CHEMICAL INHIBITION OF THE THYROID GLAND AND ITS EFFECTS ON

E. COLI O157:H7 FECAL SHEDDING PATTERNS IN SHEEP

A Thesis

by

SASHA BROOKE SCHROEDER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Animal Science

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Approved by:

Chair of Committee,	Shawn Ramsey
Committee Members,	Ken Genovese
	Jayne Zajicek
	Chris Skaggs
Head of Department,	Gary Acuff

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ABSTRACT

Chemical Inhibition of the Thyroid Gland and Its Effects on *E. coli* O157:H7 Fecal

Shedding Patterns in Sheep. (August 2005)

Sasha Brooke Schroeder, B.S., Texas A&M University

Chair of Committee: Dr. Shawn Ramsey

Due to the seasonal nature of *E. coli* O157:H7 shedding and of hormone production by the thyroid gland, two studies were initiated to determine whether chemical inhibition of the thyroid gland influences fecal shedding of *Escherichia coli* O157:H7. Twenty-four crossbred sheep (68.6 kg BW) were randomly assigned to pen and either 0.0 mg/kg BW PTU or 20 mg/kg BW PTU for 5, 11, or 14 days. Sheep were experimentally infected (d 0) with *E. coli* O157:H7 11 days prior to PTU treatment. Fecal and serum samples were collected for bacterial enumeration and for analysis of T_3 and T_4, respectively. Sheep were humanely euthanized and tissue and content samples were collected from the rumen, ileum, colon and rectum.   Detection of *E. coli* O157:H7 increased toward the terminal end of the GI tract. In the treatment group, serum T_3 levels decreased to an overall lower level than the control group. A correlation was seen between T_3 levels and daily O157:H7 bacterial shedding (P=0.003; r=0.37). In experiment 2, 12 growing lambs (41.04 kg BW) were exposed to either 0.0 mg/kg BW PTU or 40 mg/kg BW PTU for 21 days. Fecal samples were collected for analysis of generic *E. coli* and body weights were recorded on days 0, 7, 14, and 21. Feed intake was recorded throughout the experiment. Animals were experimentally infected with *E. coli* O157:H7 on day 15. Sheep were humanely euthanized on day 21 and GI tract tissue

and content was collected from the rumen, ilium, colon and rectum. A date by treatment interaction was observed for T_4 (P=0.0016) and hormone levels decreased in treated animals. Thyroxine and *E. coli* O157:H7 display a multivariate treatment (P=0.0005) and date effect (P=0.0174) but no significant interaction. Triiodothyronine and *E. coli* O157:H7 shedding have a slight date trend (P=0.065) but no significant treatment or treatment by date interaction. Generally, the treatment group shed genreric *E. coli* at higher levels throughout the study period with slightly more than a log count difference between groups at the last collection point (control = 3.8 CFU/gram of feces ($\log_{10}$); treatment = 4.9 CFU/gram of feces ($\log_{10}$)). Results from these experiments suggest that correlations exist between both *E. coli* O157:H7 and generic *E. coli* shedding in sheep.

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"Work like you don't need the money.

Dance like no one is watching.

And love like you've never been hurt."

-Mark Twain

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION AND LITERATURE REVIEW

Introduction

It is estimated that nearly 76 million people are infected with a foodborne illness in the United States every year. Recent estimates of these cases attribute 74,000 to *E. coli* O157:H7 (Mead et al., 1999; CDC, 2004). At an estimated cost of $2.9 to $6.7 billion in productivity losses and medical costs annually (Buzby et al., 1996), *Escherichia coli* O157:H7 is the leading cause of both acute kidney failure in children and bacterial bloody diarrhea. This pathogen has been isolated from cattle at all stages of production (Laegreid et al., 1999; Elder et al., 2000) and ruminants are considered primary reservoirs for these bacteria. Infected animals are typically asymptomatic while shedding these pathogens into the environment (Hancock et al., 1997; Bach et al., 2002). The seasonality of *E. coli* O157:H7 shedding patterns are well established with shedding levels higher in the summer months in both sheep and cattle (Hancock et al., 1997; Chapman et al., 2001; Barkocy-Gallagher et al., 2003).

Thyroid hormone production is also seasonal in nature with concentrations being highest during the winter months and lowest during the long days of summer (Karsch et al., 1995; Souza et al., 2002). The thyroid gland plays an important physiological role in the body. Through secretion of triiodothyronine (T_3) and thyroxine (T_4), the thyroid gland is integral in controlling the body's metabolic rate, and regulation of the growth,

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This thesis follows the style and format of the Journal of Animal Science.

energy and development of tissues, regulation of heart rate, lipid, carbohydrate and nitrogen metabolism, as well as maintenance of the immune system and myelination of nervous tissue (Wilson et al., 1998; Sherwood, 2001). Thyroid hormones are also responsible for regulation of the growth and differentiation of the epithelial cells in the gastrointestinal tract (Wilson et al., 1998).

Escherichia coli *O157:H7*

Escherichia coli O157:H7 was first recognized as a foodborne bacterial pathogen in 1982 (Barkocy-Gallagher et al., 2003). Each year nearly 74,000 people are infected with *E. coli* O157:H7 resulting in approximately 61 deaths annually. In addition, this bacteria is the leading cause of acute kidney failure in children, haemolytic uremic syndrome and bacterial bloody diarrhea in both children and adults (CDC, 2004).

In 1993, the first multistate outbreak involving *E. coli* O157:H7 occurred, spanning Washington, California, Idaho and Nevada. Of nearly 700 individuals affected, there were 195 hospitalizations and four deaths (Salyers and Whitt, 2002). Fifty-five individuals developed hemolytic uremic syndrome (HUS) as a result of consuming contaminated hamburger meat. Hemolytic uremic syndrome is a disease caused by Shiga toxin producing enterohemorrhagic *E. coli* (EHEC). Disease is characterized by microangiopathic haemolytic anemia, thrombocytopenia, renal failure and increased levels of tumor necrosis factor-α (TNF-α), an inflammatory cytokine released by macrophages, in the brain and neural cell apoptosis (Salmon and Parry, 1997). The 1993 outbreak was traced back to 73 Jack in the Box® restaurants. It was determined that contaminated ground beef was undercooked prior to being served to the

public (Salyers and Whitt, 2002). Jack in the Box® incurred $160 million dollars in losses as a result of the outbreak.

In 1994, *E. coli* O157:H7 became the first microorganism considered an adulterant of ground beef by the U.S. Food Safety and Inspection Service (FSIS) (Barkocy-Gallagher et al., 2003). Improper handling and cooking of contaminated meat is an important source of human infection. The bacteria is highly resistant to acids, yet fairly susceptible to heat (Salyers and Whitt, 2002). Therefore, the United States Department of Agriculture (USDA) mandates labeling of all meat and poultry products with safe handling instructions including thorough cooking of meat to kill bacteria (Morbidity and Mortality Weekly Report, 1994).

Costs

Since its emergence, *E. coli* O157:H7 has been responsible for $29 to $60 million dollars in medical costs annually. Losses due to decreased productivity in infected individuals results in an additional $200 to $600 million annually. Each year the total costs of *E. coli* O157:H7 in the human sector is $301 to $726 million (Buzby et al., 1996). Significant costs have resulted in the cattle sector because of this bacteria as well. Losses are due to decreased beef demand following recalls ($1.6 million annually), packer expenses ($400 million), recall costs ($100 million), plant closure and research costs (Kay, 2003).

