining the Cause of Fescue Toxicity

During the 1950s-1970s, research erroneously concentrated on possible culprits such as external plant fungi, plant alkaloids, anions, and toxins produced in the rumen, this delayed any real breakthroughs in the cause of fescue toxicity (Bush et al., 1979).

Dissection of the limbs of cattle experiencing lameness while consuming tall fescue revealed that the toxic agent in the grass caused vasoconstriction and thrombosis of the arteries, which obstructed blood flow. This, plus low environmental temperatures were determined to cause the gangrene of the extremities associated with fescue foot (Jensen et al., 1956). Chemical tests isolated several alkaloids from toxic tall fescue that were similar to those found in the ergot fungus, *Claviceps purpurea*, but no ergot sclerotia from that fungus were detected by visual examination (Trethewie et al al., 1954; Maag and Tobiska, 1956). Discovering ergot alkaloids in fescue was very important, but these early reports were mostly ignored by scientists working on fescue toxicosis at the time. Until a suitable bioassay could be perfected, progress in isolating the toxic component was very slow. The syndrome was experimentally produced in cattle by Jacobson et al. (1963), using an extract chemically fractioned from toxic forage; this method allowed them to define the physiological effects of tall fescue consumption. They also demonstrated that poor circulation resulting in gangrene of the extremities was not caused by an abrupt halt in blood flow, since gangrene only became apparent after about ten days. Their findings clearly indicated that there was vasoconstriction, but the specific causes were still unknown.

Progress began in 1973, when J. D. Robbins, J. K. Porter, and C. W. Bacon focused their research on a toxic tall fescue pasture in northern Georgia. From that single pasture, this group of scientists was able to isolate several species of fungi, which served as the basis for a multitude of grass toxicity studies. Three species were isolated from this pasture, all belonging to the tribe Balansiae: *Balansia epichloe* (Weese) Diehl, *B. henningsiana* Moell, and *Myriogensopora atrementosa* (Berk. & Curt.) Diehl. Unlike

their saprophytic or pathogenic relatives, these species are unique because they are endophytic, and not virulent to their grass hosts. The species of *Balansia* primarily associate with warm season weed and rangeland grass species, such as bentgrass, bluestem, crowngrass, and others, which are commonly found in pastures of tall fescue and contribute to the toxicity of tall fescue during the summer months (Bacon, 1995).

The most important series of toxicological studies during this time were conducted primarily by J. K. Porter. He was able to demonstrate that these endophytic species of fungi were toxic and could potentially synthesize ergot alkaloids. Fungal spores were collected and allowed to germinate, then cultures were incubated in appropriate medium so alkaloids could be produced in vitro. Inocula prepared from these cultures, containing indole compounds, proved to be toxic to chicken embryos (Bacon et al., 1975). Chemical extraction isolated four clavine-type alkaloids from cultures of *B. epichloe*: chanoclavine (I), agroclavine, penniclavine and elymoclavine, which are commonly found in species of *Claviceps*. Chanoclavine (I) was also produced by *B. claviceps, B. henningsiana* and *B. strangulans*. The lysergic acid derivatives, ergonovine and ergonovinine, were isolated from *B. claviceps* and *B. epichloe* (Bacon et al., 1979; Porter et al., 1979a, 1979b); this was the first demonstration of these compounds in fungi outside the genus *Claviceps*. These findings suggested that ergot alkaloids produced by these species may be involved in cattle toxicity syndromes where species of *Claviceps* are absent.

After the discovery of endophytic *Balansia* species in warm season grasses and the demonstration that biosynthesis of ergot alkaloids was limited to the family Clavicipitaceae, tribe Balansiae, this group of researchers began searching for a species

of *Balansia* or similar endophyte in the toxic tall fescue in the pasture in north Georgia. A 2-yr observation of the pasture grazed by cattle suffering fescue toxicity symptoms, did not reveal a specific *Balansia* taxon in the grass, but they did isolate another fungal endophyte, which at the time was believed to be *Epichloë typhina* (Bacon et al., 1977). The cattle suffering toxicosis grazed pastures with infection rates of 100%, while pastures with cattle in good condition had infection rates of 0-50%. The identity of the tall fescue endophyte, which was symptomless and nonsporulating, was originally reported to be *E. typhina* based on the work of Sampson (1933). This was challenged later by Morgan-Jones and Gams (1982), who found the endophyte to be an anamorph of *E. typhina* and not identical. To classify the anamorphs of *Epichloë*, they created the section Albo-lanosa within the genus *Acremonium* and named the endophyte *A. coenophialum*. In 1996, a phylogenic examination of species in *Acremonium* determined that the anamorphic tall fescue species was classified inappropriately and proposed the monophyletic genus *Neotyphodium* (Glenn et al., 1996). The species was reclassified again in 2014, when a nomenclatural ruling called for *Epichloë* and its anamorphs in *Neotyphodium* to be placed into one genus (Leuchtmann et al., 2014). Thus the tall fescue endophyte is currently named *Epichloë coenophiala*. Further evidence from grazing trials confirmed that this fungal endophyte was the causal agent of fescue toxicosis.

A 3-year trial in central Alabama showed that steers in paddocks with 18% *E. coenophiala* infestation, had 51% higher average daily gains (ADG) than steers in paddocks with 80% infestation (Hoveland et al., 1980). These findings were confirmed by two more trials in the following years. One demonstrated again that steers grazing fescue with low infestation rates would have higher gains. In this case, steers in paddocks

with 5% infestation, had 66% greater ADG and 28% greater gain per acre than animals in paddocks with 94% infestation (Hoveland et al., 1983). Another produced similar results by feeding steers fungus-free and infested KY-31 hay and seed; the presence of the fungus in both the hay and the seed diets decreased daily gains and feed intake (Schmidt et al., 1982). Steers eating infested grass, hay, or seed, showed typical signs of toxicosis; elevated body temperature, rough coat that did not shed, excessive salivation, and nervousness; while steers on low-endophyte grass or fungus-free feed were in excellent condition, tolerated heat, and showed no signs of nervousness (Hoveland et al., 1983; Schmidt et al., 1982). The association of endophyte infection with tall fescue toxicosis in cattle (Hemken et al., 1981; Hurley et al., 1981), sheep (Stilham et al., 1982), and horses (Monroe et al., 1988; McCann et al., 1992), was proven by several others in the following years, further confirming that endophyte-infected tall fescue is toxic to livestock.

Even though ergot alkaloids were produced in culture from the tall fescue endophyte in 1979, the toxins were not detected in plants until 1985. Using tandem mass spectrometry, significant levels of ergopeptine alkaloids were detected in extracts from tall fescue pastures with recent histories of toxicity in cattle. Ergot alkaloids were present in all aboveground parts of the plant, with 10-50% being ergopeptines. Specifically, ergovaline, ergosine, ergonine, ergoptine, and ergocornine were reported, with the predominant type being ergovaline at 84-97% of the total ergopeptine fraction (Lyons et al., 1986). Small amounts of ergotamine have also been found in endophyte infected fescue (Yates et al., 1985). Historically, ergovaline has been recognized as the major ergopeptine alkaloid produced by the endophyte, and the primary cause of reduced plasma prolactin and vasoconstriction (Dyer, 1993). However, it is unwise to overlook

the other ergopeptine alkaloids and metabolites found in infected tall fescue. In the past it has been difficult to study differences between specific alkaloids due to the lack of commercially available ergovaline.

A survey of seed and pasture from all over the United States was conducted in 1987 to detect potentially damaging levels of endophyte. They found that about 95% of tall fescue pastures showed varying degrees of endophyte infection. This widespread occurrence was largely due to the overwhelming acceptance of KY-31 from its release in 1943 into the 1960s; this cultivar also had a higher level of infection than most of the others surveyed (Shelby & Dalrymple, 1987). The species was widely dispersed in the eastern part of the country and during this time the forage industry was becoming more aware of the losses sustained when cattle grazed on infected fescue. At the time it was thought that the solution was to remove the endophyte from tall fescue altogether.

Failure of Endophyte-Free Cultivars

As researchers studied grass endophytes, they realized they were sensitive to storage conditions. When tall fescue seed was exposed to hot, humid conditions during storage, the endophyte would die, but the seed remained viable. In the past, the high demand for KY-31 seed meant that most of what was harvested in the summer was sold and planted within months, so the seed was not exposed to conditions needed to kill the toxic endophyte, which facilitated its spread. Now with a way to remove the endophyte, new cultivars of fungus free and nontoxic tall fescue were developed, the first being AU Triumph, which was released in 1982 (Hoveland et al., 1982). Thorough testing showed excellent animal performance on pastures of AU Triumph and farmers readily replaced toxic pastures with endophyte-free seed. Unfortunately, grazing practices were not altered

for these E- pastures and within a few years, farmers noticed that unlike the famous KY-31, the new cultivar could not persist through drought and over grazing. Even pastures planted with other cultivars containing low levels of endophyte infestation (<25%) were outcompeted by pastures that were highly infected (90+%), during periods of stress. The loss of stand in the low-infested pastures indicated a competitive advantage for infected tall fescue (Read and Camp, 1986). Selection for more persistent endophyte free varieties was attempted but was unsuccessful, and it became apparent that agronomic performance was enhanced by a mutualistic relationship between tall fescue and the endophyte fungus. While endophytes had previously been considered parasitic, evidence that they conferred superior fitness to the host plant and that these benefits were multifaceted were piling up.

Symbiotic Relationship

Tall fescue research up to this point had mostly focused on the detrimental effects the endophyte had on livestock production, so the biological significance of the fungus was not fully understood. This was quite apparent after the premature release of the E- tall fescue cultivars and their subsequent failure in stressful environments. The ultrastructural association between grass and fungus, as well as the growth and survival benefits granted to infected grasses, suggested that a symbiotic relationship formed early in their respective evolutions (Bacon and Seigel, 1988). *E. coenophiala* has a relatively simple life cycle. The species is asexual, so the only route of dissemination is by maternal transmission through host embryos. Fungal hyphae colonize seeds in inflorescences early in development, and occupy the embryonic axis, including the shoot atypical meristem and the scutellum (Christensen and Voisey, 2009). The endophyte lies dormant in the seeds until the they are planted and germinate, or it dies in storage. Once germinated,

elongated hyphal filaments colonize the extracellular spaces of leaf sheaths, meristems, and internodes of growing stems, but not roots, and absorb nutrients directly through the hyphal wall (Christensen and Voisey, 2009). In this relationship, the fungus benefits by receiving nutrients, protection, and a means for reproduction and dissemination, while the host plant receives a variety of advantages in overall vigor along with protections against biotic attack. Several ecological and physiological processes in the fescue plant were found to be influenced by symbiosis with the endophyte; but a single mechanism by which the endophyte promotes host fitness is not apparent (Belesky and West, 2009).

Since its accidental introduction, tall fescue has been celebrated for stand persistence and tolerance of grazing during dry conditions, these are major benefits of endophyte infestation. The mechanisms enabling this ability are complex and not fully understood. During severe water deficit, endophyte infected plants showed enhanced tiller density and survival, which conferred population stability and proved advantageous in the following year (West et al., 1993). Greenhouse experiments showed that E+ plants were more productive than E- plants of the same cultivar at mild moisture stress (-0.05 MPa). During severe moisture stress (-0.5 MPa), 75% of the E- plants died, while all the E+ plants survived. Infected plants often showed greater shoot mass and tiller numbers than endophyte-free, and previously drought stressed infected plants also had much greater regrowth after harvest (Arachevaleta et al., 1989). This fitness helped tall fescue spread until it was the premier grass in the United States and prevail as a cool-season species in states south of its typical area of adaptation. The transition zone in the United States represents a progression, from north to south, of increasing summer water deficit caused by high evaporative demand and low soil water-holding capacity, such as the

rocky soils in the Ozark Highlands and southern Appalachian Mountains. The endophyte helps tall fescue compete and survive in this transition zone when growing conditions favor other plant species (Belesky and West, 2009).

Indirectly, the endophyte also enhances host persistence by deterring herbivory. The production of certain chemicals, including alkaloids, deters grazing animals, insects, and nematodes. This allows the plant to retain high leaf area and strong root systems, thus avoiding energy depletion and maintaining the ability to acquire water and nutrients in stressful conditions. Infected fescue gains resistance to herbivorous insects which would deplete the leaves (Clay et al., 1985) and nematodes which would destroy the root mass (West et al., 1988). These findings led to a greater understanding of the complex relationship between the endophyte and tall fescue and had monumental implications for cultivar development and pasture management.

Success of Nontoxic Endophyte Cultivars

In the late 1980s, Joe Bouton listened to a talk given by Gary Latch, a researcher from New Zealand, about endophytic fungi infection in tall fescue's close relative, perennial ryegrass. Afterwards, while Bouton and Latch discussed their research, Latch mentioned that he had identified endophytes in perennial ryegrass that did not produce alkaloids toxic to livestock, which were the cause of ryegrass staggers in sheep. What made this discovery unique was that while the ryegrass endophyte didn't produce the toxic alkaloid lolitrem B, it still produced peramine, a deterrent against the main insect pest of perennial ryegrass in New Zealand, the Argentine stem weevil (Latch, 1997). This was unlike the recently developed tall fescue that was endophyte-free, so could not produce any protective alkaloids, reducing its survivability and use as a pasture grass.

Bouton was able to visit New Zealand several years later in order to work with Latch and his associates; together they isolated a naturally occurring, nontoxic strain of endophyte. The result was the AR542 endophyte, which does not produce ergot alkaloids, now marketed as MaxQ in the United States (Bouton et al., 2002). The strategy was to kill the toxic endophyte in seed from the best available tall fescue cultivars, then reinfect seedlings with the non-ergot alkaloid producing endophyte, thus eliminating the toxic effects in livestock, while maintaining the symbiotic benefits between endophyte and grass. The main commercial "novel" product on the market is Jesup MaxQ, the result of reinfecting the tall fescue cultivar Jesup. This novel cultivar demonstrated stand survival equal to toxic E+ tall fescue, but decidedly better than E- fescue (Bouton et al., 2002). Jesup MaxQ also did not express the effects of ergot alkaloids on grazing animals, so had strikingly positive effects on animal performance, compared to E+ pasture (Bouton et al., 2002; Parish et al., 2003a; 2003b). To date, this cultivar has produced no animal toxicity problems. Currently, the most dependable method for eliminating animal losses from tall fescue toxicosis is replacing toxic tall fescue pastures with novel cultivars. Replacement of pastures is a substantial up-front cost, but improvement in animal production will generously repay the investment.

Ergot Alkaloids and Animal Toxicodynamics

Alkaloids in Medicine

Alkaloids are organic, N-containing, basic compounds, which are naturally produced by a variety of organisms, most often plants. Many plant-derived alkaloids are familiar, as they have important medical uses, such as morphine (analgesic), quinine (antipyretic/antimalarial), atropine (anticholinergic), and vincristine (antitumor). Others

are addictive like cocaine, heroin, nicotine, and caffeine; or very toxic like strychnine and coniine. The multitude of ergot alkaloids were named for the ergot fungus Claviceps purpurea, their first known producer and cause of several epidemics during the middle ages. Ergot alkaloids all contain a tetracyclic ergoline ring system or a biosynthetic precursor; enzymatic modification of the ergoline ring results in a plethora of bioactive natural products that can be used as pharmaceutical treatments for a variety of ailments (Gerhards et al., 2014). They have a strong affinity for serotonin, dopamine and adrenergic receptors because of structural similarities with neurotransmitters, so they can be potent drugs; for example, methylergometrine is used to stop bleeding after childbirth, ergotamine to treat migraines, and bromocriptine is used to treat Parkinson's disease (Gerhards et al., 2014). Those that have been isolated from grass infected with the endophyte *Epichloë coenophiala* are considered the causal agents of animal disorders associated with tall fescue consumption and include clavines, lysergic acid, simple lysergic acid amides, and ergopeptines (Bush and Fannin, 2009).

Synthesis and Structure

Biosynthesis of ergot alkaloids was first studied in Claviceps purpurea, the fungus responsible for ergotism and initial suspect for the cause of fescue toxicosis. The precursors of ergot alkaloids are tryptophan and the mevalonic acid derivative dimethylallyl pyrophosphate (DMAPP), which originates from acetate metabolism (Bush et al., 1997; Garner et al., 1993). These are converted to dimethylallyl-tryptophan (DMAT) by DMAT synthase, in what is probably the limiting step in ergot alkaloid formation (Bush et al., 1997). N-methylation and C-oxidation of DMAT leads to formation of a diene, which is then epoxidized, resulting in spontaneous cyclization of

the C-ring and release of an α-carboxyl group, yielding chanoclavine I; the first of the clavines (Schardl and Panaccione, 2005). The cyclization of the D-ring of chanoclavine I is catalyzed sequentially by chanoclavine I dehydrogenase, a flavine-dependent oxidoreductase, then a reductase, to produce the tetracyclic clavine alkaloid, agroclavine (Florea et al., 2017). Agroclavine is transformed into lysergic acid by the NADPH-dependent cytochrome P450, CloA, which is responsible for three rounds of 2-electron oxidations (Florea et al., 2017). The only difference between the clavines and the derivatives of lysergic acid are the isomerization of the double bond in the D ring of agroclavine from the 8, 9 to the 9, 10 position when the lysergic moiety is formed (Hill, 2005).

A variety of amide derivatives of lysergic acid have been characterized in endophyte-infected plants; the simplest being ergolines and the most complex being ergopeptines. Ergoline alkaloids have a simple chemical structure attached to the 8 carbon; lysergic acid has a carboxylic acid and lysergol has a methyl alcohol in that position, while others may include hydrazide, azide, or amide groups (Rutschmann and Stadler, 1978). Ergopeptine alkaloids are created when a tricyclic peptide ring is attached via a carbonyl at the 8 position in the D ring of lysergic acid (Hill, 2005). Three amino acids form a tricyclic peptide, proline always occupies position 3, while the amino acids in positions 1 and 2 will differ depending on the alkaloid formed (Bush and Fannin, 2009). Biosynthesis of ergopeptide lactams, the immediate precursors of ergopeptines, are catalyzed by the nonribosomal peptide synthetase (NRPS) enzyme, lysergyl peptide synthetase (LPS), which consists of two subunits (Florea et al., 2017). The subunit LpsB, has a single module that activates D-lysergic acid, and the subunit LpsA has three

modules that each specify a hydrophobic L-amino acid or an analog; once these four substituents are linked, they are released from the NRPS and the amino acids in position 2 and 3 are cyclized to form the piperizinedione ring (Florea et al., 2017). EasH, a dioxygenase, catalyzes the oxidative cyclization of the amino acid in position 1, forming the final cyclol ring of an ergopeptine (Florea et al., 2017). Condensation of D-lysergic acid with alanine, valine, and proline for example, forms ergovaline, which as mentioned previously, is the most prevalent ergopeptine in E+ tall fescue. The R_1 position can be occupied by alanine, valine or L-2-aminobutyric acid and the R_2 position can hold valine, isoleucine, leucine, or phenyalanine (Berde and Stürmer, 1978), in order to produce other ergopeptines in E+ fescue, which include ergotamine, ergosine, ergocornine, ergocristine, ergokryptine, ergonine (Yates et al., 1985).

Absorption

Alkaloids must overcome several protective mechanisms the body has in place before they cause disruption in the physiological systems of an animal. These natural barriers include mechanisms of influx and efflux, biotransformation, impediments during transport such as the blood-brain and placental barriers, and physical elimination from the body (Strickland et al., 2011). In vivo evidence of ergot alkaloid absorption has relied on analysis of excretions and intake estimates in the live animal. Studies using radiolabeled ergot alkaloids in isotopic experiments in rats, dogs, and monkeys, found that the route of excretion of the ergot alkaloids are dependent on the molecular weight and polarity of the compound (Eckert et al., 1978). Alkaloids with a molecular weight of less than 350 Da (lysergic acid and its amides) are excreted in the urine, those between 350 and 450 Da are excreted in urine and bile, and those above 450 Da (ergopeptines) are excreted in the bile

and eliminated through feces (Eckert et al., 1978). Samples of urine and bile taken from cattle grazing endophyte infested tall fescue demonstrated that 96% of the ergot alkaloids consumed will be excreted in the urine as lysergic acid derivatives and very little in the bile as ergopeptines (Stuedemann et al., 1998). The urine collected contained most of the ergot alkaloids consumed, suggesting that the excretory form of ergot alkaloids is either intact lysergic acid derivatives or biotransformed ergopeptine alkaloids. Ergopeptines are the major ergot alkaloid in tall fescue, not lysergic acid derivatives, so it is unlikely that the concentration in the forage is high enough to account for the differences between urinary and biliary excretion patterns (Stuedemann et al., 1998). Recovery of ergovaline was determined using samples taken from the abomasum and feces of sheep fed E+ and E- fescue seed; 47 to 63% was recovered in abomasal digesta, indicating a significant reduction in the foregut, but only 6 to 7% was recovered from the feces, indicating most of the remaining alkaloids were absorbed or metabolized in the intestines (Westendorf et al., 1993). Ergopeptines analyzed via HPLC in the feces of ergot-exposed dairy cattle showed that almost 24% of their ergopeptine intake was excreted in the feces (Schumann et al., 2009). A variety of factors could have contributed to the greater fecal recovery of ergopeptines by Schumann et al. (2009), such as digesta flow rates, rumen pH, and differing alkaloid profiles. These in vivo studies supported the conclusion that extensive absorption of ergot alkaloids from the gastrointestinal tract occurs in ruminants.

While many toxins and drugs are absorbed across the gastrointestinal epithelia through passive transport, the rate and magnitude of ergot alkaloid absorption is greatly affected by their solubility within the digestive tract and the extent of ionization, which in turn determines the partitioning of alkaloids between water and lipid phases (Eckert et al.,

1978). Most of the ergot alkaloids are weak bases and possess both polar and nonpolar components, so their water/lipid partitioning and absorption are affected by the pH of the surrounding environment (Strickland et al., 2011). Since ergopeptines are charged at low pH, it's assumed that they would not be absorbed in the acidic environment of the abomasum and gastric stomach, meaning absorption is confined to the forestomach, small intestine, and large intestine in ruminants, and the small intestine and large intestine in nonruminants (Strickland et al., 2011). The rumen of a forage fed animal is functionally more like the intestine than the gastric stomach. Ruminal pH is near neutral and the compartment lacks a mucosal layer, which is necessary in the gastric stomach to protect the lining from digesta. This permits nutrient transport across the epithelium of the rumen and the small intestine, making them the likely site of alkaloid absorption.

Measuring absorption across the epithelia has proven difficult and has not been done directly in vivo, but in vitro studies have provided indirect evidence to support this theory. Ruminal, reticular, and omasal tissue from endophyte naïve sheep were used in parabiotic chambers by Hill et al. (2001) to determine absorption potential for several ergot alkaloids across forestomach tissue. Equimolar proportions of ergot alkaloids were added to the mucosal side of the tissues and the amount of alkaloid that appeared over a 4 h period on the serosal side was measured. The rumen appeared capable of transporting approximately 25% more alkaloids than the omasum and approximately 600% more than the reticular tissues. The results also suggested that the ergoline alkaloids — lysergic acid, lysergol and ergonovine — were more likely to cross digestive barriers than ergopeptine alkaloids, specifically ergotamine and ergocryptine. Lysergic acid was transported at the highest rate in all tissues. This movement appeared to be through active

transport because alkaloid transportation was reduced in the presence of sodium azide, which killed the tissue (Hill et al., 2001). These findings were supported by Ayers et al. (2009) who again demonstrated that the primary alkaloid transported across forestomach tissues is lysergic acid and additionally found that ergovaline was not transported across ruminal or omasal tissues. This suggested that the small intestine may be the most important site for ergopeptine absorption in ruminants. However, studies examining the role of the ruminant hindgut in alkaloid degradation and absorption have not been conducted, making it difficult to definitively determine the path of ergot alkaloids through the body.

Three potential routes were proposed by Klotz and Nicol (2016) for intact ergopeptines to take once they reach the small intestine. The first is that they go unabsorbed through the small intestine and continue to the large intestine, where they face possible metabolism by microbes in the hindgut but will eventually be excreted in the feces. The second is that ergopeptines are absorbed and transported via the mesenteric veins to the portal vein and the liver, where they undergo hepatic detoxification or excretion back into the intestines as bile. The alternative path is that ergopeptines are absorbed in the small intestine and transported via the lymphatic system, through the thoracic duct and into venous circulation at the subclavian vein; this would bypass first-pass detoxification by the liver and allow ergopeptines to be systemically distributed via arterial blood, before detoxification by the liver. Considering lipids are regularly absorbed from the small intestine through lacteals leading to the lymphatic system in the form of micelles, and ergopeptines are lipophilic in nature, this route of absorption is likely to occur in ruminants (Klotz and Nicol, 2016).