Prevalence

Sheep and cattle are considered major reservoirs for *E. coli* O157:H7 and are typically asymptomatic while shedding these pathogens into the environment (Kudva et al., 1996; Hancock et al., 1997; Bach et al., 2002). *Escherichia coli* O157:H7 has been isolated from cattle at all stages of production (Laegreid et al., 1999; Elder et al., 2000). Elder et al. (2000) examined the overall prevalence of this bacteria at slaughter and found a 28% prevalence rate on beef carcass swabs, much higher than previously estimated for *E. coli* O157:H7 in cattle. Chapman et al. (2001) reported a much lower prevalence rate of 12.9% for beef cattle at slaughter. Obviously, the presence of bacteria at slaughter affects the possibility of contamination of meat products going to the consumer. Hides are considered a major source of carcass contamination with up to 51% of feedlot cattle hides found to be positive for *E. coli* O157:H7 (Barkocy-Gallagher et al., 2003). It has been demonstrated that the bacteria can be transferred from the hide to the carcass during processing (Barkocy-Gallagher et al., 2003). Alternately, the prevalence of *E. coli* O157:H7 in sheep at slaughter was determined to be 1.4% (Chapman et al., 2001). However, contamination at the retail level for beef and sheep products was found to be 0.44% with sheep products contaminated more often than beef (Chapman et al., 2001), indicating slaughter techniques and sanitation methods are largely successful in eliminating this pathogen at the abattoir. Kudva et al. (1996) demonstrated prevalence levels of up to 4% in naturally infected sheep carcasses and up to 35% in free ranging sheep.

Season

Reported human cases of *E. coli* O157:H7 infection tend to be highest in the warmer months of the year (Barkocy-Gallagher et al., 2003). A surveillance of foodborne disease outbreaks by the Center for Disease Control (CDC) revealed outbreaks of *E. coli* O157:H7 frequently occur between March and August. In addition, beef was the most common vehicle of transmission of foodborne illness (Morbidity and Mortality Weekly Report, 2000). This lead researchers to examine the seasonal prevalence of *E. coli* O157:H7 in sheep and cattle. At commercial abbatoirs and at various production settings, *E. coli* O157:H7 is isolated at higher rates from May to September in both sheep and cattle (Kudva et al., 1996; Hancock et al., 1997; Chapman et al., 2001; Barkocy-Gallagher et al., 2003). An intermittent shedding pattern for this bacteria has also been documented (Kudva et al., 1996; Hancock et al., 1997; Chapman et al., 2001; Barkocy-Gallagher et al., 2003). When sampling carcasses at slaughter, prevalence rates were highest during the spring and summer and lowest during the winter months for hides, preevisceration, and postintervention beef carcasses (Barkocy-Gallagher et al., 2003). In order to estimate the overall herd prevalence of *E. coli* O157:H7 before reaching slaughter, fourteen dairy cattle herds were sampled monthly for one year. A seasonal pattern with the majority of samples testing positive in the summer months was reported by Hancock et al. (1997). The same trend has been seen in free range ewes with 31% testing positive in June, 5.7% positive in August, and 0% testing positive in November (Kudva et al., 1996).

Thyroid Gland

Located caudal to the larynx and lying across the trachea, the two-lobed thyroid gland acts via hormone receptors present in all tissues of the body (Sherwood, 2001). The thyroid gland is an important endocrine gland responsible for secretion of the hormones triiodothyronine (T_3) and thyroxine (T_4). In the body, T_3 and T_4 help to regulate the basal metabolic rate, the growth, energy and development of tissues, as well as lipid, carbohydrate and nitrogen metabolism. These hormones are also responsible for regulation of the heart rate, maintenance of the immune system, myelination of nervous tissue and, in the gastrointestinal tract, regulation of the growth and differentiation of the epithelial cells (Wilson et al., 1998).

Hormone Synthesis and Secretion

The thyroid hormones T_3 and T_4 have a variety of functions in the body. Genetically, the synthesis of T_3 and T_4 is efficiently regulated by the thyroglobulin gene (Wilson et al., 1998). Within the thyroid gland itself, the availability of exogenous iodine is crucial to the synthesis of T_3 and T_4. Iodine participates in metabolic reactions within the thyroid gland to produce the active hormones. During iodine deficiency, levels of type II iodothyronine 5' deiodinase increases in the brain and enhances the conversion of T_4 to T_3 (Oppenheimer and Schwartz, 1997). Hormone synthesis begins via oxidation of iodide by hydrogen peroxide and thyroid peroxidase (Wilson et al., 1998). Initially, the reactions result in the incorporation of iodine intermediates into monoiodotyrosine (MIT) and diiodotyrosine (DIT), two inactive tyrosine molecules

(Wilson et al., 1998). The active hormone, thyroxine is formed by the coupling reaction in which two DIT molecules are joined via an ether bridge (Wilson et al., 1998) (Figure 1).

In the body, thyroid hormone levels are maintained via two pathways. The hypothalamohypophyseal negative feedback loop is activated when levels of T_3 increase and inhibit thyrotropin-releasing hormone (TRH) secretion from the anterior pituitary (Cole et al., 1994; Sherwood, 2001). Hormone levels are also maintained by 3 iodothyronine deiodinase enzymes that activate or metabolize T_3 and T_4 (O'Shea and Williams, 2002). The three enzyme system consists of type I (D1), type II (D2), and type III D3) deiodinases. Type I enzymes are responsible for cleaving one iodine from T_4 to form an active T_3 molecule. Type II enzymes form the inactive rT_3 from T_4 and convert rT_3 to its active form. Lastly, the type III enzyme system converts T_3 to T_2. Each enzyme system is important for maintaining the hormone balance within the system (Malik and Hodgson, 2002). The type I deiodinases are responsible for roughly 30-40% of the extrathyroidal production of T_3 and are located primarily in the liver and kidney. The remainder of the extrathyroidal production of T_3 can be attributed to the type II enzyme system located in the pituitary, the central nervous system and in the skeletal muscle (Malik and Hodgson, 2002).

Inactive Iodine (I⁻) + Thyroid Peroxidase (TPO) + Hydrogen Peroxide (H_2O_2)

$\downarrow$

Active Iodine (I^+) $\longrightarrow$ I^+ + Thyroglobulin

$\downarrow$

Diiodotyrosine (DIT) and $\longrightarrow$ Coupling Reaction (TPO + H_2O_2)

$\downarrow$

T_4 and T_3

$\downarrow$

T_4 + Type I 5' deoidinase $\longrightarrow$ T_3

Figure 1. Thyroxine and triiodothyronine biosynthesis.

Thyroid Hormones

      The chemical structures of T_3 and T_4 are presented in Figure 2. Thyroxine is produced at a much higher rate in the body than T_3. There are three to four T_4 molecules produced per mole of thyroglobulin. Thyroxine is the predominant hormone in the

peripheral circulation and is directly secreted by the thyroid gland (Wilson et al., 1998).

This hormone is more stable in the peripheral circulation and helps to stabilize and

regulate circulating levels of T_3. However, it is often thought of as a "prohormone"

because it does not have the metabolic effects of T_3 (Capuco et al., 2001).

Triiodothyronine is more metabolically active than T_4 because of its affinity for its

nuclear receptor.   The hormone binds more strongly to the T_3R receptor and therefore

remains in the circulation for a longer period of time (Oppenheimer and Schwartz,

1997).

Thyroxine   **Triiodothyronine**
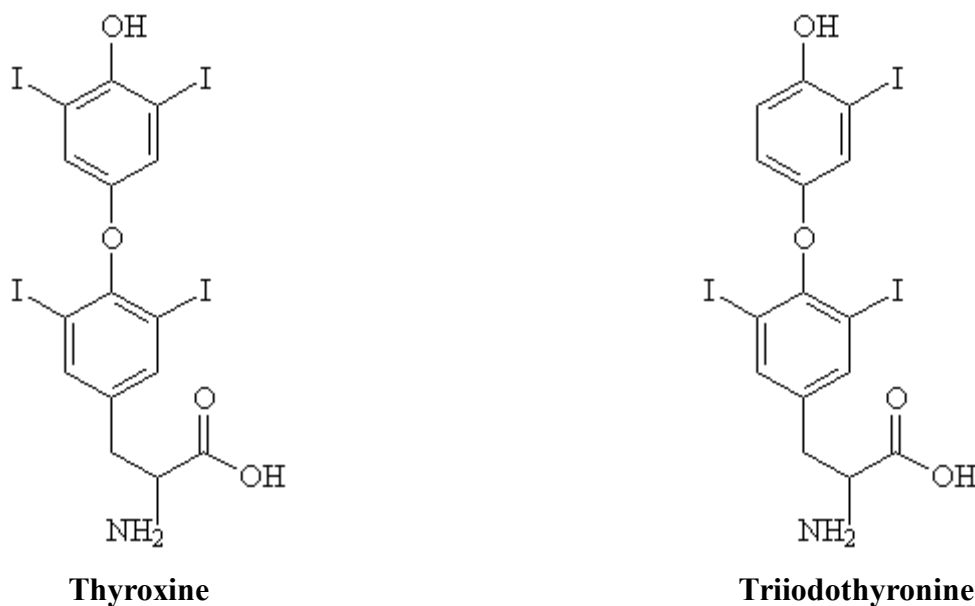
Figure 2. Chemical structures of the two major thyroid hormones thyroxine (T_4) and

triiodothyronine (T_3).

Muscle Tissue

In the body, thyroid hormones have a variety of functions and act on all tissues. Thyroid hormones are responsible for maintaining the basal metabolic rate, lipid metabolism, carbohydrate metabolism, nitrogen metabolism and regulation of energy, growth and development (Sokkar et al., 2000). In muscle tissue, hormones repress or activate myosin heavy chain (MHC) genes. Mysosin heavy chain I genes are responsible for slow twitch fibers while MHC II genes control fast twitch fibers. Changes in plasma T_3 alter the maximum shortening velocity and also the isometric twitch contraction and/or relaxation times. In muscle tissue, thyrotoxicosis results in a shift toward MHC II. Alternatively, hypothyroidism causes a shift to MHC class I. Hypothyroidism also results in decreased calcium transport in the muscle sarcoplasmic reticulum (O'Shea and Williams, 2002). Hyperthyroidism induces an increase in the amount of sarcoplasmic reticulum present in the muscle tissue, and also the percent of fibers expressing the slow type of sarcoplasmic reticulum (SERCa2). In the heart, muscle growth, cardiac output, conductance and heart rate are all affected by thyroid hormone levels. Low hormone levels lead to bradycardia, or slow heart rate, while higher hormone levels lead to tachycardia, or fast heart rate.

Central Nervous System

In the central nervous system, T_3 affects the brain, neurons and glial cells in a positive manner. Hypothyroid individuals experience depressed myelination of nervous tissue, decreased proliferation and migration of cells, decreased branching of the dendrites and axons, and inhibition of the synapse formation responsible for conduction

of signals across nerve cells within the nervous system (Gilbert, 2004). Normal brain development during fetal and neonatal life is dependent upon healthy thyroid hormone levels as well. Late brain development in particular is influenced by thyroid hormones (Schoonover et al., 2004). Myelination of nerve cells is a key function of thyroid hormones. Thyroidectomy of rats has been shown to depress cerebral myelination. In sheep infected with Border disease, a pestivirus that infects the thyroid gland, depressed myelination of the central nervous system is a characteristic symptom (Anderson et al., 1988). During late brain development, neurons begin to mature and become myelinated. This process is of particular importance in the interhemispheric space between the two halves of the brain. Information transfer across this space is crucial for higher brain functions such as learning and memory (Schoonover et al., 2004). In hypothyroid individuals, the number of myelinated axons in the interhemispheric space as well as the thickness of the myelin sheath is decreased. Myelination in the interhemispheric space is controlled by oligodendrocytes. The proliferation, survival and myelin production by these cells is dependent upon thyroid hormones (Schoonover et al., 2004).

Temperature Regulation

Triiodothyronine is also responsible for metabolism, regulation of body temperature, and thermogenesis (Golozoubova et al., 2004). Mice lacking all viable thyroid hormone receptors (TR ablated mice) were examined to determine the role of thyroid hormones in temperature acclimation and metabolism. Mice lacking hormone receptors displayed a depressed body temperature and a significantly decreased basal metabolic rate. Traditionally, when mammals are exposed to cold temperatures the

secretion of thyroid hormones increases (Dauncey, 1990). However, TR ablated mice are unable to adequately produce heat at low temperatures and thus become cold sensitive (Golozoubova et al., 2004). During hypothyroidism, there is a reduced consumption of oxygen and lower heat production (O'Shea and Williams, 2002). Alternately, during hyperthyroid states, an increased oxygen consumption trend is seen. Thus, the excessive weight loss in hyperthyroid individuals can be attributed to accelerated catabolism of food and an increased metabolic rate (Dauncey, 1990).

Liver

The liver is the main peripheral organ for metabolism and storage of thyroid hormones (Miller et al., 1978). Cirrhosis of the liver is a disease state characterized by an increase of scar tissue within the organ that replaces normal healthy tissue, which inhibits normal blood flow through the liver. Cirrhosis of the liver results in a decrease of total T_3 and T_4. This reduction is similar to that observed in chronically ill patients and most likely reflects a reduction in type I deiodinase activity (Malik and Hodgson, 2002). Interestingly, when assessing 118 patients suffering from cirrhosis of the liver, a 17% increase in thyroid gland size was observed (Bianchi et al., 1991). Triiodothyronine accumulates in the liver more quickly than T_4 because it is not as strongly bound in the serum (Miller et al., 1978). As a result, there is more T_3 secreted in the bile than T_4. Bile is secreted into the GI tract by the liver and is responsible for digestion of fat and fat soluble vitamins. The liver is also the main site of cholesterol and triglyceride metabolism (Malik and Hodgson, 2002). Triiodothyronine affects hepatic uptake and synthesis of cholesterol. During states of hypothyroidism, serum

cholesterol increases (O'Shea and Williams, 2002). Low density lipoprotein (LDL) receptors are enhanced in the presence of thyroid hormones. In addition, the activity of lipid-lowering liver enzymes is increased which lowers serum LDL levels. In the presence of T_3 and T_4, the expression of high density lipoproteins (HDL) is enhanced (Malik and Hodgson, 2002). This affect of thyroid hormones in the liver may help reduce the onset of atherosclerosis (hardening of the arteries) if managed correctly (Malik and Hodgson, 2002).

Immune System

Thyroid hormones also have strong effects on the immune system. During prolonged cold stress, hormones increase in order to counteract the negative effects of glucocorticoids (Davis, 1998). This mechanism allows for maintenance of immune system homeostasis. Thyroid hormones also stimulate the thymus, which is responsible for T cell production, and the bone marrow, responsible for B cell production (O'Shea and Williams, 2002). This stimulation maintains the innate and cell mediated immune systems. During cold stress, hormones also stimulate lymphocytes, the spleen and lymph nodes (Davis, 1998). In sheep, thyroid status at birth has been shown to dramatically affect passive immunity. Hyperthyroid lambs were less able to absorb immunoglobulin G (IgG) from maternal colostrum and thus had depressed passive immunity in the first twelve hours of life (Cabello et al., 1983). A decrease in circulating lymphocytes was linked to hypothyroidism (Comsa et al., 1979). In calves, infectious bovine rhinotracheitis virus challenge was shown to depress plasma T_3 levels and circulating lymphocytes. When treated with T_3 injections, infected calves responded

with higher antibody titers when compared to controls (Cole et al., 1994). In addition, the phagocytic immune cells, neutrophils, are strongly affected by thyroid hormone levels. The ability of neutrophils to kill and digest bacteria is enhanced when they degrade T_3 and T_4 (Inan et al., 2003).