Metabolism

Ergot alkaloid metabolism in ruminants cannot be fully explained without considering the role played by the microorganisms responsible for pre-gastric fermentation of feedstuffs. The diverse microbial community of the rumen can catabolize substrates, like cellulose, and liberate compounds that are not otherwise accessible for mammalian digestion. Toxicants appear to be among those compounds. In vitro and in vivo fermentation studies have been used to elucidate the actions of microbes on fescue digestion and alkaloid metabolism in the rumen. When endophyte infested fescue was incubated with viable microbes in vitro, the concentration of soluble ergovaline decreased linearly as fermentation progressed, with only 5% remaining after 48 h of incubation (Moyer et al., 1993). The total amount of ergot alkaloids has been found to increase over time, with lysergic acid being the primary alkaloid in both in vitro and in vivo ruminal fluids. (Ayers et al., 2009). The digestion of ergovaline and production of lysergic acid has been investigated in vivo by De Lorme et al. (2007) as well. Sheep were fed endophyte infested hay then rumen fluid was sampled 0, 6, and 12 hours after feeding and analyzed for ergot alkaloids using HPLC; this was repeated on day 0, 3, and 28. Ruminal ergovaline concentrations increased from d 0 to 3 and d 3 to 28. It was not different before feeding to 6 hours but increased from 6 to 12 hours post feeding. Lysergic acid in the rumen fluid increased from day 0 to 3 but not between day 3 and 28; it also increased in the first 6 hours post feeding but not between 6 and 12 hours. Urine and fecal samples were also taken from these sheep and evaluated using HPLC. The results of this experiment supported those reported by Stuedemann et al. (1998). The amount of ergovaline excreted in the feces was less than what was consumed by the

lamb. Lysergic acid also appeared in the urine and the feces in greater amounts than what was in the feed. This indicated that lysergic acid was produced during digestion of fescue, most likely in the rumen, which previous research has shown is the likely place of absorption (De Lorme et al., 2007). These actions by microbes in the rumen increase the concentrations of ergot alkaloids available for absorption by the animal's gastrointestinal tract and subsequently increase the chance of intoxication from fescue consumption.

Alkaloids absorbed from the rumen and across the epithelium of the small intestine enter the blood stream, where they are quickly removed from circulation by the liver and eliminated from the body. Research into the rate of clearance of these compounds for livestock has been limited, but it has been demonstrated in sheep that ergovaline injected intravenously disappeared from the blood within 1 hour of administration (Jaussaud et al., 1998). It should also be noted that lysergic acid in particular, begins to appear in the urine of steers that have been moved from E- fescue pasture to E+ pasture within 24 hours, demonstrating the speed of alkaloid metabolism and elimination in ruminants (Stuedemann et al., 1998). Pharmacokinetic studies conducted in nonruminant species using bromocriptine have found that extensive first-pass biotransformation of this peptide occurred in the liver (Oliver, 1997). The liver possibly provides additional alteration and degradation of ergot alkaloids in ruminants but there is little information concerning that route of metabolism in livestock available. In light of this, it is assumed that biotransformation of ergot alkaloids in ruminants is similar to that reported in laboratory species and humans.

Primary oxidation of a wide variety of steroids, fatty acids, and prostaglandins, as well as drugs and environmental pollutants in the liver is catalyzed by the superfamily of

cytochrome P450 enzymes (Peyronneau et al., 1994). In humans, biotransformation of ergot alkaloids is generally mediated by the CYP3A subfamily of cytochrome P450, which consists of 3 genes: CYP3A4, CYP3A5, and CYP3A7 (Ball et al., 1992). CYP3A4 is the predominant P450 enzyme in the human liver and intestine and has a very active role in the metabolism of drugs and toxicants (Strickland et al., 2011). The ergopeptines, bromocriptine, ergocryptine, and dihydroergotamine, were found to strongly interact with rat liver microsomes by Peyronneau et al. (1994), as a result of the ergopeptines binding to a protein site close to the heme. They also demonstrated that the tripeptide portion of ergopeptines was essential for these alkaloids to be recognized and bound to CYP3A, and alkaloids that lack the moiety, such as lysergic acid derivatives, failed to bind with CYP3A. Ergotamine has been shown to undergo enzymatic modification by CYP3A in cattle microsomes as well, being converted to more hydrophilic metabolites called M1 and M2 (8-hydroxy derivatives), which then undergo a second hydroxylation to become M3 and M4 (8, 9-dihydroxy derivatives) (Moubarak and Rosenkrans, 2000). Little is known about these resulting metabolites, their effects on the body or the role they play in fescue toxicosis. Further research is needed to illuminate the process undergone by ergot alkaloids once they reach the liver.

Downstream Effects on Receptors

As previously mentioned, consumption of endophyte infected tall fescue and subsequent liberation of ergot alkaloids into an animal's gastrointestinal tract can cause a variety of physical effects down the line, such as fescue foot, summer slump, and fat necrosis. These diverse biological effects are directly related to the structural similarities between the tetracyclic ergoline ring of ergot alkaloids and the biogenic amine

neurotransmitters norepinephrine, dopamine, and serotonin (Berde and Stürmer, 1978). The biogenic amides are associated with transmembrane G protein-coupled receptors which are located throughout the body in varying populations depending on tissue type (Klotz, 2015). As ligands, ergot alkaloids can stimulate these receptors by acting as agonists or partial agonists or they can stifle them by acting as antagonists (Pertz and Eich, 1999). To add to the complexity of the issue, serotoninergic, dopaminergic and adrenergic receptors are not homogenous. At least 14 distinct subtypes of serotonin receptors, 5 subtypes of dopamine receptors, and at least 10 subtypes of adrenoceptors have been identified based on structural, transductional, operational, and functional differences (Pertz and Eich, 1999). Additionally, slight structural differences between alkaloids can vary their specificity to receptor systems and selectivity among the various subtypes (Klotz et al., 2010; Choudhary et al., 1995). The non-competitive antagonism created by ergot alkaloids binding to these receptors prevents them from performing correctly. Persistent binding of alkaloids results in receptor desensitization from prolonged stimulation, in turn prompting reduced signal transduction (Millan et al., 2008). Moreover, agonist occupancy has been shown to enhance the rate of receptor internalization (Tan et al., 2004) and excessive binding creates an opportunity for alkaloids to accumulate in tissues, which may be released during tissue turnover (Klotz, 2015). Continuous consumption of toxic fescue and sustained exposure to ergot alkaloids can have huge repercussions on biological processes if the rate of alkaloid accumulation on receptors exceeds the rate of receptor turnover. Animals exposed to ergot alkaloids exhibit a diverse and variable set of symptoms which depend on the type and location of

the receptors affected, the quantity and structure of the alkaloid, and any environmental stressors that accompany consumption.

Serotonin Receptors

Serotonin or 5-hydroxytryptamine (5-HT) receptors are located throughout the mammalian body in the central and peripheral nervous system, particularly in blood vessels, the gastrointestinal tract and on platelets (Oliver, 1997). They are responsible for modulating the release of many neurotransmitters and hormones, which influence biological processes like aggression, appetite, mood, nausea and thermoregulation. The first demonstration of ergot alkaloids directly acting on 5-HT receptors in bovine vasculature was from Dyer (1993), who exposed bovine uterine and umbilical arteries to ergovaline, and showed the alkaloid elicited a contraction of both arteries. Additionally, they significantly antagonized contractions to ergovaline using ketanserin, a 5-HT_2 receptor antagonist, confirming the contraction of vascular smooth muscle was associated with activation of 5-HT_2. Later reports showed that the response of lateral saphenous veins to 5-HT_{2A} and 5-HT_7 agonists were altered in cattle that grazed endophyte infected tall fescue (Klotz et al., 2012). A series of in vitro studies investigated the constrictive potency of selected ergot alkaloids. They found that ergovaline was the most potent alkaloid, followed by ergotamine with a very similar contractile dose response curve to ergovaline, then intermediate responses from ergocryptine, ergocristine, ergocornine, and ergonovine, and lastly the least potent was lysergic acid (Klotz et al., 2006; 2007; 2008; 2010). 5-HT_2 receptors have also been found in lung tissue and have been shown to be involved with pulmonary vasoconstriction and bronchoconstriction in cattle (Oliver, 1997). This may help explain the pronounced breathing difficulties experienced by cattle

on E+ pasture, especially during the summer when animals are exposed to high heat situations. While vasoconstriction has been the focus for much of the serotonergic receptor research, the effects of ergot alkaloids on the variety of 5-HT receptors in the body have not been fully examined. Increased serotonin is known to suppress appetite by acting on the hypothalamic satiety center (Rossi-Fanelli and Cangiano, 1991), and it is involved in the regulation of gastrointestinal motility (Talley, 1992). These are possible routes for ergot alkaloids to reduce feed intake and negatively affect motility and passage rate.

Adrenergic Receptors

Adrenergic receptors are located on blood vessels throughout the body and generally stimulate the sympathetic nervous system, which is responsible for the fight-or-flight response. When these receptors are triggered, heart rate and blood pressure increase, and blood flow is diverted from non-essential organs to skeletal muscle. Cattle receiving endophyte-infected fescue hay or seed have shown large variations in plasma concentrations of norepinephrine and epinephrine, which resulted in nervous and highly excitable animals (Schmidt et al., 1982). Disruption of adrenergic receptors occurs quickly after consumption of endophyte infected fescue and causes persistent vasoconstriction and dysfunction of blood vessels in the extremities. Heifers showed reduced blood flow and narrowing of the lumen of the caudal artery just four hours after eating E+ fescue (Aiken et al., 2007). This can result in thickening and damage to the vessel's endothelial lining, as well as edema and thrombosis (Vuong and Berry, 2002). Tissue necrosis or dry gangrene is a direct result and a severe consequence of endophyte consumption.

There are two groups of adrenoreceptors, α (subtypes: α_1 and α_2) and β (subtypes: β_1, β_2, and β_3). There is no evidence that ergot alkaloids either stimulate or block β adrenoreceptors (Salzmann and Bucher, 1978). Both α_1 and α_2 adrenergic receptors have been found on the dorsal pedal vein of cattle (Solomons et al., 1989). Lateral saphenous veins from cattle grazing E+ and E- pastures showed no difference in contractile response when exposed to phenylephrine, an α_1 receptor agonist, proving α_1 receptors are not affected by ergot alkaloid consumption. Conversely, when veins from E+ animals were exposed to BHT-920, an α_2 receptor agonist, they had enhanced reactivity and greater contractile response, demonstrating that the agonist effect of many ergot alkaloids is greater on α_2 receptors (Oliver et al., 1998). E+ fescue consumption has been found to decrease blood flow to core and peripheral tissues, including rib and leg skin, adrenal glands, cerebellum, duodenum, and colon (Rhodes et al., 1991). These types of changes were expected to be accompanied by increased blood flow to other areas but the blood flow to other tissues remained unchanged. This led to the conclusion that E+ fescue reduced the cardiac output in cattle (Rhodes et al., 1991). During continuous heat challenge, animals consuming E+ fescue experienced a shift in core body temperature from 38.8°C to 39.2°C; this was accompanied by increases in respiration rate, respiratory vaporization, skin vaporization, and skin temperature but not a significant change in heat production (Al-Haidary et al., 2001). Researchers believe that vasoconstriction mediated by α-adrenergic receptors in subcutaneous areas reduces evaporative heat loss from the skin in heat stress situations, thus causing the increased core temperature seen in cattle afflicted with fescue toxicosis. α_2 receptors are also present on blood platelets, and when they are stimulated by ergot alkaloids platelet production of thromboxane increases,

which in turn induces platelet aggregation and arterial constriction (Oliver, 1997). This combined with bronchial constriction caused by alkaloid influence on serotonergic receptors, results in reduced blood O_2 saturation and tissue oxygenation. This can explain the increased respiration rate seen by Al-Haidary et al. (2001).

Dopamine Receptors

The five subtypes of dopamine receptors are divided into D1-like (D1 and D5) and D2-like (D2, D3, and D4) subfamilies. D1 and D2 receptor subtypes are located in the neostriatum of the brain, where dopamine is important for motor function. D2 receptors are also found at high levels in the pituitary gland. D3 receptors are in the limbic regions of the brain, where they control aspects of behavior, emotion, motivation, and cognition. D4 receptors are sometimes found in the brain but are more common in the cardiovascular system (Pertz and Eich, 1999). Ergot alkaloids are agonistic to D2 receptors, which results in a significant reduction in both circulating and releasable prolactin from the anterior pituitary, a classic indication of fescue toxicosis (Schillo et al., 1988). Prolactin is essential for regulating metabolism, the immune system, and pancreatic development, as well as regulating milk synthesis and secretion. Decreased milk production in cows and ewes, and agalactia in mares grazing E+ pasture is a result of the decline in circulating prolactin (Freeman et al., 2000). At the onset of spring, longer days typically elevate prolactin levels to initiate shedding of the winter hair coat, but animals suffering from ergot alkaloid poisoning have prolactin levels that are too low to trigger shedding. These animals will maintain a shaggy coat, even in the heat of summer, which further exacerbates heat stress and elevates core body temperature triggered by alkaloid-induced vasoconstriction (Aiken et al., 2011). Researchers have

suggested that decreased prolactin levels are also involved in the reduced reproduction seen in seasonal breeding livestock species grazing E+ fescue pastures (Strickland et al., 2011). Intravenous administration of ergotamine to cattle has resulted in decreased plasma insulin concentrations and increased glucagon within 1 hour of dosing (Browning et al., 2000). The exact mechanism for these effects is still unknown. It has been shown that D2 receptors may be involved in pancreatic function and associated insulin regulation (Liang et al., 1998). Disruption of these dopamine receptors by agonists has resulted in decreased insulin response and glucose intolerance (García-Tornadú et al., 2010). More research in this area could provide the mechanism by which ergot alkaloids affect insulin secretion in cattle.

Solutions for Fescue Toxicosis

Consumption of endophyte infected tall fescue results in a multitude of physiological disruptions in livestock, leading to major losses in weight gains for stocker animals, which limits the usefulness of fescue pastures for producers. Several pasture management strategies have been developed to regulate alkaloid load and animal performance while grazing tall fescue. These methods include increasing biodiversity and favoring other grass species, removing infected tall fescue and replacing it with nontoxic endophyte cultivars, clipping seed heads before grazing, moving cattle off toxic fescue pastures during the summer, and feeding concentrates to improve weight gains during hotter months (Strickland et al., 2011; Aiken et al., 2006). Another approach to improve cattle performance is the use of steroidal implants, which have been shown to increase daily gains in animals grazing infected pasture (Brazle and Coffey, 1991; Beconi et al.,

1995; Aiken et al., 2006; Carter et al., 2010). These implants have been used for decades to enhance feed efficiency, rate of gain, and muscle gain in feedlot cattle.

Estrogenic and androgenic steroids are strong growth promoters through stimulation of growth hormone and IGF-1 secretion. Estradiol acts directly upon skeletal muscle through estrogen receptors (ER) as well as indirectly through stimulation of growth hormone secretion, GH-receptors in the liver, and IGF-1 secretion into the blood (Meyer, 2001; Dayton and White, 2014). Steers treated for as few as 7 days with a combined TBA and estradiol implant have shown increased IGF-1 mRNA levels in longissimus muscle compared to nonimplanted steers (Pampusch et al., 2008; Johnson et al. 1998b, White et al., 2003). The exact mechanism for implant-stimulated growth in beef cattle is uncertain, but studies indicate their effect on muscle satellite cells may play an important role (Johnson et al., 1998a; Kamanga-Sollo et al., 2008b; Pampusch et al., 2008).

Since the number of muscle fibers in cattle is fixed at birth, postnatal muscle growth results from hypertrophy of existing muscle fibers, this in turn requires an increase in myogenic satellite cells which fuse to the periphery of the muscle fiber (Moss and Leblond, 1971). Muscle samples taken from steers implanted with a combined trenbolone acetate and estradiol implant contained a higher number of satellite cells than samples taken from nonimplanted steers, suggesting that having increased numbers of satellite cells available to fuse with muscle fibers contributes significantly to steroid-induced muscle growth in meat-producing animals (Johnson et al., 1998a). Local production of IGF-1 in skeletal muscle is thought to play a role in supporting normal muscle growth and may be partially responsible for the increased number of satellite

cells, increased hypertrophy and increased muscle growth seen in animals treated with steroidal implants (Kamanga-Sollo et al., 2008). IGF-1 is also an important stimulator of the mTOR pathway, which is responsible for protein synthesis and muscle gain in growing animals (Yoon, 2017).

While research has shown that implants are a useful tool for producers that graze stocker cattle on tall fescue, none have investigated the effects of implantation on protein synthesis pathways in the muscle of cattle or how ergot alkaloids can interfere with these pathways. Intraperitoneal injections of ergot alkaloids in rats have been shown to inhibit the growth hormone axis (Flückiger et al., 1978) and structurally similar chemicals (indoles) have been shown to inhibit the mTOR pathway in cancer cells (Ahmad et al., 2013). It has also been demonstrated that cattle grazing endophyte-infected pastures not only had lower rates of gain compared to controls, but also exhibited a slight decrease in IGF-1 responsiveness to estradiol-17β (Davenport et al., 1993). It is currently unknown if ergot alkaloids can affect protein synthesis and the mTOR pathway or if these negative effects are alleviated by implantation with anabolic agents.

Introduction to mTOR

Eukaryotic cells use the mechanistic target of rapamycin (mTOR) signaling pathway to respond to environmental cues, such as growth factors and nutrients, as well as cellular energy levels and stress. This allows cells to regulate growth, metabolism, and maintain homeostasis in changing conditions. The mTOR pathway (Figure 2.2) occupies a central role in regulating protein synthesis, autophagy, and many other essential cell processes. Disruption of these processes caused by deregulated mTOR signaling, has been implicated in cancer, diabetes, and neurodegeneration (Saxton and Sabatini, 2017).

Development of targeted therapies to control these conditions and reverse mTOR disruption is a major focus of future research concerning the mTOR pathway.

The study of mTOR began with a Canadian expedition to the South Pacific Island of Rapa Nui (also known as Easter Island) in 1964. Scientists on this expedition hoped to identify natural products from plants and soil on the island that had therapeutic properties. In 1972, Suren Sehgal isolated a compound from the bacterium Streptomyces hygroscopicus, which was present in soil samples taken from the island. He reported that it possessed potent antifungal activity and named it rapamycin (Vézina et al., 1975). Other strains of S. hygroscopicus have also been used to produce several medically important chemical compounds and enzymes, including the anthelmintic milbemycin (a precursor of moxidectin), anti-cancer drugs - called indolocarbazoles, and numerous antibiotics. Further testing revealed that rapamycin also had immunosuppressive and cytostatic anti-cancer activity.

The target of rapamycin (TOR) protein was originally discovered in yeast in 1991 by Joe Heitman, Rao Movva, and Mike Hall, by using genetic screens for rapamycin resistance. Through this method, they were able to identify three target proteins for rapamycin. The first being the small FKBP12 protein, which serves as a cellular receptor for the drug, and together they form the FKBP12-rapamycin complex (Heitman et al., 1991). Mutants without FKBP12 were viable and completely resistant to rapamycin, proving that the protein was essential. They also uncovered the TOR1 and TOR2 proteins, which were demonstrated to be directly targeted by the FKBP12-rapamycin complex, hence the name - target of rapamycin (Heitman et al., 1991). A few years later, several groups described the direct target of the FKBP12-rapamycin complex in

mammals and sequence analysis found it to be a homolog of yeast TOR genes (Saxton and Sabatini, 2017). This protein was named the mechanistic (formerly “mammalian”) target of rapamycin, or mTOR.

The mTOR protein is a 298 kDa serine/threonine protein kinase that belongs to the PI3K-related kinase (PIKK) family; it forms the catalytic subunit of the protein complexes mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) (Saxton and Sabatini, 2017). mTORC1 has five components: mTOR, Raptor (regulatory protein associated with mTOR), mLST8 (mammalian lethal with Sec13 protein 8), PRAS40 (proline rich Akt substrate of 40 kDa), and DEPTOR (DEP domain containing mTOR interacting protein). Raptor and mLST8 promote mTOR function. Raptor is responsible for recruiting substrate to mTORC1 and keeping the complex correctly localized within the cell (Nojima et al., 2003), while mLST8 associates with the catalytic domain and may provide stabilization of the kinase activation loop (Yang et al., 2013). PRAS40 and DEPTOR inhibit mTOR function, possibly by directly inhibiting substrate binding (Laplante and Sabatini, 2009). mTORC2 has six components: mTOR, Rictor (rapamycin insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase interacting protein), Proctor1/2, DEPTOR, and mLST8. DEPTOR and mLST8 are believed to be the same in both complexes, Rictor likely has a similar function to Raptor, and mSIN1 and Proctor1/2 are considered regulatory subunits. The FKBP12-rapamycin complex directly inhibits mTORC1, but mTORC2 is notably insensitive to short term treatment. Long term exposure will eventually terminate mTORC2 signaling, not by directly inhibiting or binding to mTORC2, but likely by binding to solitary mTOR, preventing its incorporation into new mTORC2 complexes (Lamming et al., 2012).

Upstream of mTORC1

Activation of mTORC1 (Figure 2.2) should only occur after feeding, when sufficient energy and chemical building blocks are present, making increased anabolism possible. Through endocrine and other chemical signals, mTORC1 stimulates growth and energy storage in tissues, such as the liver and skeletal muscle, but is inhibited when resources are limited during fasting (Saxton and Sabatini, 2017). Mechanisms for upstream signaling of mTORC1 include growth factors, amino acids, energy status, oxygen levels, and DNA damage. An important regulator of mTORC1 activity is the heterotrimeric complex TSC (tuberous sclerosis complex), comprised of TSC1, TSC2, and TBC1D7 (Dibble et al., 2012). The complex functions as a GTPase-activating protein (GAP) for the GTP-binding protein Rheb (Ras homolog enriched in brain), which directly stimulates mTORC1 activity when activated (Sancak et al., 2007). TSC downregulates mTORC1 by converting Rheb to its inactive, GDP-bound state. Excessive inactivation of TSC1/2 is linked with the presence of numerous benign tumors, composed of disorganized and enlarged cells; this disease is known as tuberous sclerosis.

Growth Factors

Multiple growth factor pathways cause phosphorylation and inactivation of TSC. The insulin/insulin-like growth factor (IGF) pathway does so through multisite phosphorylation from protein kinase B/Akt, which causes TSC2 to dissociate from the lysosomal membrane and limits its contact with Rheb (Menon et al., 2014). This pathway is activated when insulin binds to receptors on the cell surface; this promotes the tyrosine kinase activity of the insulin receptor, the recruitment of IRS1 (insulin receptor substrate 1), the activation of PI3K (phosphoinositide 3-kinase), the production of PtdIns(3,4,5)P3,

and the recruitment and activation of Akt at the plasma membrane. Receptor tyrosine kinase-dependent Ras signaling also acts on TSC2 through the MAP kinase, ERK1/2 (extracellular regulated kinase 1/2) and its effector, p90RSK1 (ribosomal S6 kinase 1), but it's unclear how this pathway inhibits GAP activity (Laplante and Sabatini, 2009). Exactly how these signals are coordinated by the TSC complex and their corresponding impact on mTORC1 in different situations has not been fully illustrated.

Energy Status and Oxygen Levels

Intracellular and extracellular stresses, such as low ATP levels and hypoxia, also elicit a response from mTORC1. When glucose is scarce, cellular energy is reduced (low ATP:ADP ratio) and the stress responsive regulator AMPK (AMP-activated protein kinase) is activated. Activated AMPK can directly inhibit mTORC1 by phosphorylating Raptor or indirectly inhibit it by phosphorylating and activating TSC2, which results in the previously mentioned downstream effects (Saxton and Sabatini, 2017). It has been suggested that mTORC1 can sense glucose deprivation through other mechanisms as well, because cells lacking AMPK still show mTOR pathway inhibition, through inhibition of the Rag GTPases (Kalender, et al., 2010). Oxygen deprivation also affects mTORC1 activity through multiple pathways. During mild hypoxia, ATP levels are reduced, which activates AMPK and inhibits mTORC1 in the same manner as glucose deprivation. This state also activates TSC2 through the induction of REDD1 (Regulated in DNA damage and development 1) (Brugarolas et al., 2004). In situations where growth factors are present, but oxygen levels are low, REDD1 disrupts the interaction between TSC2 and inhibitory 14-3-3 proteins, freeing TSC2 to then inhibit mTORC1 activity

because oxygen levels are not high enough to generate the cellular energy necessary for the pathway (DeYoung et al., 2008).