During most chronic illness, the metabolism of thyroid hormones is affected (Malik and Hodgson, 2002). A condition known as sick euthyroid syndrome is characterized by a reduction in D1 enzyme activity causing normal T_4 levels, but depressed T_3 levels. It is thought that this reduction in circulating T_3 levels is beneficial and actually increases survivability of affected individuals. The decrease in circulating thyroid hormone levels reduces the basal metabolic rate, thereby reducing caloric requirements of chronically ill individuals (Malik and Hodgson, 2002).

Intestinal Immune System

Thyroxine has been shown to affect intestinal intraepithelial lymphocytes (IEL), a distinct population of CD8 T cells. Interestingly, T_4 supplementation negatively affected developing, but not mature IEL in mice. In addition, $\gamma\delta$ T cells, the predominant T cells in the GI tract of ruminants, directly stimulate the development and growth of intestinal enterocytes (Wang and Klein, 1996). The immunosuppressive effects of T_4 were not observed in peripheral T cells. The small intestine is an important site of thyroid stimulating hormone (TSH) secretion (Wang et al., 1997). Thyroid stimulating hormone is inhibited by T_4 and is capable of altering antibody production by B cells (Wang and Klein, 1996). This could have a profound effect during times of infection when B cells must target invading cells with the proper antibodies.

Gastrointestinal Tract

Thyroid hormones have a profound effect on the gastrointestinal (GI) tract. The lumen of the rat gut contains 1950% as much T_3 and 60% as much T_4 as the entire periphery circulation (Hays, 1988). Thyroid hormones are secreted into the GI tract via bile and through the mesenteric capillary bed (Hays et al., 1992). The GI tract is clearly an important site of hormone absorption. The colon and ileum are the most efficient sites of absorption while these hormones are incompletely absorbed in the jejunum and duodenum (Miller et al., 1978). A variety of factors affect the rates of absorption of thyroxine in the gut such as intraluminal proteins, gut flora, germ-free intestinal contents and other dietary substances. These factors do not seem to play a role in T_3 absorption (Miller et al., 1978). Thyroxine indirectly affects the maturation of intestinal enzymes responsible for efficient functioning and digestion (Morisset, 1993). Stimulation of cell mitosis and growth of the intestinal mucosa crypt zones is an important function of T_3 and T_4 (Middleton, 1971). These hormones also regulate the growth, differentiation, and barrier function of the mucosa of the GI tract (Hodin et al., 1996). This helps to control the ingestion of food and the actual chemical and physical presence of food in the gut (Morisset, 1993). Hyperthyroidism results in increased jejunal secretions and intestinal mucosa hypertrophy (Miller et al., 1978). Triiodothyronine in adults is trophic for crypt cells and is able to alter the expression of brush border enzymes (Hodin et al., 1996). Removal of the thyroid gland early in development has been shown to prevent normal maturation of the small intestine (Middleton, 1971). The small intestine is lined by epithelium, 95% of which are enterocytes. These enterocytes are constantly being

renewed and are responsible for nutrient digestion and absorption. The most important regulator of enterocyte growth and differentiation is T_3 (Hodin et al., 1996). Miller et al. (1974) demonstrated that in cattle, T_4 enhances alimentary tract mobility. Cattle with damaged thyroid glands consistently have higher rumen fill. Throughout the experimental period, thyroid damaged cattle consumed less and so it can be assumed that the greater rumen fill is due to prolonged feed retention (Miller et al., 1974). The motor activity of the GI tract is greatly increased during periods of hyperthyroidism and depressed during hypothyroidism. Depressed activity during hypothyroidism can lead to constipation, atrophy and/or obstruction of the bowels. The duodenum and colon can become enormously distended and structurally altered during hypothyroidism (Middleton, 1971). Kennedy et al. (1977) demonstrated that GI tract digestibility was altered during cold stress as well. An increased rate of passage was found to be due in part to increased levels of thyroid hormones in the gut (Kennedy et al., 1977).

Factors Affecting Thyroid Hormone Levels

Other Hormones

Physiological thyroid hormone levels are affected by a large number of factors. Other circulating hormones, for instance, can influence T_3 and T_4. Melatonin, a pineal hormone released at night, is generally considered to be responsible for regulation of the circadian rhythm. It is often used in humans to regulate sleep disorders and as a remedy for "jet lag". Melatonin acts directly on the hypothalamus and the pituitary glands to inhibit the secretion of follicle stimulating hormone (Wilson et al., 1998). Altered thyroid physiology has been observed in species undergoing daily melatonin injections.

Daily melatonin injections reduced serum T_4 levels. It is hypothesized that T_4 is reduced when melatonin acts directly on the hypothalamus altering the hypothalamic-pituitary-endocrine axis (Champney, 2001). Additionally, in lactating dairy cattle, administration of somatotropin increased serum concentrations of T_4 by 12% (Capuco et al., 2001).

Nutrition

Plane of nutrition has been shown to affect circulating levels of T_3 and T_4. During starvation, tumor necrosis factor-α decreases deiodinase activity and reduces conversion of T_4 to T_3 (Abecia et al., 2001; Capuco et al., 2001). In lambs, a decrease in circulating T_3 and T_4 has been observed during restrictive feeding. Interestingly, during refeeding, T_3 levels rose to control levels within two weeks, but T_4 levels remained low. This is perhaps due to an inhibition of T_4 synthesis during restrictive feeding (Wester et al., 1995). In cattle, restricted nutrition has had a similar effect on circulating hormone levels (Hammond et al., 1984; Murphy and Loerch, 1994; Hersom et al., 2004). A 36% reduction in T_4 was observed in cattle on restricted diets. In addition, T_3 rebounded more completely during the refeeding phase (Hayden et al., 1993). The reduction in plasma T_3 levels during restriction feeding may serve to reduce the maintenance energy requirements and protein degradation. This may help to explain the increased feed efficiency observed during this time (Murphy and Loerch, 1994; Hersom et al., 2004).

Stress

The thyroid gland has been shown to respond to various stressors (Hennessy and Prichard, 1981). Serum T_3 levels decrease in response to feed and water restriction, protein deficiency, heat, parasitic infection, dexamethasone injections, and non-thyroidal

illness (Cole, 1994). Immediately following stressful situations that cause an increase in

adrenal hormone secretion, thyroid hormone levels increase (Falconer and Jacks, 1975).

Although, short term administration of physiological levels of corticosteroids to sheep

was unable to increase circulating thyroid hormone levels. It was thus determined that

corticosteroids do not directly act to increase thyroid hormone levels (Falconer and

Jacks, 1975). During heat stress, thyroid hormone levels in sheep have been shown to

drop below levels observed in nutrient restricted sheep. However, heat stressed sheep

demonstrate a faster recovery period, with T_4 levels rebounding more quickly than in

feed restricted sheep (Valtorta et al., 1982). According to Little (1985), the decrease in

thyroid hormones during illness may serve to prolong survival by conserving metabolic

energy and maintaining homeostasis. In fact, T_4 supplementation during infection with

S. pneumoniae increased mortality rates when compared to controls (Little, 1985).

Similar results have been seen in other species. In chickens, supplementing T_3 and T_4 in

the feed resulted in lower thyroid gland weights and decreased ability to fight off

infection. *Escherichia coli* endotoxin resulted in an increased rate of mortality in T_4

treated chicks (Heller and Perek, 1972). The thyroid gland is often infected during

disease progression depressing circulating thyroid hormone levels (Anderson, 1987;

Sawyer and Osborn, 1993). Parasitic infection of sheep is able to depress circulating

levels of T_4 (Hennessy and Prichard, 1981). Sepsis conditions can also affect thyroid

hormone levels. Richmond et al. (1980) demonstrated a portion of the drop in thyroid

hormone levels during sepsis is due to the depressed nutritional status of septic patients.

While Little (1985) demonstrated increased mortality during thyroid hormone

supplementation, Inan et al. (2003) found an increased level of survival during supplementation of septic individuals. Thyroid hormones target bacterial killing via neutrophils. Interestingly, Inan et al. (2003) found extremely low levels of serum T_4 increased mortality from 64 to 84%. Therefore, the ability of thyroid hormones to affect mortality during sepsis does not depend upon the mere presence or absence of hormones, it depends on the level present in the circulation.

Season

The seasonal effects of thyroid hormones are well known, particularly in species known as "seasonal breeders" like sheep. Circulating concentrations of T_4 have been shown to be inversely correlated with ambient temperature. Valtorta et al. (1982) examined the effects of heat stress and feed restriction on circulating thyroid hormone levels. Sheep stressed at 35°C had decreased feed intake when compared to controls and lower thyroid hormone levels. In order to determine if the depressed hormone level was due to decreased feed intake or heat, the feed stressed group was fed at the level of intake of the heat stressed sheep. While the feed restricted sheep did display a decrease in circulating hormone levels, the heat stressed sheep exhibited a larger decrease (Valtorta et al., 1982). The authors concluded the decrease in hormones was due to heat rather than feed intake (Valtorta et al., 1982). Hormone levels have been shown to be higher during the winter months when temperatures are lower and decrease in the summer months when temperatures are high (Souza et al., 2002). Changes in ambient temperature during a 24 hour period also affect hormone secretion with the highest levels detected in the afternoon hours (Souza et al., 2002). In sheep, thyroidectomy

inhibits normal seasonal changes in the reproductive cycle (Karsch et al., 1993; Souza et al., 2002). In rams, the absence of thyroid hormones routinely suspends normal reproductive function. Thyroxine is thought to regulate this response by inhibiting an essential energy source, hexosemonophosphatase, during steroidogenesis and by actually depressing the number of gonodotropin receptors in testicle cells (Souza et al., 2002). Interaction between the thyroid and the reproductive neuroendocrine axis is also important in the ewe. Triiodothyronine and thyroxine are necessary for inhibition of gonadotropin releasing hormone (GnRH) secretion that is responsible for transition to anestrus. This occurs via a change in the GnRH neurosecratory system. In thyoidectomized ewes, the pulsatile secretion of GnRH into the hypophyseal portal blood circulation during anestrus is not blocked by estradiol. This allows for an extended breeding season (Moenter et al., 1991). Like rams, ewes experience the highest levels of circulating thyroid hormones in the winter months when anestrus occurs (Thrun et al., 1997). In addition, thyroid hormones are only required during the late stages, the last 2 months, of the breeding season for anestrus to occur (Thrun et al., 1997). Photoperiodic cues are necessary for anestrus to begin following the breeding season. Thyroxine must also be present at this time for successful transition to occur (Webster et al., 1991).

Chemical Inhibition

A variety of naturally occurring substances have antithyroid properties. Contaminated drinking water has been found to harbor strains of *E. coli* that produce antithyroid compounds (Vought et al., 1974). Propylthiouracil (PTU) is a known

chemical inhibitor of thyroid function in many species (Figure 3). It is used in humans to control hyperthyroidism and is often used experimentally to induce hypothyroid states (Sherwood, 2001). During hypothyroid states, the release of thyrotropin-releasing hormone (TRH) is inhibited in the hypothalamus. Propylthiouracil is able to create a hypothyroid state by both decreasing the release of T_4 and T_3 from the thyroid gland and inhibiting the conversion of T_4 to T_3 in the peripheral circulation (Achmadi and Terashima, 1995). The release of T_4 and T_3 from the thyroid is reduced because PTU directly affects thyroid utilization of iodine. In the periphery, PTU acts by inhibiting the type I 5'-monodeiodination. This type I monodeiodinase is responsible for activating the reverse T_3 (rT_3), an inactive intermediate in the conversion of T_4 to active T_3 (Villar et al., 1998). Sheep and goats are much more resistant to the effects of the antithyroid drug than cattle (Achmadi and Tershima, 1994; Villar et al., 1998). Serum thyroxine has been reduced in cattle using doses as low as 4 mg PTU/kg BW, whereas in sheep, higher doses are required. Propylthiouracil doses from 20 to 40 mg/kg of BW have been used for varying time periods to sufficiently lower serum thyroxine (Hernandez et al., 2003). It is hypothesized that the dose of PTU may affect hormone circulation in different ways. Low doses of PTU are thought to act on circulating levels of T_4 and T_3 and inhibit the type I 5'-monodeiodinase, thereby decreasing circulating T_3 levels (Villar et al, 1998). On the other hand, high PTU doses are thought to act directly on the thyroid gland and reduce circulating levels of T_4 (Villar et al., 1998). Serum hormone levels tend to recover quickly following termination of PTU treatment; with serum levels of T_3 rebounding more quickly than T_4 levels (Wells et al., 2003). In addition, high doses of

PTU tend to affect younger lambs more than mature sheep as indicated by greater

decreases in circulating T_4 levels (Wells et al., 2003).
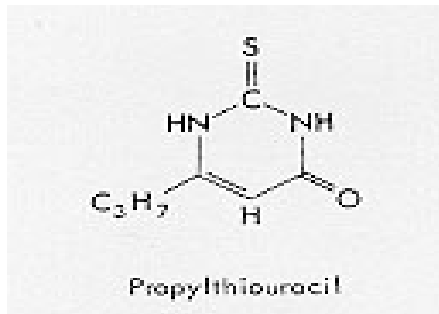
Figure 3. Chemical structure of anti-thyroid compound propylthiouracil (PTU).

Hypothesis and Objective

The seasonal patterns displayed by *E. coli* O157:H7, with levels being highest in

the summer months and lowest in the winter months is inversely correlated with seasonal

fluctuations in thyroid hormone levels. This led us to hypothesize that thyroid function,

and more specifically, production of T_3 and T_4, may play a role in the seasonality of *E.*

coli O157:H7 shedding in ruminants. Therefore we designed two studies with the

objective of determining if chemical inhibition of the thyroid gland in sheep

experimentally infected with *E. coli* O157:H7 would affect fecal shedding or gut

populations of this pathogen.

MATERIALS AND METHODS

Animal Care and Use

Experiments were conducted at the USDA, ARS, Food and Feed Safety Research Unit in College Station, TX. In experiment one, twenty-four 12-16 month old crossbred ewes (average weight of 68.6 kg) were purchased locally from a commercial source. Sheep were allowed to adjust to pens, diet and photoperiod for 16 days. All animals were individually housed in 2.74 m long and 1.83 m wide concrete floored pens with visual access to neighboring sheep and a controlled photoperiod consisting of 16h light and 8h dark for both experiments. Pens were washed daily at 0700 hours after fecal sample collection for experiment 1 and at 1400 hours for experiment 2. Sheep were fed and received fresh water following cleaning. The diet consisted of a moderate quality coastal grass hay, and 1.13 kg per head per day of a 15% protein commercial sheep and goat pellet (Producers Cooperative Association, Bryan, TX) (Table 1), and ad libitum water.