Amino Acids

Dietary amino acids play a vital role in protein synthesis by strongly stimulating the mTOR pathway, as well as providing energy and carbon for other metabolic processes. Recent advancements have shown that mTORC1 activation by amino acids is a complex pathway, tightly coupled to dietary amino acid concentrations, and independent of TSC1/2. Lysosomes are now considered active participants in the process of sensing amino acids in the cell, and mTORC1 uses them as a platform for activation. Rag GTPase proteins attached to the lysosomal membrane are stimulated by intercellular amino acids, which converts them to their active nucleotide-bound state, so they can bind to Raptor and move mTORC1 to the lysosomal surface, where Rheb is located. This relies on Rheb not being inactivated by the TSC1/2 complex, ensuring that mTORC1 is activated only when both nutrient and growth factor conditions are suitable.

Regulating mTORC1 through nutrients occupies a significant amount of protein space in the cell; so far 26 proteins have been identified in the nutrient sensing arm of the pathway and mutations of these proteins cause the mTORC1 hyperactivation seen in cancer and neurological diseases (Sabatini, 2017). Many multiprotein complexes respond to nutrients and regulate the nucleotide state of Rag GTPase proteins, which are the keystone of lysosomal and cytosolic amino acid sensing for mTORC1. SLC38A9 is an important regulator and is required for lysosomal arginine to activate mTORC1; it is also responsible for transporting amino acids and signaling their presence (Jung et al., 2015). The cytosolic signaling pathway to mTORC1 contains the key protein complexes

GATOR1 and GATOR2. GATOR1 is tethered to the surface of lysosomes by the KICSTOR complex and has an inhibitory effect on mTORC1 by acting as a GAP for RagA/B. GATOR2 interacts with the tethered GATOR1 and is a positive regulator of mTORC1 signaling (Bar-Peled et al., 2013). Sestrin2 is a direct leucine sensor that interacts with GATOR2 and works to inhibit it in the absence of leucine. When leucine is available and binds to Sestrin2, it dissociates from GATOR2, allowing it to block GATOR1's inhibitory effects on mTORC1 (Saxton and Sabatini, 2017). CASTOR1 (Cellular Arginine Sensor for mTORC1) acts on GATOR2 in a similar way, except it inhibits mTORC1 in the absence of arginine. Additional Rag-dependent and independent nutrient sensing pathways have also been described, namely those involving methionine and glutamine. How the mTORC1 pathway detects other nutrients has yet to be discovered.

Upstream of mTORC2

Unlike mTORC1, relatively little was known about the upstream signaling and cellular functions of mTOR Complex 2 until recently, and there is still much to be discovered. While it is insensitive to nutrients, growth factors such as insulin, insulin like growth factor (IGF), and hormones stimulate mTORC2 signaling through PI3K, using a signaling mechanism distinct from the pathways that activate mTORC1. The subunit mSIN1 in the mTORC2 complex is essential for insulin-dependent regulation of mTORC2 because it contains a phosphoinositide-binding PH domain. In the absence of insulin, this domain inhibits mTORC2 catalytic activity. Signaling by PI3K stimulates mTORC2 to associate with ribosomes, which in turn, serve as a scaffold to allow mTORC2 to phosphorylate substrates and ensure mTORC2 is in the proper location (Oh

and Jacinto, 2011). The mSIN1 subunit can be phosphorylated by Akt, which creates a positive-feedback loop, as partial activation of Akt encourages activation of mTORC2, which then phosphorylates and fully activates Akt (Yang et al., 2015)

Due to a negative feedback loop between mTORC1 and insulin/PI3K signaling, mTORC2 can also be regulated by mTORC1. This occurs when mTORC1 phosphorylates and activates Grb10 (growth factor receptor-bound protein 10), which negatively regulates insulin/IGF-1 receptor signaling upstream of mTORC2 and Akt (Yu et al., 2011). S6K1, a substrate of mTORC1, also stifles mTORC2 activation by degrading IRS1, this is further discussed in the following section.

Downstream of mTORC1

After upstream activation of the mTOR Complex 1 occurs, there are a multitude of downstream pathways that the complex controls in order to maintain cellular balance in response to environmental conditions. mTORC1 positively regulates anabolic processes necessary for growth and division, such as biosynthesis of proteins, lipids, and organelles, by limiting catabolic processes like autophagy (Saxton and Sabatini, 2017).

Protein Synthesis

Initiation of protein synthesis promotes ribosome biogenesis and mRNA translation; it is predominantly controlled through two key proteins that are directly phosphorylated by mTORC1, S6K1 (p70 ribosomal S6 kinase 1) and 4E-BP1 (eIF4E binding protein 1). S6K1 is phosphorylated on its hydrophobic motif site, Thr389, allowing further phosphorylation and activation by the enzyme PDK1 (3-phosphoinositide-dependent protein kinase-1). Once this occurs, S6K1 can then

phosphorylate and regulate several downstream substrates such as eIF4B (eukaryotic translation initiation factor 4B), which is a positive regulator of the 5′cap binding eIF4F complex, as well as eEF2K (eukaryotic elongation factor 2 kinase), and ribosomal protein S6, both of which subsequently promote translation initiation and elongation (Mao and Zhang, 2018; Saxton and Sabatini, 2017). PDCD4 (programmed cell death protein 4), a protein known to bind eIF4A, inhibit translation initiation, and act as a tumor suppressor, is also phosphorylated by S6K1, which promotes degradation of the protein and stops inhibition (Dorrello et al., 2006). Interaction between S6K1 and the enzyme SKAR (S6K1 Aly/REF-like substrate) increases the translation efficiency of spliced mRNA (Ma et al., 2008). S6K1 can also modify the level of input from growth factors, such as insulin and IGF-1 as part of an auto-regulatory pathway, called the S6K1-dependent negative feedback loop. Activated S6K1 directly phosphorylates and destabilizes IRS1, hindering its ability to associate with the insulin receptor, and desensitizing tissues to insulin (Manning, 2004). An increase in IRS1 phosphorylation suppresses mTORC2 activation and reduces Akt activity, which further affects downstream deactivation of TSC and FoxO, leading to inhibition of mTORC1 and disruption of mTORC2 activity, respectively. This regulatory pathway is of utmost importance when studying metabolic disorders characterized by insulin resistance, such as type 2 diabetes, where excess amino acid consumption and high fat diets cause mTORC1 to be chronically activated, and S6K1 to constantly shut down insulin response in certain cells (Manning, 2004).

The other downstream substrate of mTORC1 is 4E-BP1, the action of this protein is not related to S6K1. 4E-BP1 is responsible for inhibiting translation by binding eIF4E (eukaryotic translation initiation factor 4E) and preventing the assembly of the eIF4F

complex. The complex assembly is necessary for recruiting 40S ribosomal subunits to the 5’ end of mRNA. Multiple phosphorylations by mTORC1 cause 4E-BP1 to dissociate from eIF4E, allowing mRNA translation to occur (Saxton and Sabatini, 2017).

Lipid and Glucose Metabolism

Lipids are essential for construction of new membranes and cell expansion. De novo lipid synthesis in the cell is promoted by mTORC1 through SREBPs (sterol responsive element binding protein). These transcription factors control the expression of genes responsible for fatty acid and cholesterol biosynthesis. In the inactive state, SREBPs are attached to the membranes of the nuclear envelope and endoplasmic reticulum. When sterol levels are low in a cell, SREBPs are cleaved and translocated to the nucleus, where they bind to specific DNA sequences and upregulate the synthesis of sterol synthesizing enzymes. High sterol levels inhibit SREBP cleavage and reduce synthesis through a negative feedback loop. SREBP can also be activated independently by mTORC1 through S6K1, which mediates activation of transcription factors (Düvel et al., 2010). Another substrate, Lipin1, is an inhibitor of SREBP, but can be phosphorylated and inactivated by mTORC1, positively affecting SRBEP activity (Peterson et al, 2011). Activation of SREBP in this manner leads to increased flux through the oxidative pentose phosphate pathway, which uses carbons from glucose to generate NADPH and other metabolites needed for growth and proliferation. mTORC1 also encourages a shift in glucose metabolism from oxidative phosphorylation to glycolysis, this promotes growth through the incorporation of nutrients into new biomass. The shift occurs because mTORC1 activation stimulates increased translation of the transcription factor HIF1α (hypoxia-inducible factor-1α), which in turn facilitates the

expression of several glycolytic enzymes such as PFK (phospho-fructo kinase) (Düvel et al., 2010).

Regulation of Protein Turnover

The mTORC1 pathway also works to suppress protein catabolism, particularly autophagy, as another method for promoting cell growth. An early autophagic step is the formation of autophagosomes, driven by the activation of the kinase ULK1, which forms a complex with ATG13, FIP2000, and ATG101. When sufficient nutrients are available, mTORC1 is active and phosphorylates ULK1, preventing its activation by AMPK, a key promoter of autophagy (Kim et al., 2011). Regulation of autophagy also occurs when mTORC1 phosphorylates and inhibits the nuclear translocation of TFEB, a transcription factor that promotes the expression of genes for lysosomal biogenesis and autophagic machinery (Saxton and Sabatini, 2017).

Protein turnover is also controlled by the ubiquitin-proteasome system (UPS). The UPS is a major intercellular protein degradation system that selectively targets proteins for 20S proteasome degradation by covalent modification with ubiquitin. Contradicting results from recent studies have demonstrated that the exact mechanism used by mTORC1 to regulate UPS has yet to be defined. Two studies found that acute mTORC1 inhibition expediates proteasome-dependent proteolysis, either by an increase in protein ubiquitylation or an increased number of proteasomal chaperones, through inhibition of Erk5 (Rousseau and Bertolotti, 2016; Zhao et al., 2015). Yet a study done a couple years earlier, demonstrated that genetic hyper-activation of mTORC1 elevated expression of proteasome subunits and increased proteasome activity (Zhang et al., 2014). Further research is needed to illuminate the regulation of this system, but it is possible that acute

mTORC1 inhibition promotes proteolysis to restore free amino acid pools, while chronic mTORC1 activation drives an increase in protein turnover to compensate for an increase in protein synthesis (Saxton and Sabatini, 2017).

Downstream of mTORC2

While some aspects of mTORC2 signaling remain elusive, it has been identified as a key regulator in various cellular processes such as cell survival, anabolism, proliferation, and cytoskeleton organization. mTORC2 phosphorylates several protein kinases from the AGC family, including Akt, SGK, PKA, PKG, and PKC. These include enzymes that regulate the actin cytoskeleton (PKCα), and various aspects of cytoskeletal remodeling and cell migration (PKCδ, PKCζ, PKCγ, and PKCε) (Saxton and Sabatini, 2017). It could be said that the most important role of mTORC2 is the phosphorylation and activation of Akt. Identifying Akt as a substrate of mTORC2 linked the mTORC2 protein to glucose homeostasis, diabetes, adipogenesis, and cancer. Full activation of Akt requires phosphorylation by PDK1 at Ser308 and mTORC2 at Ser473, respectively (Sarbassov et al., 2005). Active Akt promotes cell survival, proliferation, and growth by phosphorylating and inhibiting several substrates. These include the metabolic regulator GSK3β, the mTORC1 inhibitor TSC2, and the transcription factors FoxO1 (forkhead box protein O1) and FoxO3a, which control gene expression for stress resistance, metabolism, cell-cycle arrest and apoptosis (Laplante and Sabatini, 2009).

Physiological Roles of mTOR

Multicellular organisms face constant changes in energy availability because of periods of fasting or feeding. This pushed for the evolution of the ability to adjust whole body metabolism in order to maintain homeostasis and survivability. During periods of

fasting, available nutrients and circulating growth factors decline, requiring induction of a catabolic state to mobilize energy stores and maintain crucial body functions. Alternatively, during the fed-state there is an abundance of available nutrients, which requires a switch to anabolic growth and energy storage. Metabolic regulation in the organism relies on mTOR signaling to coordinate anabolic and catabolic processes, just as it does at the cellular level. Proper modulation of the mTOR pathway is necessary to avoid negative physiological outcomes and a variety of disease states are associated with poorly regulated mTOR, which prevent organisms from responding to changing environments.

Glucose Homeostasis

When an animal's blood glucose drops, the liver responds by promoting autophagy, gluconeogenesis, and the use of other energy sources such as ketone bodies. There is evidence suggesting the liver's response to dietary changes is dependent on regulation of mTORC1 signaling, and disruption of the pathway will also disrupt proper liver function. Mice lacking liver TSC1, a crucial down-regulator of mTORC1, suffer from perpetually activated mTORC1 signaling, which in turn suppresses the master transcriptional activator of ketogenic genes, PPARα (peroxisome proliferator activated receptor α), so the animal fails to generate ketone bodies (Sengupta et al., 2010). Autophagy in the liver during fasting is essential for supplying free amino acids for gluconeogenesis. Sustained mTORC1 activity during this period has been shown to prevent autophagy in the liver of mice, resulting in fatal hypoglycemia when fasted (Efeyan et al., 2013). Conversely, when blood glucose rises following a meal, and insulin is released from β-cells in the pancreas, hepatic mTORC2 typically stimulates glucose

metabolism via Akt signaling. Mice lacking Rictor in the liver - thus lacking sufficient mTORC2 expression and subsequent Akt phosphorylation - have impaired glycolysis and lipogenesis (Mao and Zhang, 2018). Additionally, deletion of hepatic Sestrin3, an upstream regulator of mTORC2 similar to Sestrin2, results in insulin resistance and glucose intolerance

Regulation of pancreatic β-cell development and function can also be affected by improper mTORC1 signaling. Mice without mTOR or Raptor in β-cells or pancreatic progenitor cells have reduced beta cell mass, abnormal postnatal islet development, hypoinsulinemia, and glucose intolerance (Mao and Zhang, 2018). Mice lacking β-cell TSC2 exhibit hyperactivation of mTORC1, which has a biphasic effect on β-cell function. This means that young TSC2 knock out mice show increased β-cell mass, higher insulin levels, and improved glucose tolerance; but the effect is reversed in older animals, which gradually lose β-cell mass due to increased apoptosis, in turn triggering hyperglycemia (Mori et al., 2009; Shigeyama et al., 2008). This demonstrates that high mTORC1 activity in the pancreas is beneficial at first, when β-cells expand in response to an increased glycemic load and produce more insulin, but eventually this will lead to exhaustion and a rapid decline in β-cell function over time. The progression of diet-induced (type 2) diabetes is similar to this biphasic effect. Obese or high fat diet treated mice show increased mTORC1 signaling in the pancreas and other tissues, likely due to increased levels of circulating insulin, amino acids, and proinflammatory cytokines (Khamzina et al., 2005), which also contributes to increased feedback inhibition of insulin/PI3K/Akt signaling, resulting in peripheral insulin resistance. Unlike mTORC1, mTORC2 activity declines in β-cells in the diabetic state.

Skeletal Muscle and Whole-Body Metabolism

A large portion of the total body lean mass is comprised of skeletal muscle, and this organ plays a multi-faceted role in regulating whole-body metabolism. Firstly, it is the main organ responsible for insulin-induced glucose uptake. Defects in this process, such as insulin resistance, result in the development of type 2 diabetes. Mice lacking muscle TSC1 are lean but also develop insulin resistance and glucose intolerance. Even when consuming a high fat diet, these mice have reduced glucose uptake in the muscle, as well as reduced glycogen and lipid deposition in the liver (Guridi et al., 2016). This occurs because these mice do not have the proper controls in place through TSC to negatively regulate mTORC1, so the pathway is constantly activated. This results in S6K1 activation, which directly phosphorylates IRS1 and causes its degradation. The consequence is interrupting PI3K-Akt activation, causing negative downstream effects on glucose uptake, glycogen accumulation and lipid deposition.

Autophagy in the skeletal muscle is also affected by mTORC1 activity. Constant activation of mTORC1 promotes short-term muscle growth, but eventually inhibits the body's ability to induce autophagy in the tissue. Without the ability to turn over old or damaged tissue, muscle growth is stunted, resulting in reduced muscle mass and muscle fiber size in older animals, leading to myopathy, low body mass, and early death (Castets et al, 2013). This suggests that optimal muscle health and function occurs when there are alternating high and low periods of mTORC1 activation, like what occurs with typical feeding and fasting cycles (Saxton and Sabatini, 2017).

Adipogenesis and Lipid Homeostasis

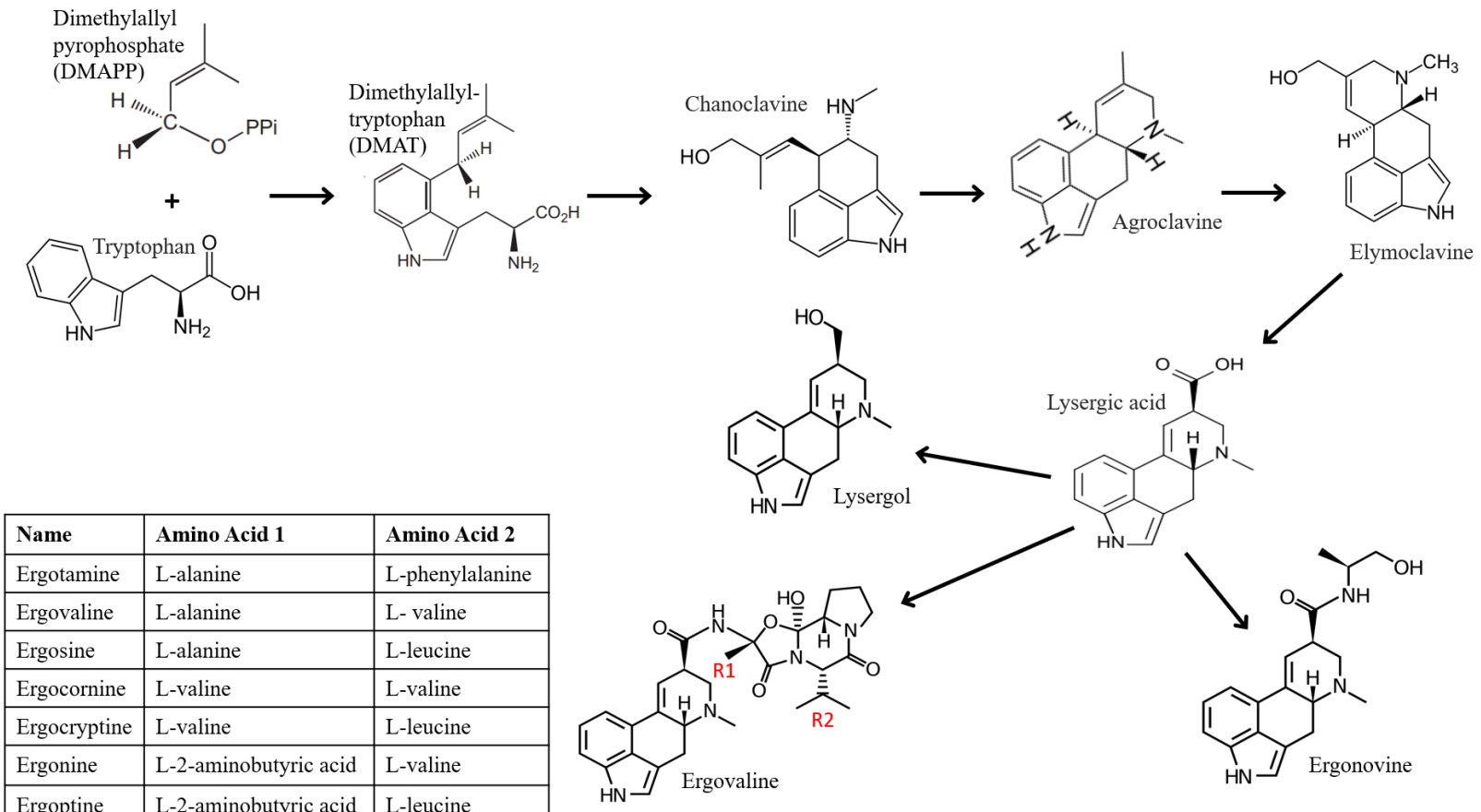
Another organ that contains a critical regulatory aspect of the mTOR pathway is adipose tissue. Development of obesity and insulin resistance occurs there, and mTOR is involved in multiple components of adipose tissue biology, including adipogenesis, maintenance of fat tissues, and lipid synthesis in response to feeding and insulin. When mice adipocytes do not have functional mTOR, they show reduced tissue mass, insulin resistance, and fatty liver, highlighting the important role of mTOR in the adipose and systemic energy metabolism (Shan et al., 2016). Deregulation of mTORC1 through deletion of TSC2 stimulates adipocyte differentiation but is countered by regulation of the translation of PPARγ by 4EBP1. Mice lacking S6K1 or Raptor in adipocytes will both remain lean and demonstrate resistance to weight gain on a high fat diet; this is due to a hindered ability to generate new adipocytes (Lamming and Sabatini, 2013).

Adipogenesis may also be regulated by mTORC1 through the regulation of SREBPs. While rapamycin treatment has been shown to impair nuclear accumulation of SREBPs, the exact regulatory mechanism is still uncertain, but S6K1 is thought to be involved. Another path may be through lipin1. mTORC1 phosphorylates lipin1, causing it to remain inactive in the cytoplasm and not move to the nucleus where it represses SREBP dependent gene transcription and upregulation of sterol synthesis (Lamming and Sabatini, 2013). Additionally, regulation of mTORC1 occurs indirectly through direct phosphorylation and mediation of Akt activity by mTORC2 in response to growth factors, making it an important facet of the pathway in adipose tissue. Loss of mTORC2 activity in adipocytes weakens Akt signaling to mTORC1, resulting in defective SREBP activation and lipogenesis, and ultimately insulin resistance, which is why mTORC2

signaling in adipocytes is of primary importance. Although further research is necessary to discern the finite molecular mechanisms of the mTOR pathway in adipose tissue, the overall importance of mTORC1 and mTORC2 to adipocyte function and lipid metabolism has been demonstrated.

Conclusion

The story of tall fescue is tightly woven with that of the endophyte *Epichloë coenophiala*. Modern grazing operations cannot ignore the benefits and drawbacks of the relationship. Novel endophyte cultivars have been developed to replace toxic tall fescue, but the reality remains, that a majority of pastures in the United States still produce toxic ergot alkaloids that cause biological issues for livestock. It is known that because of their structural similarities to neurotransmitters, alkaloids can bind to receptors throughout the body and cause a variety of physiological issues in livestock. Further investigation into the absorption and disposal of ergot alkaloids by the animal's body as well as their impact of on digestion and metabolic pathways are necessary to fully understand this disease. The objective of the current study was to determine if fescue-derived alkaloids have an inhibitory action on the mTOR pathway and decrease muscle protein synthesis by a direct effect on signal proteins.



Name	Amino Acid 1	Amino Acid 2
Ergotamine	L-alanine	L-phenylalanine
Ergovaline	L-alanine	L- valine
Ergosine	L-alanine	L-leucine
Ergocornine	L-valine	L-valine
Ergocryptine	L-valine	L-leucine
Ergonine	L-2-aminobutyric acid	L-valine
Ergoptine	L-2-aminobutyric acid	L-leucine

Figure 2.1 Ergot alkaloids produced by endophytic fungi of the genus *Epichloë* in infected plants

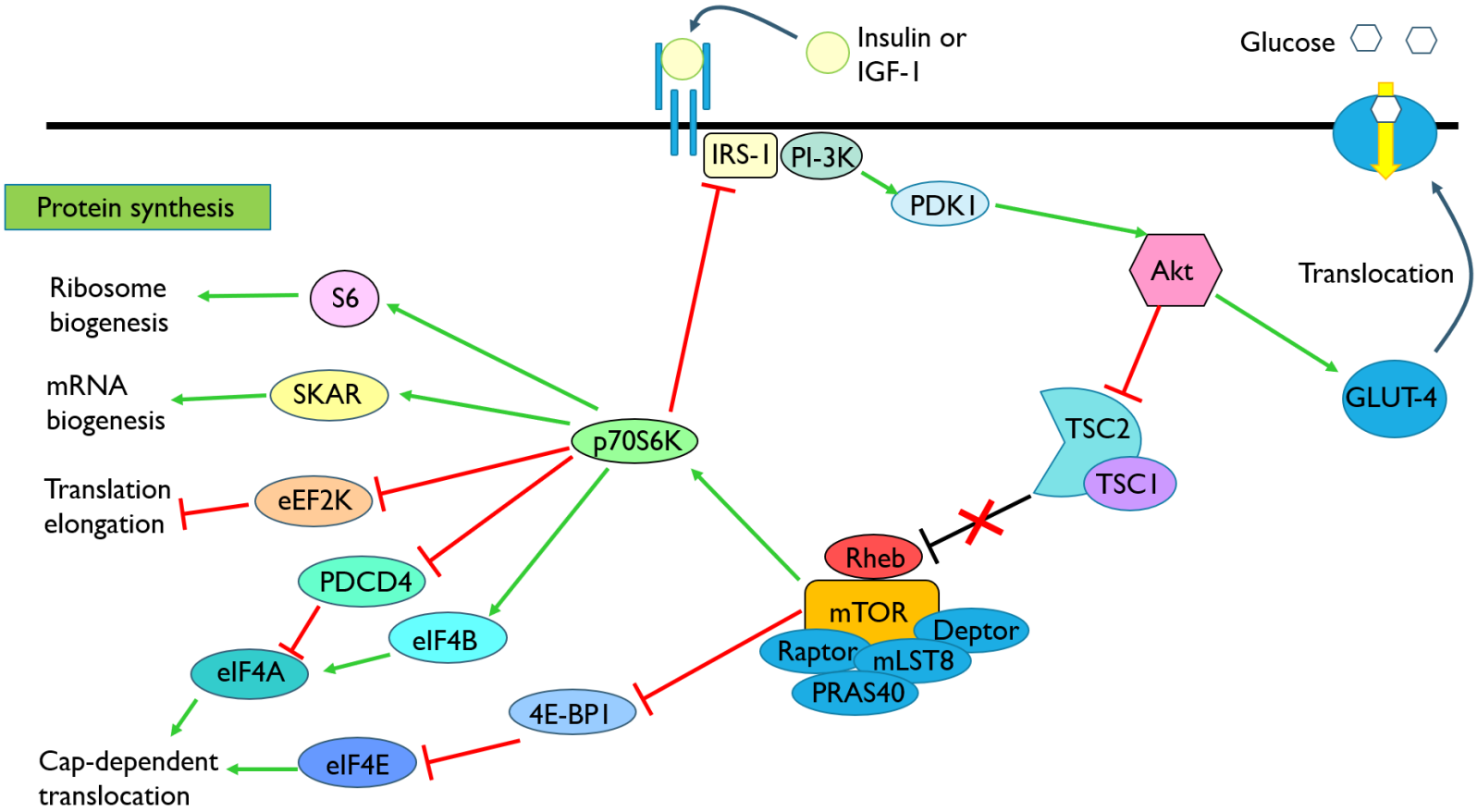


Figure 2.2 Signaling pathways contributing to muscle protein synthesis
This figure is a simplified overview of signaling molecules involved in protein synthesis. An arrow indicates activation, a perpendicular line indicates inhibition, and lines marked with a cross indicate that the action of this signaling molecule on its target is blocked under anabolic conditions.