In experiment 2, twelve 5-7 month old growing whether lambs of mixed breeding (average BW of 41.04 kg) were purchased from a local commercial source. Sheep were all individually housed in 2.74 m long and 1.83 m wide concrete floored pens with visual access to neighboring sheep and a controlled photoperiod consisting of 16h light and 8h dark. Animals were allowed to adjust to diet for 6 days before beginning treatments. Individual feed intake was recorded following pen cleaning and all animals received

fresh feed and water at this time. The study diet consisted of an 80% concentrate, 20% forage diet. The concentrate portion of the diet was comprised of a 15% protein commercial sheep and goat pellet (Producers Cooperative Association, Bryan, TX) (Table 1) and moderate quality coastal grass hay at 3.8% of body weight on an as fed basis with ad libitum access to water.

Table 1. Diet composition for experiment 1 and 2 sheep.

Ingredients	Guaranteed Analysis
Crude Protein	(Min) 15.00%
Crude Fat	(Min) 2.50%
Crude Fiber	(Max) 14.50%
Calcium	(Min) 0.80%
Calcium	(Max) 1.30%
Phosphorus	(Min) 0.40%
Salt	(Min) 0.25%
Salt	(Max) 0.50%
Copper	(Min) 8 ppm
Copper	(Max) 13 ppm
Selenium	(Min) 0.3 ppm
Vitamin A	(Min) 15,000 IU/LB

Experiment 1

Treatment and sheep were randomly assigned to pen. Treatment consisted of 0.0 mg/kg BW PTU (CON) or 20 mg/kg BW 6-N-propyl-2-thiouracil (PTU) for 5, 11, or 15 days. Following the 16 day acclimation period, sheep were experimentally infected

(d 0) via oral gavage with *E. coli* O157:H7 (strain 2029; 10 mL of 1.2x 10^{10} CFU/mL).

The inoculation strain was selected for resistance to naladixic acid and rifampicin

(Sigma Chemical Company, St. Louis, MO) in our laboratory through successive

cultivation in tryptic soy broth (TSB) (Difco™, Sparks, MD) containing 20 µg naladixic

acid/mL and 25 µg rifampicin/mL. The anti-thyroid compound 6-N-propyl-2-thiouracil

(Sigma Chemical Company, St. Louis, MO) was administered to sheep via oral gavage

in gelatin capsules at 0700 hours (Torpac, Inc., Fairfield, NJ) once daily beginning on

day 10. Sheep were experimentally infected 10 days prior to beginning PTU treatment,

so that fecal shedding of bacteria could normalize to a more physiological level of 1x 10^{2}

CFU/gram. Daily fecal samples were collected rectally from each sheep with clean

gloves and placed into individual Whirlpacks™ (Modesto, CA) from day 0 to either day

15, 21, or 24 in order to monitor shedding of the experimental strain. Sheep were

humanely euthanized (Euthasol®, euthanasia solution, Del Marva Laboratories, Inc.,

Midlothian, VA) at three separate time points, days 15 (n=8), 21 (n=8) or day 24 (n=7).

Approximately 5-10 grams of gut luminal contents and tissue from the rumen, ileum,

colon and rectum were removed at harvest for bacterial enumeration.

Experiment 2

Experiment 2 was designed to evaluate the effects of a PTU dose twice that of

the 20 mg/kg BW dose used in experiment 1. Lambs were again randomly assigned to

treatments and treatment was randomly assigned to pen. Treatments consisted of 0.0

mg/kg BW PTU (CON) or 40 mg/kg BW PTU (PTU) for 21 days. Lambs were orally

dosed daily at 0700 hours with PTU via a gelatin capsule beginning on day 0. Fecal

samples were obtained via rectal palpation with clean gloves for each lamb and placed into individual Whirlpacks™ for isolation of generic *E. coli* (GEC) on days 0, 7, 14, and 21. Body weights were also recorded at these times. All sheep were allowed to respond to PTU treatements before being experimentally infected with *E. coli* O157:H7 via oral gavage on day 15 (strain 933; 10 mL of 1.97x 10^9 CFU/mL). The inoculation strain was made resistant to naladixic acid and novobiocin (Sigma Chemical Company, St. Louis, MO) in our laboratory via successive cultivation in TSB containing 20 μg/mL naladixic acid and 25 μg/mL novobiocin. Fecal samples were obtained daily via rectal palpation with new gloves and individual Whirlpacks™ for each lamb thereafter for isolation of the experimental strain (d16-21). All sheep were sacrificed (Euthasol®, euthanasia solution, Del Marva Laboratories, Inc., Midlothian, VA) on day 21 for collection of rumen, ileum, colon and rectal tissue and content.

Bacterial Enumeration

One gram of fecal material was diluted in 9 mL of sterile phosphate buffered saline (PBS) (Sigma Chemical Company, St. Louis, MO) and then serially diluted to a 10^5 concentration. Samples were then plated onto MacConkey agar (Difco™, Sparks, MD) supplemented with 20 μg/mL naladixic acid and 25 μg/mL rifampicin for experiment one or 20 μg/mL naladixic acid and 25 μg/mL novobiocin for experiment two and incubated overnight at 37° C in a Revco B0D50A14 incubator.

For GEC quantification during experiment 2, one gram of feces was serially diluted in 9 mL of sterile PBS and plated onto CHROMager *E. coli* agar (CHROMagar Microbiology, Paris, France). Plates were then incubated lid down overnight at 37° C.

One gram of luminal content from each section of the GI tract was serially diluted and plated as previously described for each experiment. Content samples from experiment two were also enriched and plated as described for tissue samples.

Tissue samples were placed directly into 20 mL of GN Hajna broth (Difco™, Sparks, MD) supplemented with 20 µg/mL Naladixic acid and 25 µg/mL rifampicin. Samples were incubated overnight in 50 mL sterile polypropylene centrifuge tubes (Fisher Scientific International, Pittsburgh, PA) at 37° C before being plated onto MacConkey agar supplemented with 20 µg/mL naladixic acid and 25 µg/mL rifampicin for experiment one.   During experiment two, tissue samples were placed directly into 25 mL of sterile PBS to remove excess content. Samples were then enriched in 20 mL of GN Hajna broth supplemented with 20 µg/mL Naladixic acid and 25 µg/mL novobiocin and plated as previously described.

Serum Collection and Analysis

Blood was collected via jugular venipuncture for quantification of serum T_3 and T_4 using Vacutainer™ SST™ gel and clot activator tubes (Fisher Scientific International, Pittsburgh, PA). Blood was allowed to clot at room temperature for 30 minutes, centrifuged (Sorvall® legend RT centrifuge, 3000 x g, 20 minutes, 4° C) and serum was removed and stored at -20° C for later analysis. Serum T_3 and T_4 were analyzed by an independent laboratory using RIA components of commercial kits (Diagnostic Products Inc., Los Angeles, CA). The T_3 and T_4 assays were validated for use in ruminant serum as previously described (Richards et al., 1999; Wells et al., 2003). The within and between assay coefficients of variation were less than 15%.

Statistical Analysis

Gut tissue and content data was analyzed using categorical data analysis techniques using Proc Freq and Proc Logistic analysis in SAS$^{®}$ version 8.2 (Allison, 1991). Body weight, feed intake, T_3, T_4 and bacterial log count data for generic *E. coli* and *E. coli* O157:H7 was analyzed using mixed model analysis with repeated measures using Proc Mixed of SAS$^{®}$ version 8.2. A P-value less than 0.1 was declared significant.