CHAPTER 3. IMPACT OF ERGOT ALKALOID AND ESTRADIOL 17B ON WHOLE-BODY PROTEIN TURNOVER AND EXPRESSION OF MTOR PATHWAY PROTEINS IN MUSCLE OF CATTLE

Introduction

The mutualistic relationship between the grass *Festuca arundinacea* and the endophyte fungus *Epichloë coenophiala* creates an exemplary forage for livestock that has been planted on millions of acres throughout the United States. The endophyte is given nutrition, protection, and an avenue for reproduction, while the plant receives advantages in persistence, survivability, and protection against biotic attack (Belesky and West, 2009). One of these endophytic protections is the production of ergot alkaloids in the tissues of tall fescue. These compounds have been determined to cause the variety of symptoms present in animals affected by fescue toxicosis. The most prominent seen in cattle consuming endophyte infected fescue are reduced feed intake, lower weight gains, respiratory distress, vasoconstriction, and disruption of normal hormone levels (Hoveland, 2009).

Ergot alkaloids contain an ergoline ring structure, which are similar to structures present in the neurotransmitters serotonin, dopamine, norepinephrine and epinephrine, allowing them to bind to biogenic amine receptors and elicit negative effects (Strickland et al, 2011; Berde and Strumer, 1978). These biogenic amines regulate appetite, cardiovascular function, endocrine activity, gastrointestinal motility, muscle contraction, and thermoregulation in mammals (Berde and Strumer, 1978; Thompson and Stuedemann, 1993). The classic indication of fescue toxicosis is a significant reduction in circulating prolactin; this results from agonistic action on D2 dopamine receptors by ergot alkaloids (Schillo et al., 1988). Prolactin is essential for regulating metabolism, the

immune system, milk synthesis, milk secretion, and pancreatic development. Cattle suffering from fescue toxicosis have prolactin levels that are too low to trigger seasonal shedding; this may also cause seasonal breeding livestock species to have reduced reproductive efficiency (Aiken et al., 2011; Strickland et al., 2011).

In relation to nutrient metabolism, modified endocrine function and hormone secretion may also play a role in decreased livestock productivity. The synthetic ergot alkaloid bromocriptine is known to activate D2 receptors and block D1 receptors, as well as cause partial agonism of 5-HT_{2B} serotonin receptors and inhibition of 5-HT_{2A} receptors; resulting in reduced blood glucose and serum triglycerides, and decreased body weight (Kalra et al., 2011; Lopez Vicchi et al., 2016). Bromocriptine also directly activates the α_2 adrenergic receptors, inhibiting glucose-stimulated insulin secretion in pancreatic beta cells (Kalra et al., 2011; Lopez Vicchi et. al., 2016). The exact mechanism of action of ergot alkaloids at the pancreas is still not very clear. Intravenous administration of an ergotamine bolus to cattle has been shown to decrease plasma insulin concentrations and increase glucagon within 1 hour of dosing (Browning et al., 2000).

One attempt to improve the performance of stocker cattle grazing endophyte infected tall fescue is the use of steroidal implants, which have been shown to increase ADG in animals eating infected pasture (Brazle and Coffey, 1991; Beconi et al., 1995; Aiken et al., 2006; Carter et al., 2010). Androgenic and estrogenic steroids have been used for decades to enhance feed efficiency, rate of gain, and muscle gain in feedlot cattle. Combined treatment of cattle with estradiol and trenbolone acetate results in an increased number of muscle satellite cells, increased expression of IGF-1 mRNA in

muscle tissue, and increased levels of circulating IGF-1 (Dayton and White, 2014). It is currently believed that local production of IGF-1 in skeletal muscle plays a role in supporting normal muscle growth and that it is at least partially responsible for the increased number of satellite cells, increased hypertrophy and increased muscle growth observed in anabolic steroid-treated animals (Kamanga-Sollo et al., 2008b). However, Davenport et al. (1993) demonstrated that cattle grazing endophyte-infected pastures not only had lower rates of gain compared to controls, but also exhibited a slight decrease in IGF-1 responsiveness to estradiol-17β.

IGF-1 and insulin are also important stimulators of the mTOR pathway, which in turn is responsible for protein synthesis and muscle gain in growing animals (Yoon, 2017). Ergot alkaloids and structurally similar chemicals (indoles) have been shown to inhibit the growth hormone axis when given by intraperitoneal injection to rats (Flückiger et al., 1978) and the mTOR pathway in cancer cells (Ahmad et al., 2013), respectively. This led to the hypothesis that ergot alkaloids negatively affect the mTOR pathway and protein synthesis. The objective of the current study was to determine if fescue-derived alkaloids decrease muscle protein synthesis though inhibitory action on the mTOR pathway via a direct effect on signal proteins, and if these negative effects can be alleviated by implantation with anabolic agents.

Materials and Methods

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee. Experiments were conducted at the University of Kentucky C. Oran Little Research Center in the Intensive Research Building in Versailles, Kentucky.

Animal Management

32 Holstein steers (BW = 332.6 ± 54.6 kg) were used in a 6-week randomized complete block design experiment. Steers were housed indoors at thermo-neutral conditions (22°C) in individual 3 m x 3 m pens with ad libitum access to water. A 14 h:10 h, light:dark cycle was established with lights turning on at 0600 h and off at 2000 h each day. Animals were fed a corn silage-based diet (Table 3.1) that exceeded maintenance energy and protein requirements. Steers were also housed in individual 1.25 m x 2 m metabolism stalls for one week for quantitative collection of urine to measure whole-body protein dynamics.

Experimental Design and Procedures

Before the start of the experiment, steers were blocked by weight and randomly assigned within block to one of four treatments arranged in a 2 x 2 factorial structure. Treatment factors consisted of a synthetic ergot alkaloid or carrier and commercial implant or no implant. Bromocriptine mesylate (Cayman Chemical, Ann Arbor, MI) was used as the source of ergot alkaloid and was delivered via i.m. injection in an ethanol carrier (14.02 mg/mL in 95% ethanol; 0.1 mg/kg of BW; Baldwin et al., 2016; McLean et al., 2020). Bromocriptine was reconstituted as working stock in 95% ethanol and diluted with saline so no more than 40% ethanol was given in a single injection. Carrier injections consisted of 95% ethanol diluted with saline (40% and 60%, respectively). The commercial implant used was Revalor-S (Merck Animal Health, Kenilworth, NJ), which contains 120 mg of trenbolone acetate (TBA) and 24 mg of estradiol 17β. The four treatments were (1) a negative control, CON (n=8), no implant and injection with carrier only; (2) BROMO (n=8), no implant and injection with bromocriptine ; (3) IMP (n=8),

implant and injection with carrier only; (4) B+I (n=8), implant and injection with bromocriptine. Beginning with the heaviest animals, one block per week was brought into the barn, until capacity was reached on week 3 (blocks 1-3). After 37 days, the experimental period was complete and one block per week exited the barn, then was replaced with the next block until capacity was reached again (blocks 4-6). This patten was repeated when block 7 and 8 entered the barn. Animals were adapted to the individual pens for five days before the start of treatment. Treatment began on day 1. Animals assigned the estradiol implant were implanted at this time and animals assigned the ergot alkaloid received an injection of bromocriptine, with injections given every three days (day 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, and 34). Injection protocol was based on a previous study comparing the bromocriptine treatment response with the response observed in cows fed endophyte-infected fescue seed; where bromocriptine given at this dosage elicited the same gene expression response in 90% of identified differentially expressed genes in mammary tissue (n = 866) (Capuco et al., 2018).

Steers were weighed weekly to ensure injection volumes were calculated properly and feed intake was adjusted, except during the week that they were confined to tie-stalls. Blood samples were also obtained weekly from each steer via jugular venipuncture using an 18 ga needle for prolactin analysis. Samples were immediately placed on ice and centrifuged within 30 min at 2,800 × *g* for 20 min. Plasma was frozen in 3 aliquots to prevent multiple freeze-thaw cycles and stored at -20°C until analysis.

On day 27, prior to moving steers into the tie-stalls, each animal was fitted with a 14 ga jugular catheter. Before placement, the area was prepared by scrubbing with betadine, then a jugular catheter (MILA International, Inc., Florence, KY LA1415, 14 ga,

15 cm) was inserted and sutured in place. Then a sterile extension set was added and filled with heparinized saline to ensure patency. Antibiotic ointment was applied to the external insertion site and covered with gauze. The gauze and extension line were held in place with vet wrap and covered with a Velcro hernia belt/wrap. Jugular catheters were flushed on day 28, then covered until day 35 when they were checked for issues and replaced if necessary prior to muscle biopsies.

Measurement of Protein Turnover

On days 27 through 32, steers were moved to metabolism stalls for urine collection. Total urine output was collected by continuous suction using a rubber funnel system attached to the ventral portion of the abdomen, allowing urine to flow into a plastic collection vessel. Urine acidity was reduced to pH < 3 by adding 1 L of a 23.5% solution of H_3PO_4 to the collection vessel after each sampling to prevent ammonia N loss and microbial growth. In order to determine background enrichment of ^{15}N in urinary urea, steers were fitted with collection harnesses on day 27 and urine was collected for approximately 15 hours prior to isotope infusion; a representative aliquot was taken for later analysis. Whole-body protein turnover was determined using a single pulse dose of [^{15}N] glycine (98 atom percent excess; Cambridge Isotope Laboratories, Inc., Andover, MA) into the jugular vein given at 0730 h on day 28. The [^{15}N] glycine infusate was prepared by dissolving [^{15}N] glycine (3.0 mg/kg bodyweight; Duggleby and Waterlow, 2005) in physiological saline (50 mL) and sterilizing with Millipore filtration (0.45 uM m; Millipore, Bedford, MA) (Wessels et al., 1997). After isotope administration, urine was sampled every 12 hours, during which urine weight was recorded and representative aliquots (2% by weight) were taken at 12, 24, 36, 48, 60, 72, 84, and 96 hours after the

pulse dose. Urine samples were kept separate for each collection time and stored at 0° C until analysis. On day 32, after the last collection, steers were returned to individual pens.

To prepare samples for measuring ^{15}N enrichment of urinary urea, urine samples were first analyzed for urea concentration using a Technicon AutoAnalyzer II (SEAL Analytical Inc., Mequon, Wisconsin) (Marsh et al., 1965). This was used to determine the volume of urine needed for each sample to contain 100 μmoles of urea during ion exchange clean up. Polypropylene cation exchange columns (Bio-Rad, Richmond, CA), filled with AG 50W-X8 ion exchange resin (100-200 mesh, H+ form, Bio-Rad, Richmond, CA) were charged with NaOH and HCl, then used to isolate urea from ammonia. Urine samples were diluted with ddH_2O to reach the desired urea concentration, then transferred to the charged columns. The first 5 mL of filtrate was discarded, then 20 mL of ddH_2O was added to the column, and the eluent was collected into a scintillation vial. Vials were dried in a circulating air oven at 55°C for 3 days, then 2 mL 0.1 M phosphate buffer was added to each vial to solubilize the sample. 100 μL of reconstituted sample was then transferred to an Erlenmeyer flask containing 3 mL of phosphate buffer. A 5 x 25 mm strip of folded Whatman 934-AH filter paper with 30 μL of 2.5 M $KHSO_4$ was placed in a stopper cup and attached to each flask. Then 0.2 mL urease type III (100 unit/mL, Millipore Sigma, St. Louis, MO) was injected into the flasks in order to convert urea N to NH_3. Flasks shook for 1 h at room temperature. Subsequently, 0.2 mL 3 N NaOH was injected into the flasks to volatilize NH_3, and they shook again for 1 h at room temperature. After resting 24 hours, stopper units were placed in a desiccator with an open container of concentrated H_2SO_4 and allowed to dry for 24 hours. Then filter paper was enclosed in tin capsules and submitted for commercial

analysis of ^{15}N enrichment (UC Davis Stable Isotope Facility, Department of Plant Sciences, Davis, California) by automated mass spectrometry.

Calculation of Protein Turnover

The [^{15}N] glycine single dose urea end product method was used to measure whole-body protein turnover (Assimon and Stein, 1992; Wessels et al., 1997).

Total protein turnover was calculated (described previously by Waterlow et al., 1978) as:

$$Q = d/G$$

Where Q is protein turnover (g N/d), d is the rate of urea N excretion (g/d) in urine on day 28, 29, and 30, and G is the fractional recovery (%) in urinary urea of ^{15}N from [^{15}N] glycine. The ^{15}N enrichment of each urine sample, following the pulse dose, was corrected by subtracting background ^{15}N enrichment.

Whole-body protein synthesis was calculated as:

$$PS = Q - \text{N excretion}$$

Where PS is protein synthesis (g N/d), Q is protein turnover (g N/d), and N excretion is the amount of N excreted in urine.

Muscle Biopsies

Prior to muscle biopsies, on day 34, the area of the surgical site in the paralumbar fossa was prepared by clipping hair. On day 35, biopsy procedures were conducted in standing animals. The surgical site was aseptically scrubbed and anesthetized using a 19-gauge 1-1/2 inch needle to inject bupivacaine into the area using an "inverted L" block,

following the dorsal edge of the spinal processes from the 12th and 13th rib to a point slightly below the biopsy area. After blocking, the area was re-scrubbed to prepare for surgery. Muscle biopsies were taken immediately before and 60 minutes after activation of the mTOR pathway with a rapid IV infusion of 50% glucose (sterile) at a dose of 0.25 g/kg body weight (Mann et al. 2016). This dose of glucose was shown to increase muscle mTOR signaling in muRF1, Akt, 4E-BP1, and ERK 1/2 proteins in dairy cows transitioning from dry to in milk (Mann et al. 2016). A vertical incision (approx. 3 cm long) was made through the skin of the paralumbar fossa, then adipose layer and fascial layer were removed to expose the musculus obliquus externus abdominis. Using forceps and a scalpel, approximately 300 mg of muscle was removed and processed for protein analysis. The skin incision was closed using Supramid (B. Braun Medical Inc., Bethlehem, PA) and a cruciate suture pattern. Post-biopsy care included treatment with meloxicam (1 mg/kg body weight; Covetrus, Dublin, OH) for 2 days and antibiotic ointment over skin incision sites. Muscle tissue samples were processed for extraction and quantification of mTOR pathway proteins. Immediately following removal from the animal, muscle tissue was minced and weighed; an aliquot (approximately 100 mg) was added to 700 µLhomogenizing buffer (20 mM HEPES, 2 mM EGTA, 50 mM NaF, 100 mM KCL, 0.2 mM EDTA, 50 mM β-glycerophosphate) containing 14µL/mL of a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc., Waltham, MA), then processed using a Tissue-Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK). Homogenized sample was centrifuged at 10,000 x *g* for 10 minutes at 4° C. The resulting supernatant was removed and placed in three cryovials and stored at -80° C until further analysis. Any extra muscle tissue was flash frozen in liquid nitrogen and

stored at -80° C in a cryovial. Additionally, baseline blood samples (10 mL) were taken at 15 and 5 min before and 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min after completion of the intravenous glucose bolus infusion and processed as previously described, for subsequent determination of glucose and insulin concentrations. Following collection of the final blood sample, the catheter was removed, and animals were observed in individual pens for 48 h before being housed in outside pens and returned to the herd.

Western Immunoblot Analysis

Muscle samples were analyzed by western immunoblotting for the abundance and phosphorylation of proteins associated with the regulation of muscle protein synthesis (mTOR, p70 S6 Kinase, and 4E-BP1) based on previously described methods (Wagner and Urschel, 2012; Mastro et al., 2014). First, total protein content of sample homogenate was determined using a Bradford assay (Bradford Reagent; VWR International, Radnor, PA), then samples were diluted to 4 μg/μL in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA). SDS-PAGE was performed by loading 40 μg of total protein per sample, into gels containing different percentages of polyacrylamide (percentage noted below), made in laboratory (Laemmli, 1970). For each assay, all samples from one block were run at the same time in duplicate gels to eliminate interassay variation. Proteins were transferred to a 0.45 μm polyvinylidene deflouride membrane (Millipore Sigma, St. Louis, MO). Immediately after transfer, membranes were stained with Fast Green stain (0.2 g fast green, 50% methanol, 10% glacial acetic acid), which binds to all protein on the membrane, to check for success of protein transfer. A picture was taken of the stained membrane using an Azure c600 digital imager (Azure Biosystems, Dublin, CA), and this

image was later used to correct for loading errors. After imaging, Fast Green Stain was removed with destain (50% methanol, 10% glacial acetic acid) and buffer washes. Then membranes were blocked by incubation with 5% (w/v) fat-free milk dissolved in TBST for 1 hr at room temperature. After blocking solution was removed, membranes were probed with primary antibody for the phosphorylated form of each protein overnight at 4° C. All antibodies were validated in our lab for use with bovine muscle tissue (See Appendix A.1).

Quantification of mTOR. Protein immunoblot analysis was performed using a rabbit monoclonal antibody, diluted in 5% (w/v) BSA dissolved in TBST that recognizes site-specific phosphorylation of mTOR at Ser2448 or total mTOR (8% gel; 1:1000 dilution, catalog #5536 and #2983, respectively, Cell Signaling Technology, Danvers, MA). Antibodies were raised against human proteins. Band density of phosphorylated mTOR was normalized to band density of total mTOR.

Quantification of S6K. Protein immunoblot analysis was performed using a rabbit monoclonal antibody, diluted in 5% fat-free milk dissolved in TBST, that recognizes site-specific phosphorylation of S6K at Thr389 or total S6K (8% gel; 1:1000 dilution, catalog #9234 and #2708, respectively, Cell Signaling Technology, Danvers, MA). Antibodies were raised against human proteins. Band density of phosphorylated S6K was normalized to band density of total S6K.

Quantification of 4E-BP1. Protein immunoblot analysis was performed using a rabbit monoclonal antibody, diluted in 5% fat-free milk dissolved in TBST, that recognizes site-specific phosphorylation of 4E-BP1 at Thr37/46 or total 4E-BP1 (12% gel; 1:1000 dilution, catalog #2855 and #9644, respectively, Cell Signaling Technology, Danvers,

MA). Antibodies were raised against mouse proteins. Band density of phosphorylated 4E-BP1 was normalized to band density of total 4E-BP1.

After removing the primary antibody, membranes were washed and incubated with a goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (1:2000 dilution in 5% fat-free milk dissolved in TBST, catalog #7074, Cell Signaling Technology, Danvers, MA) for 1h at room temperature. Membranes were developed using a chemiluminescent kit (Amersham ECL Plus western blotting detection reagent, GE Healthcare Bio-Sciences, Pittsburgh, PA) and visualized using the Azure c600 imaging system (Azure Biosystems, Dublin, CA). Following image capture, membranes were stripped for 10 min with stripping buffer (2% SDS, 62.5 mM Tris-Base, 0.1 M β-mercaptoethanol), blocked with 5% fat-free milk blocking solution and re-probed with primary antibody for the total form of each protein.

Band densities of all blots and Fast Green stained membranes were quantified using densitometry software (AzureSpot Analysis Software, Azure Biosystems, Dublin, CA). To control for intra-assay variation, each sample band was normalized to the band density of a positive control (pooled bovine muscle homogenate samples), which was loaded onto each gel. To correct for possible gel loading errors, all band densities (phosphorylated and total protein abundance) were normalized to the value of total protein in each respective lane, determined by the densitometry software as “total lane volume” of the Fast Green stained membranes (See Appendix A.2). The band density of phosphorylated 4E-BP1, p70-S6K, and mTOR was then normalized to that of the total abundance of each respective protein. The calculated ratio of phosphorylated to total protein serves as a measure for activation status of these proteins, and relevant

differences in phosphorylated and total protein abundance will also be discussed. All obtained values are expressed as relative abundances in arbitrary units.

Plasma Analysis

Weekly blood samples were obtained from each steer via jugular venipuncture into vacutainer tubes with EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ). The samples were processed immediately and centrifuged at 2000 x g for 10 min at 4 °C, after this plasma was removed and aliquoted before being stored at -20 °C. Subsequently, plasma prolactin concentrations were measured in duplicate as described previously (Forrest et al., 1980) using reagents for bovine prolactin provided by the National Hormone and Peptide Program (Dr. A.F. Parlow, Harbor-UCLA Medical Center, Torrance, Ca). Intra- and inter-assay coefficients of variation were 5.8% and 8.3%, respectively.

Blood samples collected on the day of muscle biopsies were taken via jugular catheter 15 and 5 min before the animal received an intravenous glucose bolus and 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min after the glucose bolus infusion. All samples were collected with sodium heparin and centrifuged as soon as possible. Plasma was removed and aliquoted before storage at -20 °C. Plasma glucose concentrations were determined using a biochemical analyzer (Konelab 20XTi, Thermo Electron Corp., Waltham, MA). Intra- and inter-assay coefficients of variation were 1.1% and 3.6%, respectively.

Plasma insulin levels were assayed using a commercially available radioimmunoassay kit (Porcine insulin RIA, Millipore Sigma) which had previously been

used for analysis of bovine insulin and validated in our lab. All samples for each assay were run in duplicate and within the same day. Insulin intra-assay coefficient of variation for low and high quality controls was 2.2% and 3.13%, and inter-assay coefficient of variation for low and high quality controls was 4.65% and 4.66%, respectively.

Data Analysis

All data for dry matter intake, body weight, plasma prolactin, protein metabolism, plasma insulin and glucose, as well as baseline protein expression before glucose infusion and the change after infusion were analyzed as a randomized complete block design using mixed or glimmix procedures of SAS (version 9.4; SAS Institute, Cary, NC) with significance considered at $P < 0.05$ and statistical trends considered at $0.05 < P < 0.10$. Terms in the model included bromocriptine, implant, and the implant x bromocriptine interaction, in a 2x2 factorial structure, with block specified as a random effect. When fixed effects were significant, least square means were compared using Fisher's LSD procedures. Area under the response curve (AUC) was determined for prolactin, insulin and glucose using GraphPad Prism (v. 5.01, San Diego, CA). Area under the curve, time to peak and peak plasma concentrations were analyzed for insulin, glucose, and prolactin. The response of protein expression to glucose infusion determined by western immunoblot was analyzed as the difference between baseline (pre-infusion) values and post-infusion (60 min) values of each dependent variable. Additionally, post-infusion values were also analyzed using each animal's baseline values as covariates to determine if pre-infusion values affected post-infusion values and if this could be influenced by treatment. For covariate analysis, we first evaluated independent slope models by testing baseline values (pre) and interactions between baseline values and treatment as fixed

effects (pre, pre x bromo, pre x implant, and pre x bromo x implant) to determine which covariates were significant ($P < 0.05$). When interactions were not significant, interaction terms were dropped to evaluate common-slope covariate models. Insignificant covariates were dropped from the model. Graphs were created with GraphPad Prism (v. 5.01, San Diego, CA).

Model assumptions were assessed by evaluation of homogeneity of variances and normality of studentized residuals. For each data set, the homogeneous and heterogeneous models were compared for best fit using the BIC criterion. The model with the lowest BIC was preferred. Then, normality of the data was assessed by visual inspection of the distribution of studentized residuals, Q/Q plots, and a Shapiro-Wilk test ($P < 0.05$). Observations that disrupted normal distribution, with studentized residuals ≥ 3, were considered outliers and were removed from the datasets to obtain normal distribution. Four animal observations (two from CON, one from IMP, and one from B+I) were removed from the prolactin dataset based on distribution of residuals, all were over 3, and visual assessment of the data. One animal observation (BROMO) was removed from the weight gain dataset for d 8-15 based on a residual over 3 and weight gain that was unusually high from visual observation of the dataset. Seven animal observations (one from CON, two from BROMO, two from IMP, and two from B+I) were removed from the protein metabolism dataset based on urine collection errors and visual observation of the data. One more animal observation (B+I) was removed from the dataset based on residuals that were over 3 and irregularly high urea excretion and fractional recovery, which effected calculation of protein turnover and synthesis. Lastly, four animal observations were removed from the western immunoblot datasets based on

distribution of residuals. The following observations, with residuals over 3, were removed: one from activation status, pre-infusion mTOR (CON), one from phosphorylated, post-infusion mTOR (IMP), one from activation status, pre-infusion S6K (IMP), and one from total, post-infusion S6K (BROMO).

Results

Throughout the 35-day experiment, DMI was restricted to 1.5 times maintenance energy requirement. This was done to minimize potential differences in DMI across treatments and thus prevent the influence of DMI on the measured variables, while ensuring sufficient energy and nutrient intake to support muscle accretion. Accordingly, DMI was the same for all treatments ($P \geq 0.22$). The average BW at enrollment was 332.56 ± 54.55 kg and did not differ between treatment groups ($P \geq 0.29$). Weekly BW measurements were taken from day 1 at the start of treatment administration to day 22 (Table 3.1). There was a trend ($P = 0.07$) for implanted steers to have higher gains during the first week, but bromocriptine showed no influence ($P = 0.79$) on gain. Treatment differences were not significant until week two, during which weight gain was greater ($P = 0.01$) in implanted animals and less ($P = 0.04$) in bromocriptine treated animals. During the third week, implanted steers had higher ($P = 0.02$) gains, while those receiving bromocriptine showed no differences in gain ($P = 0.12$). Overall, total gain and ADG were higher in implanted steers ($P < .0001$), regardless of bromocriptine administration; and bromocriptine alone caused steers to gain less than control animals ($P = 0.01$). There were no treatment interactions ($P \geq 0.15$) associated with weight gain during this experiment.

Plasma Prolactin

Weekly plasma prolactin concentrations are shown in Figure 3.1 and area under the curve measuring prolactin concentration over time are shown in Table 3.1. Before treatment began, there were no differences in circulating prolactin between groups ($P \geq 0.24$) and there were no interactions. Steers that received bromocriptine injections experienced a sharp drop in prolactin after the first week of treatment (Day 8, $P = 0.04$) and maintained those depleted levels during week two (Day 15, $P = 0.001$) and three (Day 22, $P = 0.002$) (Figure 3.1). Consequently, prolactin AUC in steers treated with bromocriptine (1,803 ± 347) was significantly lower ($P = 0.002$) than the AUC of steers that were not treated with the alkaloid (3,278 ± 367). Prolactin concentrations were not different in steers treated with implant during the first 2 weeks of treatment (Day 8 & 15, $P \geq 0.45$) and there were no interactions (Day 8 & 15, $P \geq 0.34$). However, during the third week of treatment, implanted steers had significantly lower concentrations of prolactin (Day 22, $P = 0.02$) and there was a treatment interaction (Day 22, $P = 0.01$). During the fourth week of treatment circulating prolactin was not different between treatment groups (Day 29, $P \geq 0.53$) and there was no treatment interaction (Day 29, $P = 0.49$). Despite this, implant treatment had no effect ($P = 0.61$) on prolactin AUC and there were no treatment interactions ($P = 0.45$) associated with prolactin.

Protein Turnover

Whole-body protein metabolism determined by [^{15}N] glycine infusion and urine collection are shown in Table 3.2. Urinary excretion of urea (g/d) was lower ($P = 0.006$) in steers treated with estradiol implants. In contrast, bromocriptine had no effect ($P =$

0.13) on urea excretion and there was no treatment interaction (P = 0.87). The fractional recovery of labelled glycine from urine 48 hours after infusion, was lower (P = 0.03) in implanted steers, but no differences (P = 0.30) were seen in bromocriptine steers. There was no treatment interaction (P = 0.87). Protein turnover tended to be lower in steers given estradiol implants (131.7 ± 7.8 g N/d) than in those that did not receive an implant (141.9 ± 7.5 g N/d) (P = 0.10), but there was no effect (P = 0.43) of bromocriptine on turnover. However, this was not true regarding protein synthesis, since neither estradiol implants nor bromocriptine treatment were able to alter the amount of protein synthesized (P ≥ 0.47). Treatment interactions were not present for protein turnover or protein synthesis (P ≥ 0.52).

Plasma Glucose

Plasma glucose concentrations in response to an intravenous glucose bolus are shown in Figure 3.2. The maximum concentration or peak height (Table 3.3) of blood glucose occurred at 5 min post infusion and was not different between treatment groups (P ≥ 0.70) and there was no treatment interaction (P = 0.32). There was a trend (P = 0.09) for AUC to be higher in steers treated with bromocriptine (17,155 ± 687 mg/dL) in comparison to steers not treated with bromocriptine (16,206 ± 687 mg/dL). Total AUC was not affected by estradiol implants (P = 0.73) and there was no treatment interaction (P = 0.45).

Plasma Insulin

Plasma insulin concentrations in response to an intravenous glucose bolus are shown in Figure 3.3. The time to peak insulin concentration (Table 3.4) was between 15-

and 20-minutes post glucose infusion, and there was a trend (P = 0.09) for bromocriptine treated steers (18 ± 2 min) to take longer to reach peak insulin levels compared to steers that did not receive bromocriptine (15 ± 1 min). Time to peak was not affected by implant (P = 0.29) and there was no treatment interaction (P = 0.29). The maximum concentration (peak height) of insulin showed no differences (P ≥ 0.79) between treatment groups and exhibited a tendency (P = 0.07) for a treatment interaction. In the absence of bromocriptine, maximal concentration was lower for implanted steers, whereas in the presence of bromocriptine maximal concentration was higher for implanted steers. The insulin AUC of steers treated with bromocriptine (10,496 ± 818) was higher (P = 0.04) than that of untreated steers (8,052 ± 818). However, there was a tendency (P = 0.07) for a treatment interaction like that observed for peak height. In response to implant, insulin AUC was slightly decreased or unchanged in the absence of bromocriptine but was increased in the presence of bromocriptine.

Western Immunoblots

There was an overall effect of bromocriptine on total abundance of mTOR protein in the pre-infusion state (Table 3.5); steers that received bromocriptine had greater (P = 0.05) pre-glucose infusion levels of total mTOR than those that did not. Baseline levels were not affected by implant status (P = 0.55) and there was no treatment interaction (P = 0.14). Conversely, pre infusion levels of phosphorylated mTOR and the activation status (phosphorylated protein:total protein) were not affected (P ≥ 0.19) by treatments and there were no treatment interactions (P ≥ 0.12). Post-infusion values of total abundance of mTOR protein were not affected (P ≥ 0.20) by bromocriptine or implant treatment and there was no treatment interaction (P =0.76). There was a significant effect of

bromocriptine treatment on the post-glucose infusion phosphorylation of mTOR (P = 0.04) with steers receiving bromocriptine having a greater degree of phosphorylation, but following glucose administration, there was no significant effect of bromocriptine treatment on the activation status (ratio of phosphorylated to total) of mTOR (P = 0.38). The implant treatment did not affect (P ≥ 0.14) phosphorylated mTOR abundance, or activation status. There were no treatment interactions (P ≥ 0.42) for phosphorylated mTOR or activation status. Additionally, it was determined that pre glucose infusion levels of phosphorylated mTOR affected the amount of phosphorylated mTOR found post infusion, but only in animals treated with bromocriptine (P = 0.0012; pre infusion-by-bromocriptine interaction). For steers receiving bromocriptine, there was a positive relationship between pre- and post- infusion abundance; post-infusion abundance of phosphorylated mTOR increased with pre-infusion abundance (Figure 3.4). However, in the absence of bromocriptine, there was no relationship between pre- and post- infusion phosphorylated protein abundance. The pre covariate also affected (P = 0.01) post infusion values of total mTOR for all treatments; in this instance, as pre-infusion values increased, so did post-infusion values of total mTOR. There were no significant (P ≥ 0.96) covariates for activation status.

Total and phosphorylated protein abundance and activation status for S6K1 prior to glucose infusion was unaffected (P ≥ 0.12) by implant or bromocriptine treatment and there were no treatment interactions (P ≥ 0.12; Table 3.6). Similarly, post-infusion protein abundance of phosphorylated S6K1 and its activation status was unaffected (P ≥ 0.17) by treatment. Post-infusion abundance of total S6K1 was unaffected (P= 0.40) by implant, however, abundance was lower (P= 0.05) for the bromocriptine treated steers

compared with those not treated with bromocriptine. There were no ($P \geq 0.28$) post-infusion treatment interactions observed for S6K1. It was also determined that the pre covariate affected ($P < .0001$) total S6K1, so that animals treated with implants and bromocriptine had the highest pre-glucose infusion levels of total S6K1, but had the lowest abundance of post-infusion total S6K1. Animals not treated with implants and bromocriptine had low levels of total S6K1 pre-infusion and the highest levels of abundance post-glucose infusion. There were no significant covariates for activation status or phosphorylated S6K1 ($P \geq 0.17$).

Pre infusion levels of total and phosphorylated 4E-BP1 protein, as well as activation status, (Table 3.7) were not affected ($P \geq 0.30$) by bromocriptine or implant treatment. However, there was a tendency for implant to increase the abundance of total 4E-BP1 in the absence, but not in the presence of bromocriptine treatment (bromocriptine-by-implant interaction, $P = 0.06$); there were no other pre-infusion interactions ($P \geq 0.15$). Post-infusion abundance of total 4E-BP1 was lower in both the bromocriptine ($P = 0.005$) and implant ($P = 0.04$) treated animals compared to those that did not receive bromocriptine or implant, respectively. The lower total 4E-BP1 abundance for both implant and bromocriptine treatment, however, is largely explained by lower abundance for the implant treatment in the absence, but not in the presence of bromocriptine (bromocriptine-by-implant interaction, $P = 0.10$). The abundance of phosphorylated 4E-BP1 ($P = 0.02$) and activation status of 4E-BP1 ($P = 0.01$) were decreased with implant treatment, while bromocriptine had no effect on abundance ($P = 0.60$) or activation status ($P = 0.11$). There were also no treatment interactions ($P \geq 0.34$) for post-infusion phosphorylated 4E-BP1 and activation status. Additionally, it was

determined that pre glucose infusion levels of total 4E-BP1 affected ($P < .0001$) the amount of total 4E-BP1 found post infusion. Animals treated with implants and bromocriptine had the highest pre-infusion levels of total 4E-BP1 and the lowest abundance of post-infusion total 4E-BP1 protein; animals not treated with implants or bromocriptine had the lowest levels of total 4E-BP1 pre-infusion but did not demonstrate a change in protein abundance. In animals treated with bromocriptine ($P = 0.003$; pre infusion-by-bromocriptine interaction), the decline in post-infusion abundance of total 4E-BP1 was not as severe as that seen in implanted animals following glucose infusion (Figure 3.5). The pre covariate also affected ($P = 0.01$) post infusion values of phosphorylated 4E-BP1 for all treatments, in this instance, as pre-infusion values increased, post-infusion values of phosphorylated 4E-BP1 decreased. There were no significant ($P \geq 0.30$) covariates for activation status.

Discussion

The purpose of this study was to determine if fescue-derived alkaloids affect whole-body protein metabolism and decrease muscle protein synthesis by directly inhibiting the mTOR pathway via signaling proteins, and if so, if such negative effects can be alleviated by implantation with anabolic agents. Previous research has shown that cattle given an anabolic steroid implant while grazing endophyte infected fescue will have improved performance compared to cattle consuming infected pasture without an implant (Aiken et al., 2006; Beconi et al., 1995; Brazle and Coffey, 1991) — how this is achieved has not been determined. Steroidal implants are known to increase production of IGF-1 in skeletal muscle, which is at least partially responsible for the increased muscle growth and weight gain observed in implanted animals (Kamanga-Sollo et al.,

2008); this is possibly the source of improved performance in cattle affected by ergot alkaloids. IGF-1 and insulin are important stimulators of the mTOR pathway, which in turn is responsible for protein synthesis and muscle gain in growing animals (Yoon, 2017). Ergot alkaloids and structurally similar chemicals (indoles) have been shown to inhibit the growth hormone axis (Flückiger et al., 1978) and the mTOR pathway (Ahmad et al., 2013), respectively, in other species. This experiment is the first to demonstrate the effects of ergot alkaloids and estradiol implants on the mTOR pathway in the skeletal muscle of cattle.

Plasma Prolactin

Decreased circulating prolactin is one of the few measurable parameters available to diagnose fescue toxicosis (Schillo et al., 1988). Lactotrophs in the pituitary gland are responsible for the synthesis and secretion of prolactin; release of prolactin from these cells is controlled by D2-dopamine receptors and is inhibited by high levels of dopamine (Freeman et al., 2000). Ergot alkaloids are structurally similar to dopamine, allowing alkaloids to bind and activate these receptors and reduce circulating prolactin (Berde and Stürmer, 1978). Bromocriptine is a synthetic ergopeptine that also activates dopamine receptors in the pituitary, and therefore imitates the actions of naturally occurring ergot alkaloids on prolactin secretion (Fitzgerald and Cunningham, 1982; Freeman et al., 2000). A fescue toxicosis-like syndrome can be induced using bromocriptine injections and has previously been shown to decrease prolactin in steers and cows (McLean et al., 2020; Baldwin et al., 2016). It has also been demonstrated that 90% of differentially expressed genes (DEG) (n=866) in mammary tissue respond similarly in both bromocriptine treated cows and those fed fescue-derived alkaloids (Capuco et al., 2018);

indicating that bromocriptine provides a satisfactory model for studying physiological changes in cattle affected by fescue derived alkaloids. In the current study, while prolactin appeared to decrease over time with all treatments (the exception being the increase from d1 to d8 for CON), prolactin in steers treated with bromocriptine decreased very sharply on d8 and stayed at nadir levels throughout the study — resulting in an overall decrease in area under the curve (AUC) for bromocriptine treated steers. Estradiol has previously been shown to stimulate prolactin release in male rats (Seo et al., 1979) as well as increase prolactin levels in the serum and milk of dairy cattle (Tong et al., 2018). It is unclear why prolactin decreased throughout the course of the study in the steers not treated with bromocriptine. Light is an important regulator of prolactin secretion, with shorter photoperiods resulting in decreased prolactin (Freeman et al., 2000). In preparation for the experiment, animals were housed outside from April to July, when there are 12-14 hours of light per day, then during the experimental period they entered a facility with an established 14 h:10 h, light:dark cycle. Since there was no change, or even an increase in photoperiod length when animals moved inside, this effect of light on prolactin secretion cannot account for the decrease in circulating prolactin seen in animals not treated with bromocriptine. While there is no explanation for this decrease over the course of the experiment, the decrease seen with bromocriptine treatment was more severe, indicating that a fescue toxicosis-like syndrome was induced in the current experiment.

Dry Matter Intake and Weight Gain

The diet used in this study was designed to meet the recommended nutrient intake of growing steers. To prevent the results being confounded by dry matter intake, we

limited intake to 1.5x maintenance to ensure the daily feed allotment was consumed by all animals, regardless of any possible effects from treatments. This was especially important because ergot alkaloid consumption has been shown to decrease feed intake (Schmidt et al., 1982; Baldwin et al., 2016). The agonistic action of alkaloids on serotonin receptors is thought to affect satiety and gastrointestinal motility (Simansky, 1995; Talley, 1992). Although bromocriptine treated animals always consumed their daily meal, their ADG was lower than animals not treated with bromocriptine (bromocriptine steers gained 0.15 kg BW less per day than steers not treated). This demonstrates the decreased growth performance that is a common symptom of fescue toxicosis (Walker, 2009) and highlights that this impairment exists even in the absence of differences in DMI. Implants containing estradiol are commonly used to enhance steer performance and have been shown to improve the gains of cattle grazing endophyte infected pastures (Aiken et al., 2006; Beconi et al., 1995; Brazle and Coffey, 1991), but this is the first instance where the dry matter intake of animals was controlled. Implantation increased ADG in both the presence (B+I steers gained 0.19 kg BW more per day than CON) and absence (IMP steers gained 0.41 kg BW more per day than CON) of bromocriptine, demonstrating that estradiol implants can improve weight gain, even when intake is controlled. This shows that there were pronounced benefits of steroidal implantation on ADG, further demonstrating the value of implants use for growing cattle exposed to endophyte infected tall fescue.

Glucose Homeostasis

Thus far, research has primarily focused on the detrimental effects of ergot alkaloids on animal production and the beneficial growth promoting effects of hormonal

implants, but very little is known about the impact these chemical alterations have on glucose homeostasis in cattle. Bromocriptine has central and peripheral actions on the autonomic nervous system and the endocrine system in rats and humans (Kalra et al., 2011). Its effects on pancreatic β-cells, and therefore glucose homeostasis, have made it an important treatment option for type 2 diabetes in humans, as it improves glucose tolerance and insulin sensitivity (Kalra et al., 2011).

In the current study, an intravenous glucose tolerance test (IVGTT) was used to activate the mTOR pathway and assess the first phase of insulin release. The resulting insulin curve shows that bromocriptine tended to delay the rise in plasma insulin levels; Steers not treated with bromocriptine peaked at 15 min post glucose infusion, while B+I steers peaked at 16.25 min and BROMO peaked at 20 min post infusion. This delay with bromocriptine treatment cannot be attributed to a delay in glucose time to peak because time to peak for glucose was similar across all treatments.

Implanted steers tended to have increased insulin peak height in the presence of bromocriptine, but in the absence of bromocriptine there were no differences. This increase in maximal concentration, paired with decreased insulin clearance, resulted in a tendency for B+I steers to have an insulin AUC that was higher than either treatment alone (33.08% and 37.46% higher AUC than BROMO and IMP, respectively). Due to the lack of research concerning the effects of implantation combined with ergot alkaloids on glucose homeostasis, an explanation must be derived from our current understanding of the independent effects of steroidal implants and ergot alkaloids on animals.

The affinity of ergot alkaloids for the receptors of biogenic amines such as dopamine and norepinephrine in the pancreas may help explain the endocrine responses

seen in bromocriptine treated animals. Although research in this area is limited in cattle, ergot alkaloids have been shown to decrease insulin and increase glucagon in heifers within 1 h of a single dose of ergotamine tartrate injection (Browning et al., 2000). Dopamine type-2 receptors are expressed in the pancreatic β-cells of humans, rats, and mice; allowing dopamine to mediate inhibition of insulin secretion (García-Tornadú et al., 2010; Rubí et al., 2005). Insulin secretion typically starts with glucose-induced electrical activity of β-cells which leads to the opening of voltage-gated calcium channels, Ca^{2+} influx, and Ca2+-dependent exocytosis of insulin (Lang, 1999). Dopamine decreases cell membrane depolarization and cytosolic Ca^{2+} entry, resulting in blunted insulin secretion in response to glucose stimulation (Rubí et al., 2005). Ergot alkaloids and bromocriptine are agonistic to D2 receptors (Schillo et al., 1988) allowing them to activate those receptors found in the pancreas and illicit the same response. Pancreatic β-cells also express α2-adrenergic receptors, which mediate the effects of norepinephrine released from the hypothalamus, postganglionic sympathetic nerve endings and the adrenal medulla (Fagerholm et al., 2011; Lopez Vicchi et. al., 2016). Activation of these receptors by norepinephrine or α2-adrenoreceptor agonists such as bromocriptine leads to dysregulated glucose homeostasis and elevated blood glucose through potent inhibition of insulin secretion, increased glucagon secretion from the pancreas, and increased gluconeogenesis and glycogenesis in the liver (Fagerholm et al., 2011). Research in this area is limited in cattle and the complex interaction of alkaloids with various bioamine receptors make it difficult to identify a precise mode of action in this study.

Steroid hormone implants were expected to increase levels of IGF-1 in the current study, which in turn would affect insulin secretion by the pancreas. Estradiol-17β and

TBA implants have been shown to increase serum GH and IGF-1 in steers to levels comparable to intact bulls (Hunt et al., 1991; Lee et al., 1990) and steers given a Revalor-S implant had increased IGF-1 mRNA levels in longissimus muscle compared to nonimplanted steers (Pampusch et al., 2003). IGF-1 receptors have been found on rat pancreatic β-cells, which allow the hormone to mediate a suppressive action on insulin release (Van Schravendijk et al., 1990). Elevated circulating IGF-1 binds to these receptors on the surface of β-cells, activating a series of kinases that eventually activate the phosphodiesterase, PDE3B. This causes a reduction of cAMP levels in the β-cells and attenuation of insulin release from pancreatic islets (Zhao et al., 1997). In humans, high levels of IGF-1 can directly stimulate glucose transport into muscle through either IGF-1 or insulin/IGF-1 hybrid receptors (Clemmons, 2012) which may explain why glucose clearance from the blood was not affected in implanted steers.

There is limited research in cattle concerning the effects of ergot alkaloids on estradiol implants and circulating levels of IGF-1. Estradiol implants result in increased circulating IGF-1 in steers grazing pastures with low endophyte infection rates but not in steers grazing Kentucky 31 pasture with high endophyte infection rates (Davenport et al., 1993). A single bolus injection of ergotamine tartrate in cattle has been shown to decrease plasma IGF-1 (Browning, 2003) and activation of D2 receptors by bromocriptine decreases growth hormone release in acromegalic patients, which inhibits GH-mediated antagonism of insulin (Lopez Vicchi et. al., 2016). Paradoxically, ergotamine tartrate and ergonovine maleate have been shown to elevate plasma growth hormone concentrations in normal steers (Browning et al., 1997). Perhaps bromocriptine treatment removes the suppressive action of growth hormone and IGF-1 on insulin

release, and therefore B+I animals show increased insulin secretion after the IVGTT. This does not account for all the effects on glucose homeostasis seen with combined bromocriptine and implant treatment. This result is likely due to a complex interplay of estradiol, growth hormone and IGF-1, in addition to bromocriptine and its activation of dopaminergic and adrenergic receptors in the pancreas. At this time, the exact mechanism of action for this novel response is unclear and requires further research to clarify what occurred in the current study.

Interestingly, the insulin response curve also shows that bromocriptine produced larger areas under the curve (AUC). Following peak insulin concentration, bromocriptine treated steers displayed higher insulin levels post peak, which continued until the end of sampling, they also took 30 mins longer than steers not treated with bromocriptine to drop to pre-infusion levels, indicating decreased insulin clearance. The reduced clearance rate of insulin occurs at the same time as the reduced clearance rate of glucose, indicating that bromocriptine decreased insulin sensitivity and explaining the tendency for glucose AUC to be higher in bromocriptine treated steers than in those not treated with bromocriptine. In lean mice, a single intraperitoneal injection of bromocriptine one hour before an intraperitoneal glucose tolerance test (ipGTT) has been shown to elevate blood glucose levels compared to mice that received a placebo, demonstrating acutely impaired glucose tolerance (de Leeuw van Weenen et al., 2010). The results of the current study and de Leeuw van Weenen et al., (2010) appear to contradict the improvements in glucose tolerance and insulin sensitivity seen in obese insulin resistant animals and humans treated with bromocriptine (Kalra et al., 2011). This disparity might stem from the use of healthy/lean animals instead of obese insulin-resistant animals. A similar

situation occurs with the pancreatic KATP activator diazoxide, another medication used to decrease insulin secretion, that also has opposite effects on glucose tolerance in lean and obese Zucker rats (Alemzadeh et al., 1993). While de Leeuw van Weenen et al., (2010) also found that bromocriptine delayed and reduced the rise in plasma insulin levels following the glucose challenge, their resulting AUC for total insulin release in bromocriptine treated mice was lower than placebos, unlike in the current study. These conflicting results may be due to species differences, or perhaps from the difference between single/acute treatment in the mice vs multiple/chronic treatment in the steers from the current study.