RESULTS

Experiment 1

Gut Tissue and Content

Treatment consisted of 0.0 mg/kg BW PTU (CON) or 20 mg/kg BW 6-N-propyl-2-thiouracil (PTU) for 5, 11, or 15 days. Anti-thyroidal PTU treatment had minimal effect on presence of *E. coli* O157:H7 in GI tissue samples. Detection of *E. coli* O157:H7 in luminal content dilutions increased from rumen, ilium, colon to rectum (Figure 4). The greatest difference in O157:H7 isolation between groups was seen in the ileum with roughly 0.05 log CFU difference between groups. However, more O157:H7 were isolated from the rectum in both groups. Isolation method affected the probability of detecting *E. coli* O157:H7 in content samples. Enrichment of content samples increased the detection of O157:H7 bacteria in roughly 50% of the animals. In the colon, detection was 4.5 times more likely if samples were enriched before plating rather than just serially diluting samples and plating ($P=0.06$). Detection of O157:H7 increased six-fold in rectal samples if enrichment was used ($P=0 .02$). This data indicates that if sheep are shedding at low levels, enrichment of samples greatly increases the probability of detection.
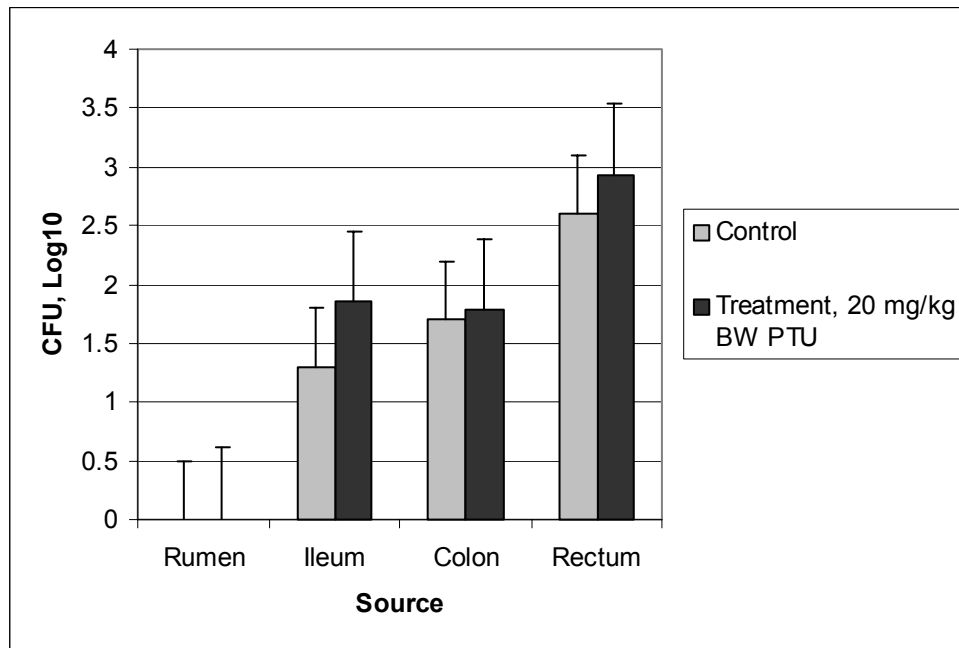
Figure 4. *E. coli* O157:H7 (CFU, Log$_{10}$) enumeration of gut content dilutions of samples collected from the rumen, ileum, colon and rectum at the time of harvest (n=22).

Serum

Prior to treatment, triiodothyronine levels for control and treated animals were similar (2.69 ± .95 ng/mL and 2.62 ± .16 ng/mL respectively) (P=0.40). In the control group, T$_3$ and T$_4$ were strongly correlated to each other (P < 0.0001) and regression analysis revealed a slope of 0.028 for the T$_3$/T$_4$ relationship (R^2=0.6). This relationship between T$_3$ and T$_4$ was not seen in the treatment group (P=0.38).

Twelve days of PTU treatment decreased treated animal's serum T$_3$ levels to 1.57 ± 0.30 ng/mL whereas control group levels remained at 2.06 ± 0.09 ng/mL. A treatment by date interaction (P=0.03) was detected in which T$_3$ levels decreased over the course of the experiment. In the treatment group, T$_3$ levels decreased to an overall lower level than the control group (Figure 5). With the exception of day 9 of serum collection, T$_3$

levels were numerically different between groups and were statistically lower in the

treated group on the final day of collection (P=0.058). Thyroxine was not affected by

treatment (P=0.39). However, there was a date effect (P=0.0006). Thyroxine levels did

not decrease from beginning to end as with T_3 levels (Figure 6). The date effect may

have been influenced by one animal sampled on the second collection day that exhibited

a very high T_4 level. This was interpreted to be a non-biological finding.

Figure 5. Serum T_3 (ng/mL) levels across time for sheep (n=22) receiving either 0.0
mg/kg BW 6-N-propyl-2-thiouracil (PTU) or 20 mg/kg BW PTU for 15 days.
*P=0.01
*P=0.058

A correlation between T_3 levels and daily bacterial shedding was seen in the

treatment group (P=0.003; r=0.37). When T_3 and shedding were modeled as a

multivariate response, there was a significant multivariate treatment effect (P=0.001), multivariate date effect (P<0.0001), as well as a significant multivariate interaction (P=0.05). There was a decreased rate of shedding across the experiment from 1.8 CFU/gram of feces ($\log_{10}$) on d1 to .3 CFU/gram of feces ($\log_{10}$) on d12 (P = 0.02) (Figure 7). Average treatment shedding was 1.54 ± 2.37 CFU/gram of feces ($\log_{10}$) while average control group shedding equaled $.82 \pm 3.0$ CFU/gram of feces ($\log_{10}$) across the experiment (P=0.06).
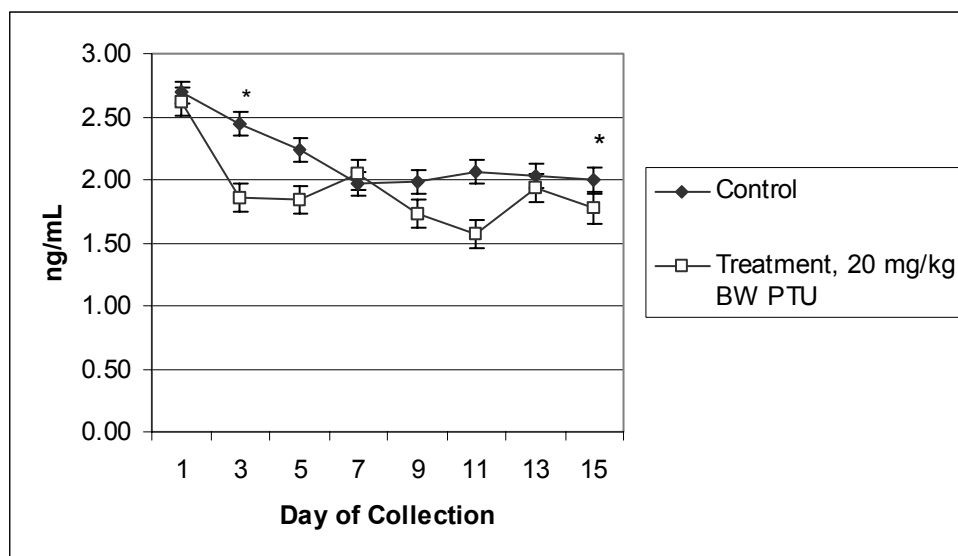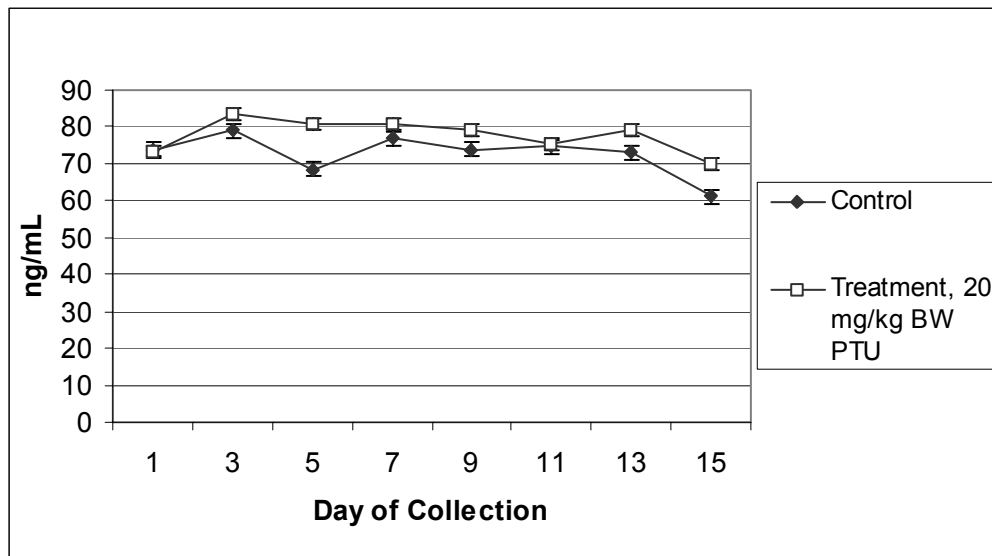