Protein Turnover

DMI was restricted with the intention of minimizing effects of treatment on intake, allowing observation of direct changes in protein metabolism in tissues. Curiously, while total weight gain was decreased by bromocriptine in both the presence and absence of implantation, urea excretion was not affected by bromocriptine treatment. To our knowledge the effect of alkaloids on nitrogen metabolism has been studied only twice before in cattle. Unfortunately, in neither of those studies was DMI controlled to the extent of the current study, making direct comparisons difficult. Those studies demonstrated that steers fed E+ or E- tall fescue hay showed no differences in urinary urea N excretion (Huntington et al., 2009) or total urinary N (Matthews et al., 2005). Matthews et al., (2005) also determined that N retention was lower in steers fed E+ hay with 13.8 g N/d vs 22.4 g N/d in E- steers. It is uncertain whether the lower N retention observed was due to ergot alkaloid induced changes in nitrogen metabolism or loss of appetite due to fescue toxicosis, which would result in lower DMI and N intake in steers

consuming E+ fescue hay. Considering fecal and urinary N were not different, reduced N retention was most likely a function of reduced intake (Matthews et al., 2005). In the current study, there were also no differences in whole-body protein synthesis or protein turnover between bromocriptine treated steers and those not treated with bromocriptine. To the best of our knowledge, these aspects of protein metabolism in alkaloid treated animals have not been documented in any species. These preliminary findings suggest that the effects of ergot alkaloids do not stem from disruption of protein synthesis or N retention.

Urinary nitrogen is consolidated from three main sources: (1) urea and ammonia from catabolism of tissue, (2) urea and ammonia from fermentation in the rumen and cecum, and (3) non-urea and non-ammonia components of microbial and host animal origin (Lobley et al., 1985). Most excess nitrogen is excreted as urea, which comes from excess protein and amino groups that are not used by the body for protein synthesis, transamination, and recycling to the gastrointestinal tract (Reeder et al., 2011). Despite similar DMI among treatments in the current study, implanted steers showed increased growth rate, and lower urea excretion than non-implanted steers (implanted steers excreted 12 g/d less than non-implanted). Thus, it appears that N retention was higher in steers treated with estradiol/TBA implants. These possible improvements in N retention indicate a decrease in amino acid catabolism and should be accompanied by an alteration in the rate of protein synthesis and/or protein degradation. Similarly, steers with restricted DMI and implants (140 mg TBA/20 mg estradiol), excreted 85 g/d urinary nitrogen and had increased nitrogen retention, whereas controls excreted 100 g/d of urinary nitrogen

(Lobley et al., 1985). To our knowledge this is the only other study to observe the effects of a combined estradiol + TBA implant on nitrogen metabolism when DMI is controlled.

Additionally, there were no differences in whole-body protein synthesis between treatments, but protein turnover tended to be over 10 g N/d lower in implanted steers. Protein turnover is the net result of the continuous synthesis and breakdown of body proteins that is necessary to maintain protein function (Chou et al., 2012). This trend for lower turnover in steers that received steroidal implants stems from the unchanged rates of protein synthesis paired with decreased protein degradation. This was also demonstrated by Lobley et al. (1985), in that implant-treated steers showed a slight decline in synthesis 5 weeks post-implant, while amino acid oxidation was significantly lower 2- and 5-weeks post-implant compared to non-implanted animals. Increased protein deposition through a decrease in protein degradation, results in less energy expenditure for the animal, compared to increased protein deposition through an increase in protein synthesis. This is because of the relatively high energetic cost of protein synthesis, which requires at least 4.5 kJ/g (Lobley et al., 1985). Manipulation of whole-body protein metabolism through the combined action of the exogenous steroids promotes protein accretion, allowing for the considerable weight gains accomplished by implanted animals.

This is possible through the strategic combination of estradiol 17β and trenbolone acetate (TBA). TBA has been shown to decrease protein synthesis and protein degradation in the muscle of female rats, but its effects on degradation seem more pronounced (Buttery, 1983). TBA is known to bind to a variety of receptors, including estrogen, progestin, androgen, and glucocorticoid (Raynaud et al., 1981). Androgenic

activity from TBA results in stimulation of growth hormone and IGF-1 secretion as well as lowering receptor sensitivity to endogenous glucocorticoids. When binding to glucocorticoid receptors, TBA almost completely suppresses tyrosine amino transferase (a key enzyme in amino acid degradation) in rat hepatocytes (Danhaive and Rousseau, 1986; 1988). The strong growth-promoting potency of TBA derives from both its anabolic activity as an androgen and anticatabolic activity as an anti-glucocorticoid.

In cattle, estrogens are known to act in all strategically important tissues to reduce nitrogen excretion, and improve mineral retention, protein anabolism and protein deposition in skeletal muscle (Meyer, 2001). Direct action of estradiol on skeletal muscle through estrogen receptors (ER) as well as indirect action through stimulation of growth hormone secretion, GH-receptors in the liver, and IGF-1 secretion into the blood are all considered important influences of estradiol (Meyer, 2001; Dayton and White, 2014). The exact mechanism of action involved for implant-stimulated muscle growth in beef cattle is still uncertain, but studies indicate their effects on muscle satellite cells may play an important role (Johnson et al., 1998a; Kamanga-Sollo et al., 2008b; Pampusch et al., 2008). It is currently believed that local production of IGF-1 in skeletal muscle plays a part in supporting normal muscle growth and an estradiol-induced increase in muscle IGF-1 mRNA expression contributes to the increased number of satellite cells, and increased rate and efficiency of muscle growth observed in anabolic steroid-treated animals (Kamanga-Sollo et al., 2008b; Dayton and White, 2014).

In the current study estradiol and TBA were used in conjunction to maximize metabolic changes and growth response. Implanted steers appeared to have higher N retention, indicating a decrease in amino acid catabolism and possibly an alteration in the

rate of protein degradation, as well as increased protein accretion and increased growth rates compared to non-implanted animals, even in the presence of bromocriptine. The detrimental effects of bromocriptine on weight gain may be due to the actions of ergot alkaloids on IGF-1 secretion. As mentioned previously, cattle given an estradiol implant and grazed on endophyte-infected pastures have been shown to not only have lower rates of gain compared to controls, but also a slight decrease in IGF-1 responsiveness to implantation (Davenport et al. 1993). Cattle injected with ergotamine tartrate have been shown to experience drops in plasma IGF-1 concentrations (Browning, 2003). Additionally, bromocriptine has also been shown to decrease growth hormone release in patients with acromegaly, which in turn leads to decreased circulating IGF-1 from the liver. Although bromocriptine may affect circulating IGF-1, there has been no research showing its effects on local production of IGF-1 in non-hepatic tissues. It is unknown if ergot alkaloids affect production of IGF-1 in skeletal muscle or muscle growth induced by IGF-1, estradiol, and TBA in cattle. Considering bromocriptine did not have any effect on nitrogen excretion, protein turnover, and protein synthesis, the stunted weight gain in bromocriptine treated cattle may not be from an effect on protein accretion but rather glucose metabolism in skeletal muscle and protein degradation signals through the mTOR pathway.

Western Immunoblots

The mTOR signaling pathway plays a central role in regulating cell growth and metabolism in eukaryotes through mediation by two multi-protein complexes, mTORC1 and mTORC2 (Saxton and Sabatini, 2017). mTORC1 is an intermediary of nutrient and energy sensing and maintains the balance between anabolism and catabolism in response

to environmental conditions. In the presence of pro-growth endocrine signals and sufficient energy, the mTORC1 pathway shifts toward increased anabolism and phosphorylates two substrates, p70 ribosomal S6 kinase 1 (S6K1) and eIF4E binding protein 1 (4E-BP1). Phosphorylation of S6K1 promotes protein synthesis and cell growth through multiple substrates, including translational regulator eIF4B, as well as eEF2K and ribosomal protein S6, both of which subsequently promote translation initiation and elongation (Mao and Zhang, 2018; Saxton and Sabatini, 2017; Ruvinsky et al., 2009). Multiple phosphorylations of 4E-BP1 by mTORC1 cause the protein to dissociate from eIF4E, allowing assembly of the eIF4F complex and mRNA translation to occur (Saxton and Sabatini, 2017; Yoon, 2017). A mTORC1-dependent shift towards anabolism can only occur when the appropriate endocrine signals and sufficient energy are present.

The main signaling pathways investigated in the current study begin with activation of insulin receptors (IR) and insulin-like growth factor type 1 receptors (IGF-1R) on the surface of skeletal muscle cells by insulin and IGF-1. The functions of the IR and the IGF-1R appear to be physiologically distinct but overlapping, in that they can produce similar cellular responses. IR is a metabolic mediator and IGF-IR is primarily a growth promoter (Kim and Accili, 2002). In mice, observations have indicated that IGF-1R can mediate glucose utilization in skeletal muscle (Di Cola et al., 1997); suggesting that IGF-1R works in tandem with IR for metabolic regulation in skeletal muscle. The main signaling pathways activated by IR and IGR-1R also overlap, in that they signal through PI3K/Akt and converge on the TSC1/TSC2 complex, culminating in mTOR mediated phosphorylation of S6K1 and 4E-BP1 (Saxton and Sabatini, 2017). IGF-1/IGF-1R signaling to Akt/mTOR has been shown to be crucial in promoting muscle

hypertrophy, with muscle growth resulting from increased protein synthesis or decreased degradation in myofibers (Glass, 2005).

As previously mentioned, bromocriptine treated steers showed decreased insulin and glucose clearance, which indicated decreased insulin sensitivity. The primary action of insulin in skeletal muscle is to stimulate glucose uptake and metabolism, with 80% of total glucose uptake occurring in skeletal muscle (Abdul-Ghani and DeFronzo, 2010). This makes ergot alkaloid induced insulin sensitivity issues potentially detrimental to growing cattle in terms of overall health and energy metabolism. The primary defect that leads to insulin resistance in skeletal muscle is likely a defect in the insulin signaling cascade at insulin receptor substrate 1 (IRS-1), wherein the insulin receptor is downregulated by chronic hyperinsulinemia (Abdul-Ghani and DeFronzo, 2010). It is currently unknown if bromocriptine effects this receptor and there is very little research concerning alkaloid effects on the mTOR pathway, although structurally similar compounds (indoles) have been shown to inhibit PI3K/Akt/mTOR/NF-κB (Ahmad et al., 2013). Lower insulin sensitivity in bromocriptine treated steers could impact IR signaling and downstream activation of pathway proteins. However, phosphorylation of mTOR at Ser2448 after glucose stimulation was higher in the presence of, suggesting some positive influence on the mTOR protein in bromocriptine treated steers. Yet this did not translate to an increase in downstream activation of S6K1 or 4E-BP1 proteins in either the basal or stimulated state, suggesting that bromocriptine did not have an inhibitory effect on activation of the mTOR pathway and consequently did not inhibit protein synthesis which is in agreement with the previous section, concerning protein turnover.

Insulin in conjunction with increased signaling by IGF-1 in implanted steers, was expected to produce downstream activation of mTOR, resulting in phosphorylation of S6K1 and 4E-BP1, which in turn would promote protein synthesis. However, an increase in phosphorylation at Ser2448 was not seen for mTOR, nor were any significant changes to S6K1 in either the basal or stimulated state in implanted steers. There did appear to be a numerical increase in phosphorylation and activation status of S6K1 in the stimulated state in implanted steers. Surprisingly, activation status and phosphorylation of 4E-BP1 were lower in implanted steers, indicating interference with skeletal muscle protein translation initiation in the presence and absence of bromocriptine. While the current study did not show any significant decreases in protein synthesis in implanted steers — despite interference at 4E-BP1 — Lobley et al. (1985) demonstrated that implant-treated steers showed a slight decline in synthesis. This suggests that hormonal implants decrease the rate of protein synthesis and as previously discussed, accomplish protein accretion instead through decreased protein turnover and degradation. These results partially contradict previous research in mice and rats showing that IGF-1 activation of mTOR stimulates protein synthesis and muscle hypertrophy through S6K1 and 4E-BP1 (Glass, 2005). Numerically, S6K1 showed the anticipated increases in activation status, but 4E-BP1 showed the opposite. This is not unexpected as it has been previously shown that while S6K1 and 4E-BP1 both possess the TOR signaling motif, which is essential for mTOR activation, mTOR exhibits differing kinase activity towards S6K1 and 4E-BP1 (Figueiredo et al., 2017). The mTOR protein also has multiple residues that can undergo phosphorylation (Figueiredo et al., 2017). Perhaps in the current study phosphorylation occurred at a site other than Ser2448, which could explain any disparity between

activation of mTOR and phosphorylation of downstream proteins. In this case, because DMI was controlled, protein synthesis may have been stimulated through a pathway independent of mTOR, such as inhibition of GSK3β by Akt (Glass, 2005). It has also been demonstrated that the stimulatory effect of IGF-1 on cell proliferation is mediated through the MEK/ERK pathway (Coolican et al., 1997; Jones et al., 2001) and inhibition of protein degradation in myotubes is through the PI3K/Akt/FoxOsa pathway (Ge et al., 2013). Since protein abundance of upstream regulators of mTOR and these independent pathways were not evaluated in the current study, further investigation is required to get an accurate representation of the effects of estradiol/TBA implants on protein metabolism in cattle.

To our knowledge the effect of long-term administration of bromocriptine on the mTORC1, S6K, and 4E-BP1 has not been previously studied in cattle. The current study indicated that bromocriptine influenced potential relationships between pre- and post-glucose infusion levels of phosphorylated mTOR and total 4E-BP1. Bromocriptine treatment appeared to permit expression of a positive relationship between pre- and post-infusion abundance of phosphorylated mTOR. In other words, bromocriptine stimulation of post-infusion phosphorylated mTOR was only seen in cases with high pre-infusion concentrations of that phosphorylated protein. This suggests that bromocriptine may encourage activation of the mTOR protein at Ser2448. Conversely, bromocriptine appeared to nullify the positive relationship between pre- and post- infusion abundance of total 4E-BP1, suggesting that bromocriptine may have some effect on the expression of the 4E-BP1 protein. These findings demonstrate that long term administration of bromocriptine affected signal transmission between glucose, insulin, and the mTOR

pathway. At this time, the exact mechanism of action for this novel response is unknown and requires further investigation of upstream signaling to mTOR and mTOR independent pathways to elucidate what occurred in the current study.

Conclusion

Injection with bromocriptine was successful in inducing a fescue toxicosis-like syndrome in steers, and treatment with a combined estradiol + TBA implant was able to improve the performance and ADG of cattle, even in the presence of fescue-derived alkaloids. Bromocriptine treatment caused reduced insulin and glucose clearance after an IVGTT, indicating decreased insulin sensitivity and possible disruption of glucose uptake and metabolism in the skeletal muscle. This suggests that ergot alkaloids are detrimental to growing cattle in terms of overall glucose homeostasis and energy metabolism. However, there were no detrimental effects of bromocriptine on protein synthesis or N retention. Implantation improved N retention, decreased protein turnover, and had no effect on protein synthesis, suggesting that steroidal implants promote protein accretion through unchanged rates of synthesis and decreased degradation, which may allow for more energy to be available for growth, even in the presence of bromocriptine. Although previous research suggested that combined estradiol + TBA treatment would increase IGF-1 signaling in implanted steers, activation of mTOR and stimulation of protein synthesis through downstream activation of S6K1 and 4E-BP1 does not appear to have transpired, and results suggest steroidal interference occurred at 4E-BP1. This is in agreement with Lobley et al. (1985) who demonstrated that implant-treated steers showed a slight decline in synthesis. This further supports the assertion that implantation accomplishes protein accretion through decreased protein turnover and degradation, even

in the presence of bromocriptine. Bromocriptine did not affect S6K1 or 4E-BP1, so does not appear to inhibit activation of the mTOR pathway or protein synthesis, which is in agreement with the results of the protein turnover analysis. This suggests that, when feed intake is controlled, the decreased performance in cattle exposed to fescue-derived alkaloids stems from issues with glucose homeostasis and skeletal muscle metabolism, disruption of upstream signals in the mTOR pathway, or disturbances in pathways independent of mTOR. These results also suggest that many of the differences in animal performance seen in cattle affected by fescue toxicosis may be attributed to differences in feed intake.

Table 3.1. Composition of experimental diet fed to steers

Item	Percentage (DM basis)
Ingredient	
Corn Silage	61.00
Cracked Corn	24.00
Supplement[1]	10.00
DDGS	5.00
Analyzed chemical components	
DM, %	49.00
Crude Protein, %	13.00
Ca, %	0.55
P, %	0.48
Calculated energy densities	
NE_m, MCal/kg	0.81
NE_g, MCal/kg	0.52

[1] Supplement included soybean meal, ground corn, limestone, trace mineral premix, fat, vitamin premix, and Rumensin 90. Trace mineral premix provided 92.9% salt, 68 ppm Co, 1838 ppm Cu, 120 ppm I, 9290 ppm Mn, 19 ppm Se, and 5520 ppm Zn. Vitamin premix supplied 1820 IU/kg Vitamin A, 363 IU/kg Vitamin D, and 227 IU/kg Vitamin E.

Table 3.2 Prolactin area under the curve, dry matter intake, body weight at enrollment, body weight change, and average daily gain in steers treated with bromocriptine and estradiol/TBA implants

	+ Bromo		- Bromo			P-values		
	+ Implant	- Implant	+ Implant	- Implant	SEM[1]	Bromo	Implant	Bromo*Implant
Prolactin AUC	1853	1754	3013	3543	492	0.002	0.61	0.45
DM Intake, kg/day	6.18	6.14	6.24	6.20	0.08	0.22	0.44	0.99
Starting weight, kg	330.9	329.9	334.3	335.1	5.6	0.29	0.99	0.82
Weight change d1-8, kg	5.68	4.66	7.61	3.47	1.88	0.79	0.07	0.26
Weight change d8-15, kg	6.31	1.50	7.05	5.51	1.19	0.04	0.01	0.15
Weight change d15-22, kg	7.22	5.80	9.21	6.42	1.19	0.12	0.02	0.41
Total gain, kg	19.21	13.58	23.87	15.40	2.04	0.01	<.0001	0.25
ADG, kg	0.92	0.65	1.14	0.73	0.10	0.01	<.0001	0.25

[1]SEM = standard error of the mean; +Bromo/+Implant (B+I) n = 8; +Bromo/-Implant (BROMO) n = 8, except for Weight change d8-15 value where n = 7; -Bromo/+Implant (IMP) n = 8; and -Bromo/-Implant (CON) n = 8.

Table 3.3 Protein metabolism in steers treated with bromocriptine and estradiol/TBA implants

	+ Bromo		- Bromo			P-values		
	+ Implant	- Implant	+ Implant	- Implant	SEM[1]	Bromo	Implant	Bromo*Implant
Urea excretion (g/day)	38.88	50.60	44.28	57.27	4.97	0.13	0.006	0.87
^{15}N Fractional Recovery	0.13	0.18	0.15	0.18	0.02	0.30	0.03	0.64
Protein Turnover (g N/day)	132.3	136.7	131.1	147.1	8.6	0.43	0.10	0.32
Protein Synthesis (g N/day)	112.4	113.0	109.4	118.6	7.5	0.85	0.47	0.52

[1]SEM = standard error of the mean; +Bromo/+Implant (B+I) n = 5; +Bromo/-Implant (BROMO) n = 6; -Bromo/+Implant (IMP) n= 6; and -Bromo/-Implant (CON) n = 7.

Table 3.4 Area under the curve and peak height of plasma glucose from steers treated with bromocriptine and estradiol implants before and following an IV glucose bolus

	+ Bromo		- Bromo			P-values		
	+ Implant	- Implant	+ Implant	- Implant	SEM[1]	Bromo	Implant	Bromo*Implant
AUC	17450	16860	16095	16318	781	0.09	0.73	0.45
Peak Height (mg/dL)	219.5	231.0	228.2	223.0	8.8	0.97	0.70	0.32

[1]SEM = standard error of the mean; n = 8.

Table 3.5 Area under the curve, peak height, and time to peak of plasma insulin from steers treated with bromocriptine and estradiol implants before and following an IV glucose bolus

	+ Bromo		- Bromo		P-values		
	+ Implant	- Implant	+ Implant	- Implant	Bromo	Implant	Bromo*Implant
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE			
AUC	11984 ± 1136	9005 ± 1136	7386 ± 1136	8718 ± 1136	0.04	0.47	0.07
Peak Height (µIU/mL)	220.5 ± 26.2	167.8 ± 26.2	162.5 ± 26.2	211.3 ± 26.2	0.79	0.94	0.07
Time to Peak (mins)[1]	16.25 ± 2.89	20.00 ± 0.95	15.00 ± 1.61	15.00 ± 0.97	0.09	0.29	0.29

[1]Data analyzed with heterogenous variance model

Data are presented as least squares means ± standard error of the mean; n = 8.

Table 3.6 Relative abundance in arbitrary units of activated, phosphorylated, and total mTOR proteins in muscle of steers treated with bromocriptine and estradiol implants before (pre) and in response (post) to an IV glucose bolus

		+ Bromo		- Bromo			P-values			Covariate P-values	
		+ Implant	- Implant	+ Implant	- Implant	SEM[1]	Bromo	Implant	Bromo* Implant	Pre	Pre*Bromo
Pre	Ratio	0.81	0.81	0.87	0.70	0.08	0.63	0.21	0.18	-	-
	Pho	0.81	1.07	0.84	0.64	0.18	0.19	0.83	0.12	-	-
	Total	0.97	1.23	0.90	0.79	0.13	0.05	0.55	0.14	-	-
Post	Ratio	1.35	1.28	1.25	1.27	0.10	0.38	0.69	0.53	0.98	0.96
	Pho	1.73	1.85	1.23	1.63	0.27	0.04	0.14	0.42	0.10	0.0012
	Total	1.26	1.42	1.20	1.46	0.21	0.94	0.20	0.76	0.01	0.21

Ratio data represent the ratio of phosphorylated to total protein (i.e. activation status). Pho data represent the abundance of phosphorylated form of each protein. Total data represent the relative abundance of the total form of each protein.

[1]SEM = standard error of the mean; +Bromo/+Implant (B+I) n = 8; +Bromo/-Implant (BROMO) n = 8; -Bromo/+Implant (IMP) n = 8, except for Post-Pho values where n = 7; and -Bromo/-Implant (CON) n = 8, except for Pre-Ratio values where n = 7.

Table 3.7 Relative abundance in arbitrary units of activated, phosphorylated, and total S6K1 proteins in muscle of steers treated with bromocriptine and estradiol implants before (pre) and in response (post) to an IV glucose bolus

		+ Bromo		- Bromo		P-values			Covariate P-values	
		+ Implant	- Implant	+ Implant	- Implant	Bromo	Implant	Bromo* Implant	Pre	Pre*Bromo
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE					
Pre	Ratio	0.08 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.82	0.24	0.12	-	-
	Pho[1]	0.10 ± 0.02	0.06 ± 0.01	0.10 ± 0.03	0.08 ± 0.02	0.63	0.12	0.63	-	-
	Total	1.32 ± 0.15	1.42 ± 0.15	1.34 ± 0.15	1.14 ± 0.15	0.20	0.60	0.15	-	-
Post	Ratio[1]	1.54 ± 0.55	0.63 ± 0.12	0.90 ± 0.36	0.75 ± 0.13	0.46	0.14	0.28	0.66	0.22
	Pho[1]	1.30 ± 0.54	0.53 ± 0.10	0.86 ± 0.37	0.67 ± 0.16	0.66	0.17	0.40	0.78	0.17
	Total	0.76 ± 0.06	0.76 ± 0.07	0.83 ± 0.06	0.92 ± 0.07	0.05	0.40	0.41	<.0001	0.17

[1]Data analyzed with heterogenous variance model

Ratio data represent the ratio of phosphorylated to total protein (i.e. activation status). Pho data represent the abundance of phosphorylated form of each protein. Total data represent the relative abundance of the total form of each protein. Data are presented as least squares means ± standard error of the mean. +Bromo/+Implant (B+I) n = 8; +Bromo/-Implant (BROMO) n = 8, except for Post-Total values where n = 7; -Bromo/+Implant (IMP) n = 8, except for Pre-Ratio values where n = 7; and -Bromo/-Implant (CON) n = 8.

Table 3.8 Relative abundance in arbitrary units of activated, phosphorylated, and total 4E-BP1 proteins in muscle of steers treated with bromocriptine and estradiol implants before (pre) and in response (post) to an IV glucose bolus

		+ Bromo		- Bromo			P-values			Covariate P-values	
		+ Implant	- Implant	+ Implant	- Implant	SEM[1]	Bromo	Implant	Bromo* Implant	Pre	Pre*Bromo
Pre	Ratio	0.71	0.58	0.58	0.62	0.08	0.42	0.38	0.15	-	-
	Pho	0.97	0.87	0.83	0.76	0.14	0.30	0.47	0.91	-	-
	Total	1.32	1.47	1.45	1.17	0.12	0.44	0.57	0.06	-	-
Post	Ratio	0.79	0.90	0.63	0.86	0.08	0.11	0.01	0.34	0.30	0.56
	Pho	0.74	0.90	0.66	0.90	0.10	0.60	0.02	0.59	0.006	0.80
	Total	0.99	1.01	0.96	1.15	0.09	0.005	0.04	0.10	<.0001	0.003

Ratio data represent the ratio of phosphorylated to total protein (i.e. activation status). Pho data represent the abundance of phosphorylated form of each protein. Total data represent the relative abundance of the total form of each protein.
[1]SEM = standard error of the mean; n=8.

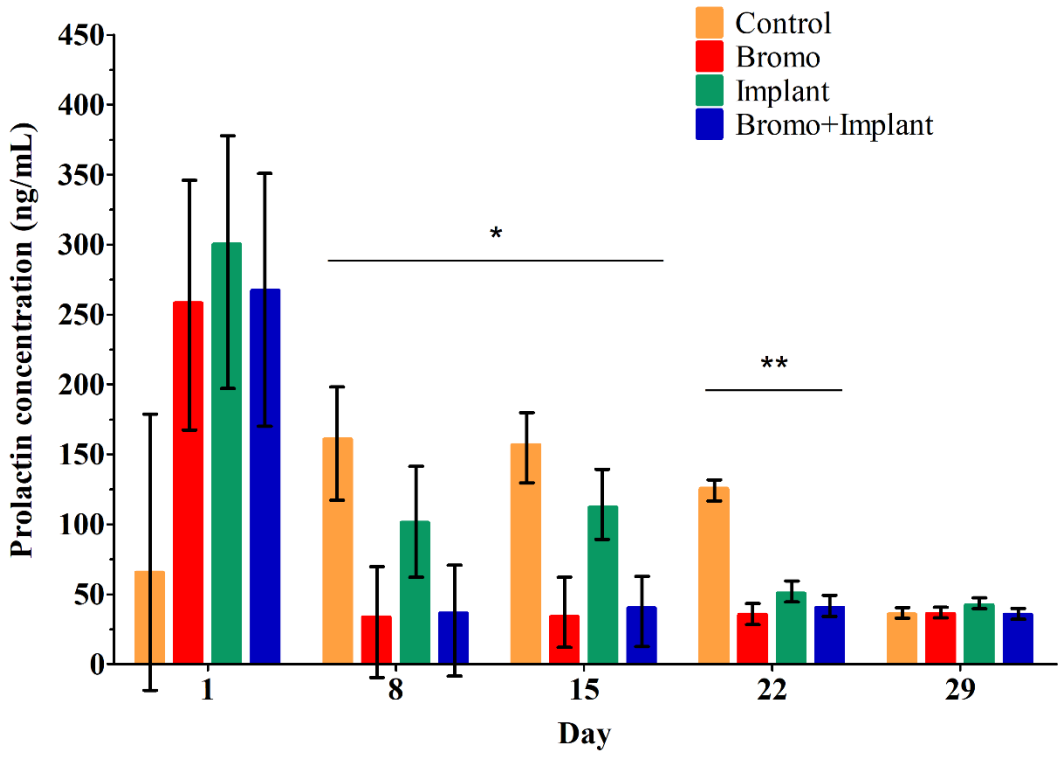


Figure 3.1 Mean plasma concentrations of prolactin (ng/mL)
Values are means ± SE of plasma prolactin concentrations for steers treated with bromocriptine injections and estradiol/TBA implants from weekly samples over 29 days. Day 1 represents sample taken immediately before treatment administration. Days denoted with * show bromocriptine treatment significantly decreased prolactin (Day 8, P = 0.04; Day 15, P = 0.001). Days denoted with ** show bromocriptine treatment significantly decreased prolactin (P = 0.002), implantation significantly decreased prolactin (P = 0.02), and a bromo x implant interaction (P = 0.01). Data for each day analyzed as a randomized complete block, terms of the model included bromo, implant, and their interactions, in a 2x2 factorial structure.
Control n = 6; Bromo n = 8; Implant n = 7; and Bromo+Implant n = 7.

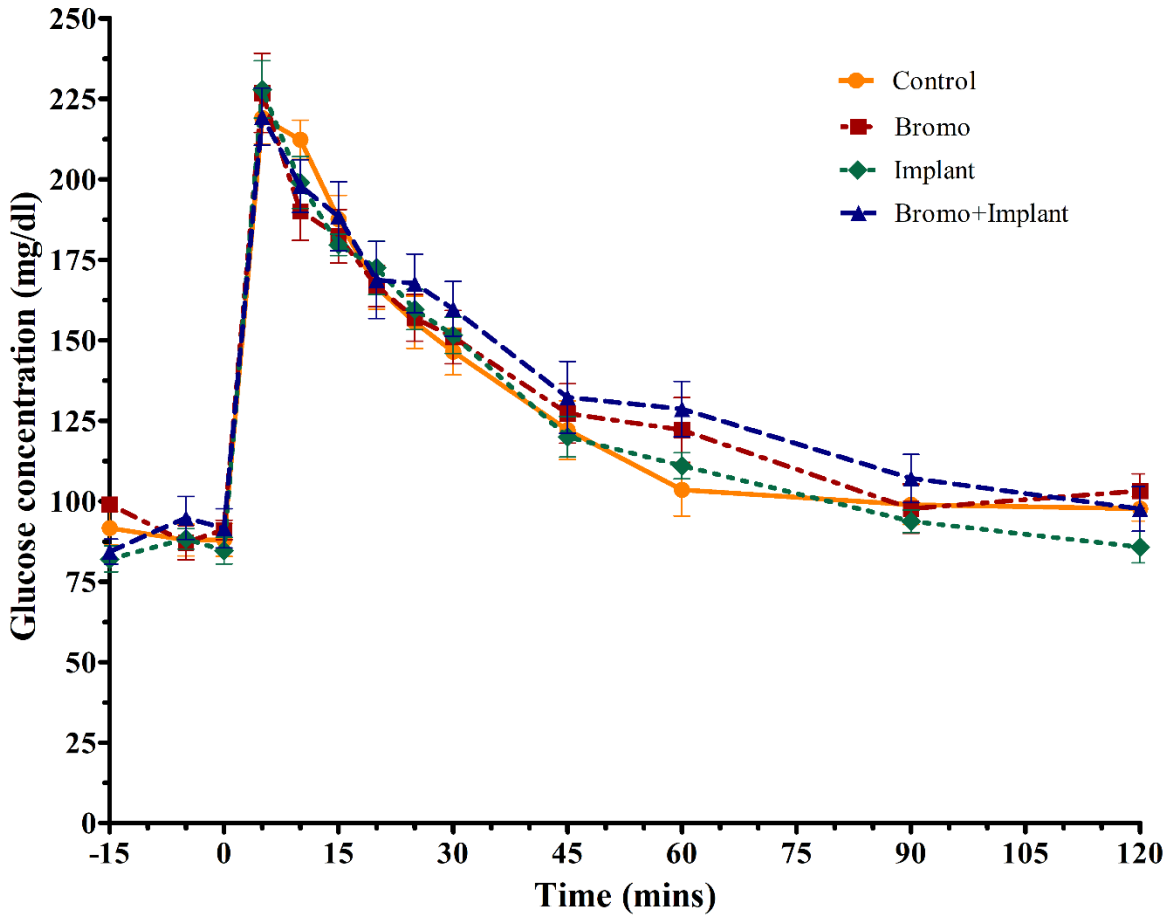


Figure 3.2 Mean plasma concentration of glucose (mg/dL)
Values are means ± SE of plasma glucose concentrations during intravenous glucose tolerance test (IVGTT) for steers treated with bromocriptine injections and estradiol/TBA implants performed on d 35. Used to determine area under the curve which tended to be higher with bromocriptine treatment (P = 0.09) and peak height. Time point 0 represents sample taken immediately before intravenous glucose bolus administration (0.25 g/kg BW). Area under the curve and peak height were analyzed as a randomized complete block, terms of the model included bromo, implant, and their interactions, in a 2x2 factorial structure.
n = 8 for all treatments.

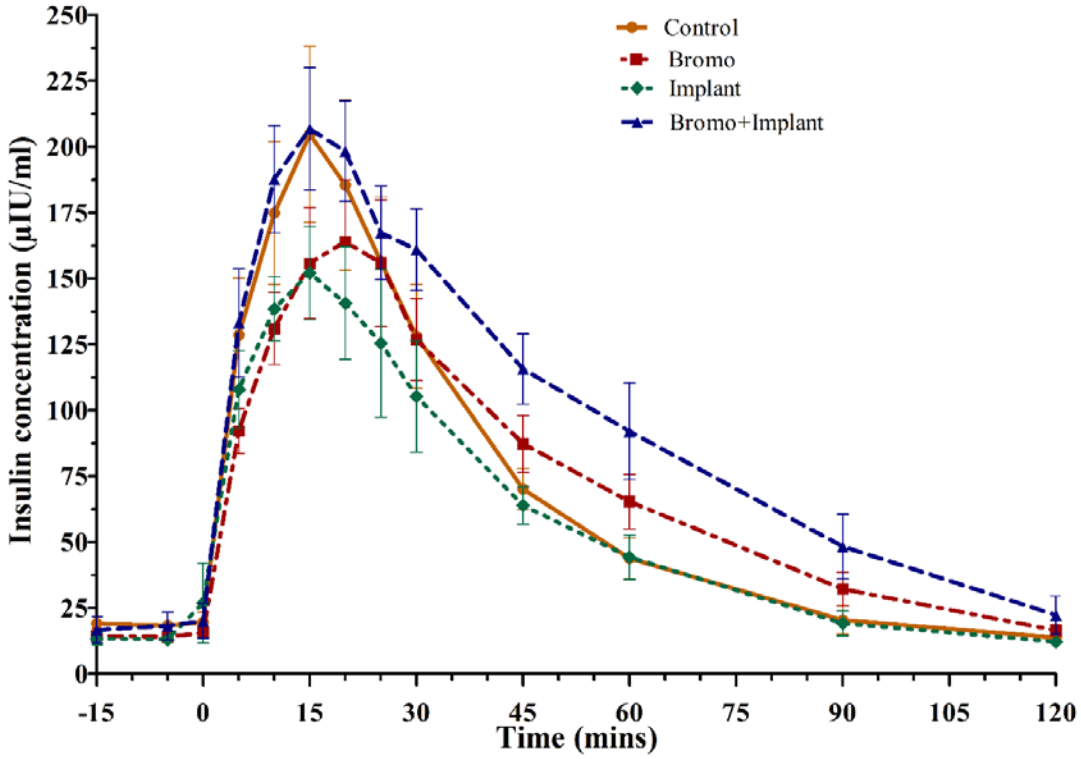


Figure 3.3 Mean plasma concentration of insulin (µIU/mL)
Values are means ± SE of plasma insulin concentrations during intravenous glucose tolerance test (IVGTT) for steers treated with bromocriptine injections and estradiol implants performed on d 35. Used to determine area under the curve, peak height, and time to peak. Bromocriptine significantly increased AUC (P = 0.04) and there was a trend (P = 0.09) for it to increase time to peak. There was a significant bromo x implant interaction (P = 0.07) which increased AUC and peak height. Time point 0 represents sample taken immediately before intravenous glucose bolus administration (0.25 g/kg BW). Area under the curve, peak height, and time to peak were analyzed as a randomized complete block, terms of the model included bromo, implant, and their interactions, in a 2x2 factorial structure.
n = 8 for all treatments.

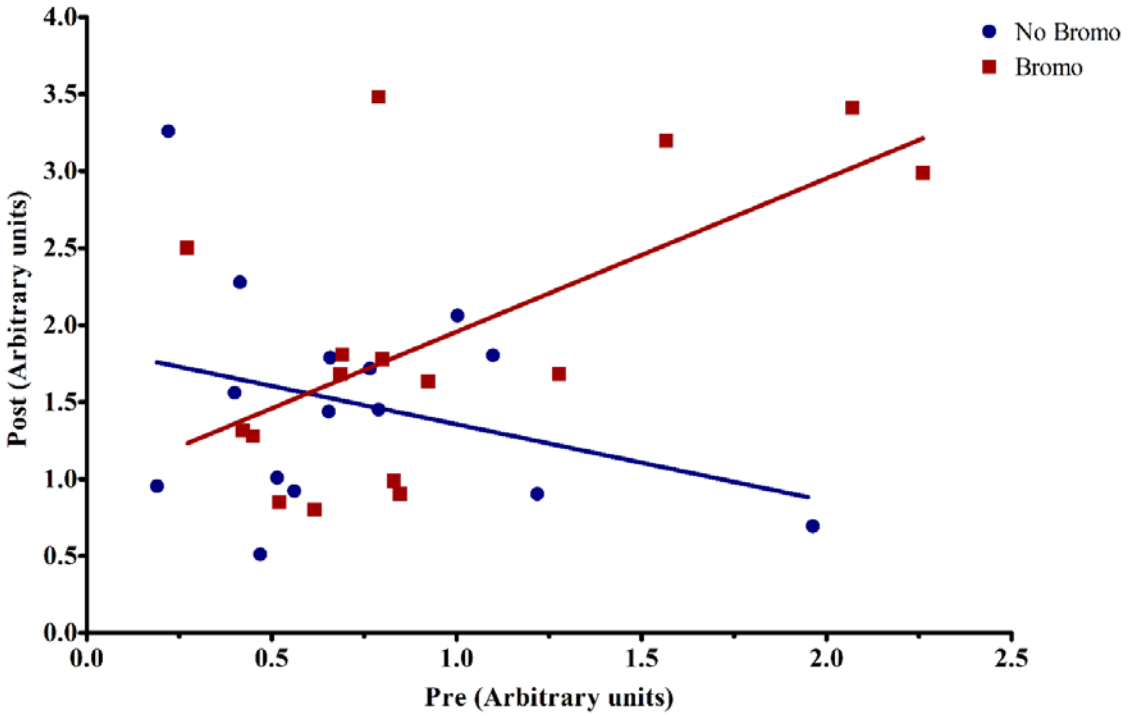


Figure 3.4 Representative graph of the interaction between pre-infusion levels of phosphorylated mTOR and bromocriptine treatment on post-infusion levels of phosphorylated mTOR

For covariate analysis, pre and its treatment interactions were first tested as fixed effects (pre, pre x bromo, pre x implant, and pre x bromo x implant) to determine which covariates were significant ($P < 0.05$). Insignificant covariates were dropped from the model. The pre x bromo interaction was significant ($P = 0.0012$), demonstrating that pre glucose infusion levels of phosphorylated mTOR affected the amount of phosphorylated mTOR found post infusion in animals treated with bromocriptine. There was a positive relationship between pre- and post- infusion abundance in bromocriptine treated steers. In the absence of bromocriptine, there was no relationship between pre- and post- infusion phosphorylated protein abundance. Each point represents an individual steer.

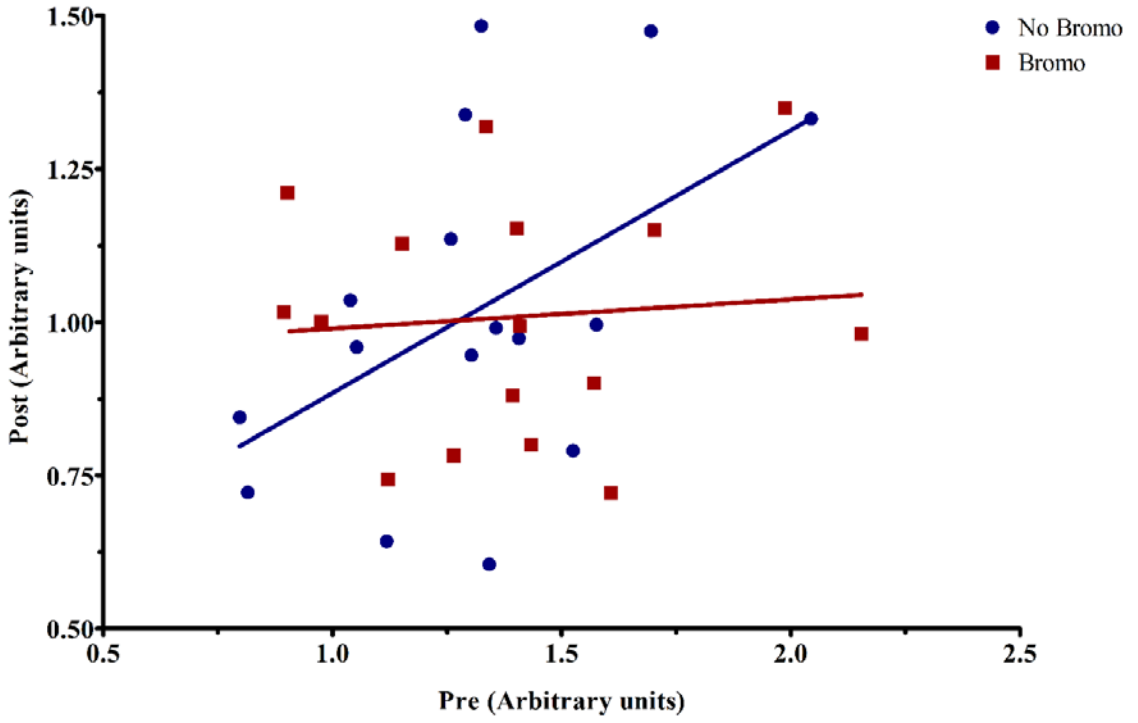


Figure 3.5 Representative graph of the interaction between pre-infusion levels of total 4E-BP1 and bromocriptine treatment on post-infusion levels of total 4E-BP1
For covariate analysis, pre and its treatment interactions were first tested as fixed effects (pre, pre x bromo, pre x implant, and pre x bromo x implant) to determine which covariates were significant ($P < 0.05$). Insignificant covariates were dropped from the model. The pre x bromo interaction was significant ($P = 0.003$), demonstrating that pre glucose infusion levels of total 4E-BP1 affected the amount of total 4E-BP1 found post infusion in animals treated with bromocriptine. There was a positive relationship between pre- and post- infusion abundance in steers not treated with bromocriptine, but in the presence of bromocriptine, there was no relationship between pre- and post- infusion total protein abundance. Each point represents an individual steer.

CHAPTER 4. SUMMARY AND CONCLUSIONS

Tall fescue (*Festuca arundinacea*) is the most important pasture forage in the United States, occupying 12 to 14 million ha and supporting millions of livestock (Buckner et al., 1979). Its vitality can be attributed to a mutualistic relationship with the endophytic fungi *Epichloë coenophiala*, which extends the grass' growing season, and enhances its adaptability and persistence under heavy grazing (Buckner et al., 1979). Soon after it rose to prominence, tall fescue gained a reputation for inducing performance issues in livestock. It was later discovered that the cause was the variety of toxic ergot alkaloids produced by the endophyte fungus. High levels of ergot alkaloids result in three syndromes: fescue foot, fat necrosis, and summer slump. Notable symptoms include, elevated respiration, failure to shed the winter haircoat, heat intolerance, reductions in weight gain and milk production, and in extreme cases, gangrene of the extremities (Hoveland, 2009). Fescue toxicosis impacts all species in the grazing industry and results in an estimated, combined annual loss of up to $1 billion per year (Strickland et al., 2011).

Animal disorders associated with tall fescue are the result of ergot alkaloids liberated from the forage in the gastrointestinal tract. The diverse effects of alkaloids are directly related to the structural similarities between them and the biogenic amine neurotransmitters norepinephrine, dopamine, and serotonin; which regulate appetite, cardiovascular function, endocrine activity, gastrointestinal motility, muscle contraction, and thermo regulation (Berde and Strumer, 1978; Thompson and Stuedemann, 1993). This allows alkaloids to bind to multiple neurotransmitter receptors throughout the body and obstruct their normal performance. Interference with serotonin and adrenergic

receptors has been shown to cause vasoconstriction of bronchial, subcutaneous and peripheral blood vessels and is believed to be the cause of the respiratory and circulatory issues that are common in affected animals (Oliver, 1997; Dyer, 1993). Animals suffering from fescue toxicosis also experience a sharp reduction in serum prolactin, caused by activation of dopamine receptors by ergot alkaloids (Schillo et al., 1988). Prolactin is essential for regulating metabolism, the immune system, milk synthesis, milk secretion, and pancreatic development. Decreased livestock productivity may also result from disruption of normal function of dopaminergic and adrenergic receptors in the pancreas as well. The synthetic alkaloid bromocriptine has been shown to reduce serum triglycerides, inhibit insulin secretion, and decrease body weight in rats and humans (Kalra et al., 2011; Lopez Vicchi et. al., 2016).

Many attempts have been made to improve the performance of stocker cattle grazing endophyte infected fescue, and steroidal implants have been shown to increase ADG in affected cattle (Brazle and Coffey, 1991; Beconi et al., 1995; Aiken et al., 2006; Carter et al., 2010). Androgenic and estrogenic steroids have been used for decades to enhance feed efficiency, rate of gain, and muscle accretion in feedlot cattle. Cattle given implants containing estradiol and trenbolone acetate have an increased number of muscle satellite cells, increased expression of IGF-1 mRNA in muscle tissue, and increased levels of circulating IGF-1 (Dayton and White, 2014). Local production of IGF-1 in skeletal muscle is thought to play a role in supporting hypertrophy and increased muscle growth observed in anabolic steroid-treated animals (Kamanga-Sollo et al., 2008b). Despite improvements seen with implant treatment, it has also been demonstrated that cattle grazing endophyte-infected pastures not only had lower rates of gain compared to

cattle grazing non-infected fescue, but also exhibit a slight decrease in IGF-1 responsiveness to estradiol-17β (Davenport et al. (1993). IGF-1 is an important stimulator of the mTOR pathway, which regulates protein synthesis and muscle gain in growing animals (Yoon, 2017). Ergot alkaloids and structurally similar chemicals (indoles) have been shown to inhibit the growth hormone axis (Flückiger et al., 1978) and the mTOR pathway (Ahmad et al., 2013), respectively, in other species, but research investigating the effects of ergot alkaloids on these metabolic pathways, particularly in cattle, is severely lacking.

The aims of this research were to determine if fescue-derived alkaloids decrease muscle protein synthesis though inhibition of the mTOR pathway by directly affecting signal proteins, and if these negative effects could be alleviated by implantation with anabolic agents. Fescue toxicosis was successfully induced using the synthetic alkaloid, bromocriptine, resulting in reduced circulating prolactin after one week of treatment. Treatment with a combined estradiol-17β and trenbolone acetate implant mitigated some of the adverse effects of bromocriptine, allowing steers to gain more weight throughout the treatment period. Bromocriptine reduced insulin and glucose clearance following an intravenous glucose tolerance test, indicating decreased insulin sensitivity which would result in disruption of glucose uptake and metabolism in the skeletal muscle. This suggests that fescue-derived alkaloids are detrimental to growing cattle in terms of overall glucose homeostasis and energy metabolism. Conversely, analysis of whole-body protein turnover demonstrated that bromocriptine does not appear to affect protein synthesis or N retention and western immunoblot analysis of skeletal muscle showed that it did not affect abundance of S6K1 or 4E-BP1, so does not appear to inhibit activation of

the mTOR pathway or protein synthesis. Implantation improved N retention, decreased protein turnover, and had no effect on protein synthesis, suggesting that steroidal implants promote protein accretion through unchanged rates of synthesis and decreased degradation, even in the presence of bromocriptine. Implanted steers may have experienced increased IGF-1 signaling, but downstream activation of mTOR, S6K and 4E-BP1, and thus increased protein synthesis, did not occur. These results also suggest that steroidal implants interfere with phosphorylation and abundance of 4E-BP1, blocking mRNA translation.

Overall, this research suggests that the poor performance of cattle exposed to ergot alkaloids originates with perturbation of skeletal muscle metabolism, specifically disruption of upstream signals in the mTOR pathway, or in pathways independent of mTOR. These results also suggest that many of the performance losses in cattle grazing endophyte infected tall fescue may be attributed to differences in feed intake, likely due to the negative effects of alkaloids on appetite and gastrointestinal motility. Further research should focus on the effects of fescue-derived alkaloids and steroid implants on cellular pathways independent of mTORC1, such as the PI3K/Akt/FoxO pathway (regulates inhibition of protein degradation in myotubes) and MEK/ERK pathway (regulates cell proliferation). Additionally, those located upstream like AMPK (senses glucose depravation) and downstream such as ULK1 (initiates autophagy), could provide further insight. Other cellular pathways could prove important in understanding the mechanisms by which ergot alkaloids cause such costly growth restrictions and health problems in cattle.

APPENDIX

A. 1 Antibody Validations for Western Blot Analysis

The antibodies used in the research chapters, total and Ser^{2448} mTOR, total and Thr^{389} p70 S6 Kinase, and total and $Thr^{37/46}$ 4E-BP1 were validated in our lab to determine cross-species reactivity of these proteins in bovine muscle tissue.

A.1.a mTOR Antibody Validation

To measure the abundance of 289 kDa mTOR, aliquots of pooled muscle sample from four randomly selected steers used in Chapter 3 were loaded into a polyacrylamide gel (8%). Identical samples were loaded in duplicate with one empty well separating the sets of pooled samples. Two molecular markers were also loaded onto the gel, Spectra to follow separation during electrophoresis and Magic Mark to verify the molecular weight of the protein bands after chemiluminescence. After separation by electrophoresis, the proteins were transferred onto a PVDF membrane and stained with Fast Green stain to verify if the protein transfer was successful. More information about the electrophoresis, transfer and staining procedures can be found in Chapter 3. Before the stain was removed with destain, the empty lanes were cut so that four pieces of membrane had an identical set of samples loaded. These membranes were placed in separate containers, washed and blocked with 5% fat-free milk dissolved in TBST for 1 hour at room temperature. During this time, the primary antibody and antigen (blocking peptide, catalog #1230, Cell Signaling Technology, Danvers, MA) mixtures were prepared and incubated for 1 hour at room temperature, per the manufacturer's suggestion to allow for antibody-antigen interaction. Rabbit monoclonal antibody specific to Ser2448 mTOR (catalog #5536, Cell Signaling Technology, Danvers, MA) was mixed as a 1:1000 dilution in 5% bovine

serum albumin dissolved in TBST and divided into four conical tubes. The first tube contained only primary antibody and was expected to bind to phosphorylated mTOR without impediment. Antigen was added to the next three tubes so that the first had a 1:5 mTOR antibody to antigen ratio, the second was 1:0.5, and the third a 1:0.05 ratio. These mixtures were then poured onto the membranes and allowed to incubate at 4 °C under gentle rocking overnight. The following day, the mixtures were removed, the membranes were washed and then probed with secondary antibody (Cell Signaling Technology, Danvers, MA, 1:2000 dilution in 5% fat-free milk dissolved in TBST) which was incubated for 1 hour at room temperature under gentle rocking. Membranes were then developed using a chemiluminescent kit (Amersham ECL Plus western blotting detection reagent, GE Healthcare Bio-Sciences, Pittsburgh, PA) and visualized using a digital imager (Azure c600, Azure Biosystems, Dublin, CA). The following figure shows that all ratios of the antibody:antigen solutions prevented the phosphorylated mTOR antibody from binding to the mTOR protein in bovine externus abdominis muscle samples, thereby validating the use of this primary antibody in cattle.

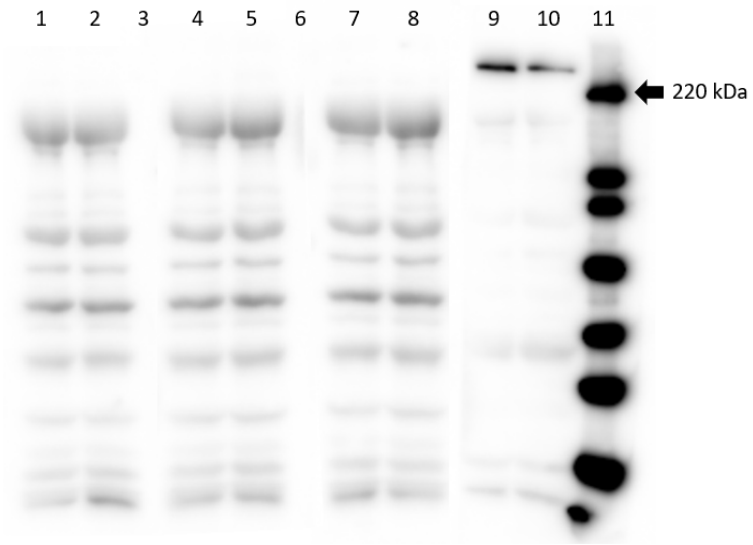


Figure A.1.1 Example gel showing the location of the band for phosphorylated mTOR and its disappearance when incubated with the antigen
Lanes contained: (1 & 2) pooled bovine obliquus externus abdominis muscle incubated with 1:5 antibody:antigen solution, (4&5) pooled bovine muscle incubated with 1:0.5 antibody:antigen solution, (7&8) pooled bovine muscle incubated with 1:0.05 antibody:antigen solution, (9&10) pooled bovine muscle incubated with typical antibody solution, and (11) molecular weight marker.

Following image capture, these membranes were stripped and re-blocked with 5% fat-free milk dissolved in TBST as described in Chapter 3, and re-probed, this time with the antibody for total mTOR (catalog #2983, Cell Signaling Technology, Boston, MA). Procedures for antigen incubation were repeated as described with the phosphorylated form but this time an antigen specific to total mTOR (blocking peptide, catalog #1072, Cell Signaling Technology, Boston, MA) was used, then the membranes were developed and visualized. The following figure shows that all ratios of the antibody:antigen solutions prevented the total mTOR antibody from binding to bovine externus abdominis muscle samples, thereby validating the use of this primary antibody in cattle.

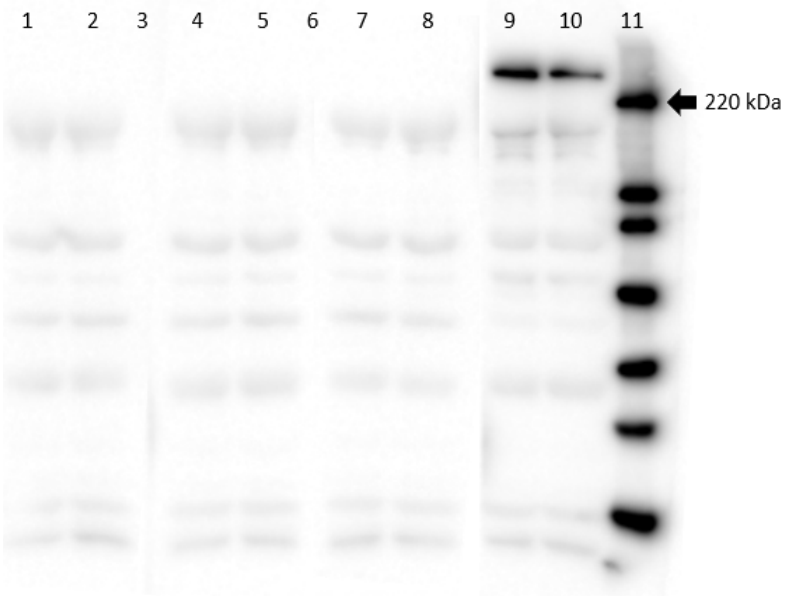


Figure A.1.2 Example gel showing the location of the band for total mTOR and its disappearance when incubated with the antigen
Lanes contained: (1 & 2) pooled bovine obliquus externus abdominis muscle incubated with 1:5 antibody:antigen solution, (4&5) pooled bovine muscle incubated with 1:0.5 antibody:antigen solution, (7&8) pooled bovine muscle incubated with 1:0.05 antibody:antigen solution, (9&10) pooled bovine muscle incubated with typical antibody solution, and (11) molecular weight marker.

A.1.b. p70-S6 Kinase Antibody Validation

To measure the abundance of 70 and 85 kDa p70-S6 Kinase, aliquots of pooled muscle sample from post glucose challenge steers (from blocks 6 and 7) used in Chapter 3, were loaded into a polyacrylamide gel (8%). Identical samples were loaded in duplicate with one empty well separating the sets of pooled samples, except for the first four lanes which were loaded without the empty well separating them, due to space limitations on the gel. Two molecular markers were also loaded onto the gel, Spectra to follow separation during electrophoresis and Magic Mark to verify the molecular weight of the protein bands after chemiluminescence. After separation by electrophoresis, the proteins were transferred onto a PVDF membrane and stained with Fast Green stain to

verify if the protein transfer was successful. More information about the electrophoresis, transfer and staining procedures can be found in Chapter 3. Before the stain was removed with destain, the membrane was cut between the sets of duplicate so that five pieces of membrane had an identical set of samples loaded. These membranes were placed in separate containers, washed and blocked with 5% fat-free milk dissolved in TBST for 1 hour at room temperature. During this time, the primary antibody and antigen (custom blocking peptide, catalog #1630, Cell Signaling Technology, Danvers, MA) mixtures were prepared and incubated for 1 hour at room temperature, per the manufacturers suggestion to allow for antibody-antigen interaction. Rabbit monoclonal antibody specific to Thr^{389} p70 S6 Kinase (catalog #9234, Cell Signaling Technology, Danvers, MA) was mixed as a 1:1000 dilution in 5% fat-free milk dissolved in TBST and divided into five conical tubes. The first tube contained only primary antibody and was expected to bind to phosphorylated p70-S6K without impediment. Antigen was added to the next four tubes so that the first had a 1:5 p70-S6K antibody to antigen ratio, the second a 1:0.5 ratio, the third a 1:0.05 ratio, and the fourth a 1:0.005 ratio. These mixtures were then poured onto the membranes and allowed to incubate at 4 °C under gentle rocking overnight. The following day, the mixtures were removed, the membranes were washed and then probed with secondary antibody (Cell Signaling Technology, 1:2000 dilution in 5% fat-free milk dissolved in TBST) which was incubated for 1 hour at room temperature under gentle rocking. Membranes were then developed using a chemiluminescent kit (Amersham ECL Plus western blotting detection reagent, GE Healthcare Bio-Sciences, Pittsburgh, PA) and visualized using a digital imager (Azure c600, Azure Biosystems, Dublin, CA). The following figure shows that all but the 1:0.005 ratio of the antibody:antigen solutions

prevented the phosphorylated p70-S6 Kinase antibody from binding to bovine externus abdominis muscle samples, thereby validating the use of this primary antibody in cattle.

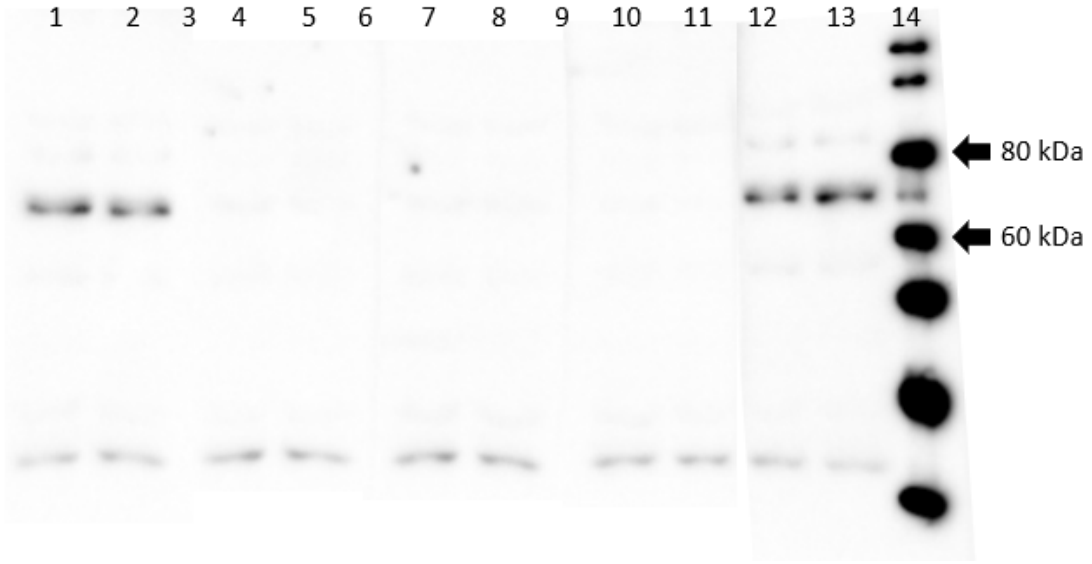


Figure A.1.3 Example gel showing the location of the band for phosphorylated p70 S6K and its disappearance when incubated with the antigen
Lanes contained: (1&2) pooled bovine obliquus externus abdominis muscle incubated with 1:0.005 antibody:antigen solution, (4&5) pooled bovine muscle incubated with 1:0.05 antibody:antigen solution, (7&8) pooled bovine muscle incubated with 1:0.5 antibody:antigen solution, (10&11) pooled bovine muscle incubated with 1:5 antibody:antigen solution, (12&13) pooled bovine muscle incubated with typical antibody solution, and (14) molecular weight marker.

Following image capture, these membranes were stripped and re-blocked with 5% fat-free milk dissolved in TBST as described in Chapter 3, and re-probed, this time with the antibody for total p70-S6 Kinase (catalog #2708, Cell Signaling Technology, Danvers, MA). Procedures for antigen incubation were repeated as described with the phosphorylated form but this time an antigen specific to total p70-S6 Kinase (catalog #1205, Cell Signaling Technology, Danvers, MA) was used, as well as a different ratio of antibody to antigen. The first tube contained only primary antibody and was expected to

bind to total p70-S6K without impediment. Antigen was added to the next four tubes so that the first had a 1:2 antibody to antigen ratio, the second a 1:0.2 ratio, the third a 1:0.02 ratio, and the fourth a 1:0.002 ratio. Following incubation with secondary antibody the membranes were developed and visualized. The following figure shows that all ratios of the antibody:antigen solutions prevented the total p70-S6 Kinase antibody from binding to bovine externus abdominis muscle samples, thereby validating the use of this primary antibody in cattle.

Figure A.1.4 Example gel showing the location of the band for total p70 S6K and its disappearance when incubated with the antigen
Lanes contained: (1&2) pooled bovine obliquus externus abdominis muscle incubated with 1:0.002 antibody:antigen solution, (4&5) pooled bovine muscle incubated with 1:0.02 antibody:antigen solution, (7&8) pooled bovine muscle incubated with 1:0.2 antibody:antigen solution, (10&11) pooled bovine muscle incubated with 1:2 antibody:antigen solution, (12&13) pooled bovine muscle incubated with typical antibody solution, and (14) molecular weight marker.

A.1.c. 4E-BP1 Antibody Validation

To measure the abundance of 15-20 kDa 4E-BP1, aliquots of pooled muscle sample from steers from block 4 used in Chapter 3, were loaded into a polyacrylamide gel (12%). Identical samples were loaded in duplicate with one empty well separating the

sets of pooled samples. Two molecular markers were also loaded onto the gel, Spectra to follow separation during electrophoresis and Magic Mark to verify the molecular weight of the protein bands after chemiluminescence. After separation by electrophoresis, the proteins were transferred onto a PVDF membrane and stained with Fast Green stain to verify if the protein transfer was successful. More information about the electrophoresis, transfer and staining procedures can be found in Chapter 3. Before the stain was removed with destain, the empty lanes were cut so that four pieces of membrane had an identical set of samples loaded. These membranes were placed in separate containers, washed and blocked with 5% fat-free milk dissolved in TBST for 1 hour at room temperature. During this time, the primary antibody and antigen (blocking peptide, catalog #1052, Cell Signaling Technology) mixtures were prepared and incubated for 1 hour at room temperature, per the manufacturers suggestion to allow for antibody-antigen interaction. Rabbit monoclonal antibody specific to $Thr^{37/46}$ 4E-BP1 (catalog #2855, Cell Signaling Technology, Danvers, MA) was mixed as a 1:1000 dilution in 5% fat-free milk dissolved in TBST and divided into four conical tubes. The first tube contained only primary antibody and was expected to bind to phosphorylated 4E-BP1 without impediment. Antigen was added to the next three tubes so that the first had a 1:5 4E-BP1 antibody to antigen ratio, the second was 1:0.5, and the third a 1:0.05 ratio. These mixtures were then poured onto the membranes and allowed to incubate at 4 °C under gentle rocking overnight. The following day, the mixtures were removed, the membranes were washed and then probed with secondary antibody (Cell Signaling Technology, 1:2000 dilution in 5% fat-free milk dissolved in TBST) which was incubated for 1 hour at room temperature under gentle rocking. Membranes were then developed using a chemiluminescent kit

(Amersham ECL Plus western blotting detection reagent, GE Healthcare Bio-Sciences, Pittsburgh, PA) and visualized using a digital imager (Azure c600, Azure Biosystems, Dublin, CA). The following figure shows that all ratios of the antibody:antigen solutions prevented the phosphorylated 4E-BP1 antibody from binding to bovine externus abdominis muscle samples, thereby validating the use of this primary antibody in cattle.

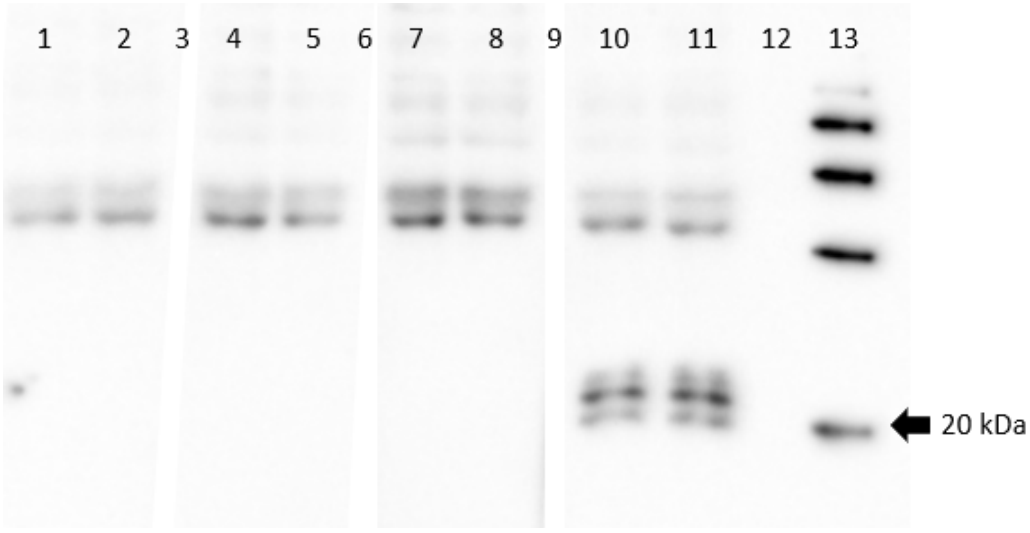


Figure A.1.5 Example gel showing the location of the band for phosphorylated 4E-BP1 and its disappearance when incubated with the antigen
Lanes contained: (1&2) pooled bovine obliquus externus abdominis muscle incubated with 1:0.05 antibody:antigen solution, (4&5) pooled bovine muscle incubated with 1:0.5 antibody:antigen solution, (7&8) pooled bovine muscle incubated with 1:5 antibody:antigen solution, (10&11) pooled bovine muscle incubated with typical antibody solution, and (13) molecular weight marker.

Following image capture, these membranes were stripped and re-blocked with 5% fat-free milk solution as described in Chapter 3, and re-probed, this time with the antibody for total 4E-BP1 (catalog #9644, Cell Signaling Technology, Danvers, MA). Procedures for antigen incubation were repeated as described with the phosphorylated form but this time an antigen specific to total 4E-BP1 (catalog# 1053, Cell Signaling

Technology, Danvers, MA) was used, then the membranes were developed and visualized. The first tube contained only primary antibody and was expected to bind to total 4E-BP1 without impediment. Antigen was added to the next three tubes so that the first had a 1:5 antibody to antigen ratio, the second a 1:0.5 ratio, and the third a 1:0.05 ratio. Following incubation with secondary antibody the membranes were developed and visualized. The following figure shows that all ratios of the antibody:antigen solutions prevented the total 4E-BP1 antibody from binding to bovine externus abdominis muscle samples, thereby validating the use of this primary antibody in cattle.

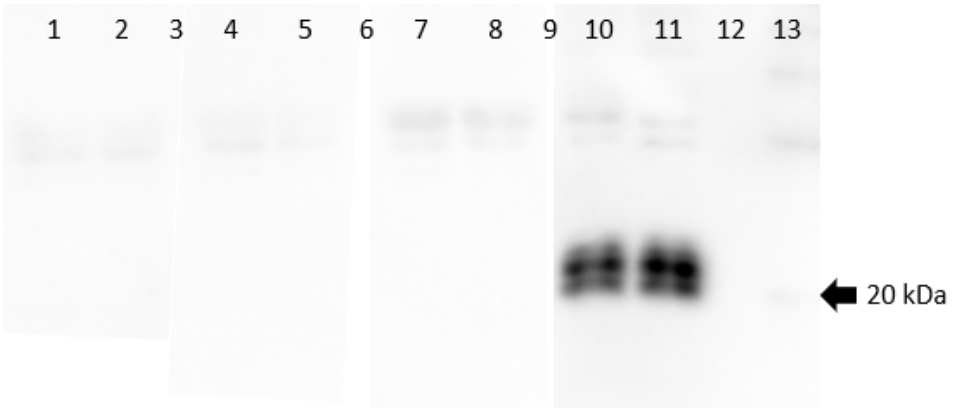


Figure A.1.6 Example gel showing the location of the band for total 4E-BP1 and its disappearance when incubated with the antigen
Lanes contained: (1&2) pooled bovine obliquus externus abdominis muscle incubated with 1:0.05 antibody:antigen solution, (4&5) pooled bovine muscle incubated with 1:0.5 antibody:antigen solution, (7&8) pooled bovine muscle incubated with 1:5 antibody:antigen solution, (10&11) pooled bovine muscle incubated with typical antibody solution, and (13) molecular weight marker.

A.2. Normalization to Total Protein

Normalization to total protein was used as a loading control to reduce variation due to concentration (Aldridge et al., 2008). Total protein stains have been shown to accurately correct loading errors and to have no limitation of dynamic range, in addition

to being completely reversible (Romero-Calvo et al., 2010). To achieve the total protein stain, PVDF membranes were washed in Fast Green FCF solution (0.2g fast green, 50% methanol, 10% glacial acetic acid). Fast Green is an anionic dye that binds to all protein present on the membrane; making it useful when evaluating transfer efficiency and quantifying total protein (Luo et al., 2006). After electrophoresis and protein transfer, membranes were incubated with Fast Green for approximately 2 min, then washed with destain buffer (50% methanol, 10% glacial acetic acid) until the background was white and only the protein bound stain was visible. Once this occurred, an image of the total protein stain was obtained using a digital imager (Azure c600, Azure Biosystems, Dublin, CA). The membranes were then washed until all remaining Fast Green stain was removed before blocking and immunodetection as described in Chapter 3. The image of the stain was later quantified using the AzureSpot Software (Azure Biosystems, Dublin, CA); where each individual lane (representing different muscle samples) was quantified, and the background was subtracted, to determine the total amount of protein in each lane. The sample lane values were then normalized to the total protein signal for the positive control that was loaded onto each gel (pooled bovine muscle sample). Subsequently, these normalized total protein values were then used to normalize the band densities obtained for each protein measured as described in Chapter 3.

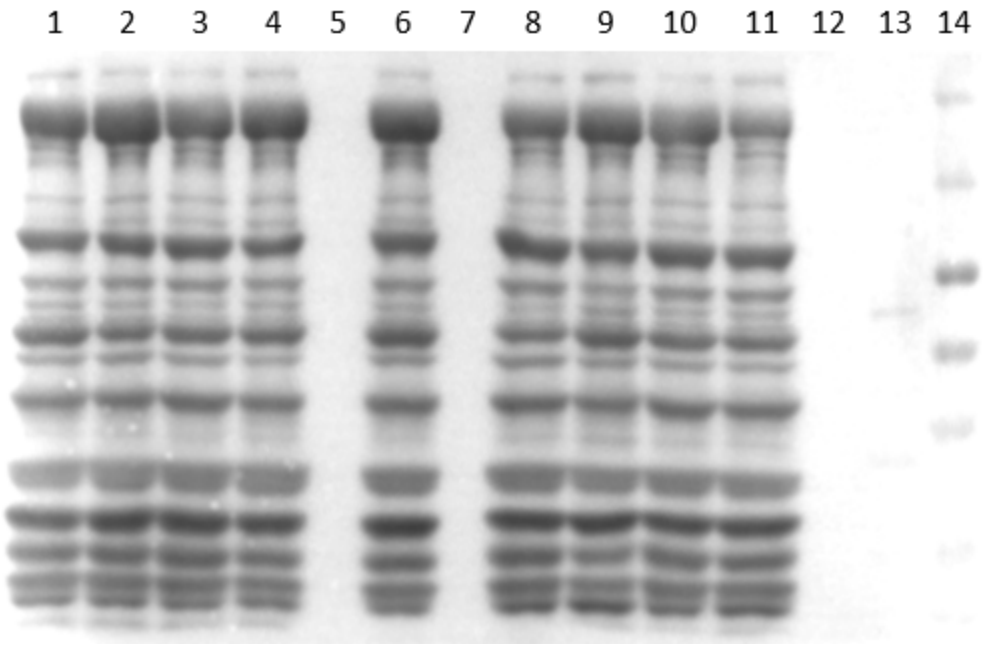


Figure A.2.1 Representative image of the total protein stain
Lanes 1-4 are bovine muscle samples obtained from one block of steers post glucose challenge, lane 6 represents the pooled sample of bovine muscle as the positive control, and lanes 8-11 are bovine muscle samples obtained from the same block of steers before the glucose challenge. Lane 13 contains Magic Mark (only visible after chemiluminescence) and lane 14 contains Spectra, both are molecular weight markers. Lanes 5, 7, and 12 are empty lanes.

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