Figure 6. Serum T_4 (ng/mL) levels across time for sheep (n=22) receiving either 0.0 mg/kg BW 6-N-propyl-2-thioruacil (PTU) or 20 mg/kg BW PTU for 15 days.
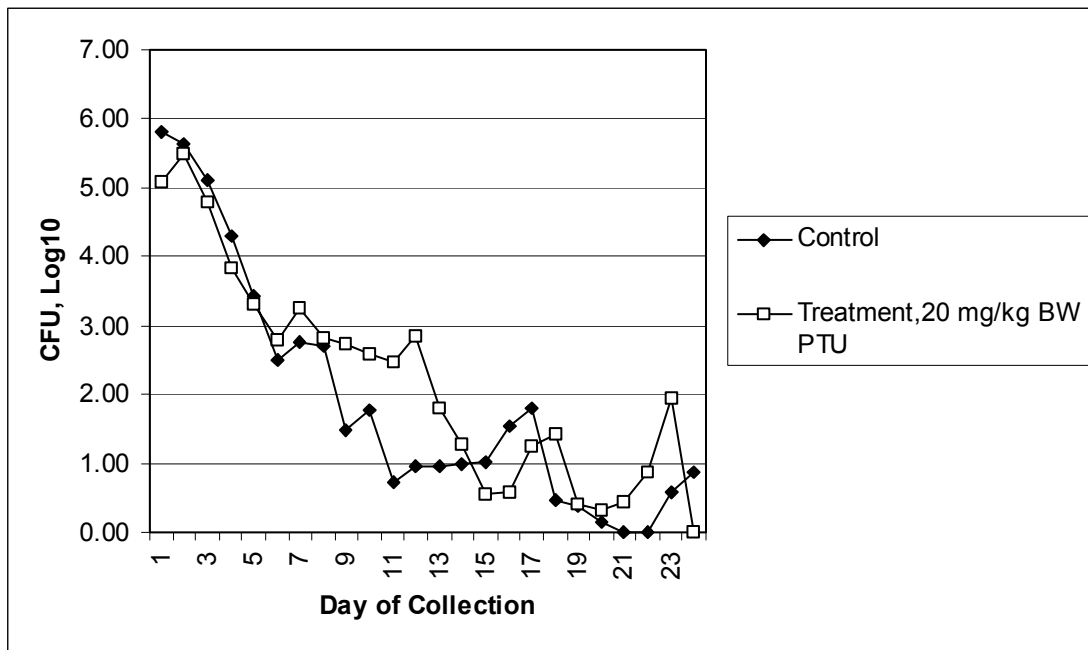
Figure 7. *E. coli* O157:H7 (CFU, Log$_{10}$) fecal shedding patterns from d 0 to d 24 for sheep (n=22) receiving either 0.0 mg/kg BW PTU or 20 mg/kg BW PTU.

Experiment 2

Gut Tissue and Content

There was no statistical difference across treatments for bacteria for gut tissue or content samples. Mean bacterial levels in the rumen, ileum and rectal samples were numerically, but not statistically, higher in the treatment group (Figure 8). Enrichment of samples increased the likelihood of detecting *E. coli* O157:H7 by a factor of 6 in the rumenal content samples (P=0.05) and by a factor of 15 in the colon content samples (P=0.02). When comparing tissue and content samples, the likelihood of detecting *E. coli* O157:H7 was greater in enriched rectal content samples (P=0.05) than in rectal tissue samples.
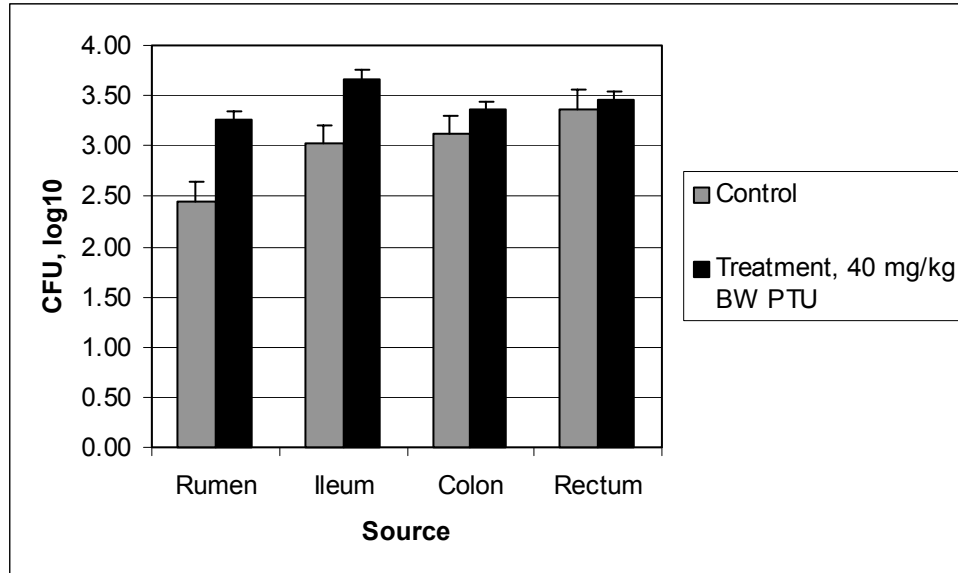
Figure 8. Bacterial (*E. coli* O157:H7) counts from gut content dilutions collected at the time of harvest from sheep (n=12) receiving either 0.0 or 40 mg/kg BW 6-N-propyl-2-thiouracil (PTU).

Body Weight and Intake

Body weight data displayed a treatment by date interaction (P=0.036) in both groups by initial weight loss in the first two weeks followed by weight gain in the following weeks. Treated animals had higher average body weights (41.5 ± 6.4 kg) compared to control animals (36.8 ± 4.7 kg) with both groups gaining weight over the experimental time course (Control=39.9 kg; Treatment=44.7 kg) than beginning weights (Control=36.2 kg; Treatment=41.7kg) (Figure 9).

Figure 9. Recorded body weights of sheep (n=12) receiving 0.0 or 40 mg/kg BW 6-N-propyl-2-thiouracil (PTU).

Average recorded daily feed intake in both groups was similar throughout the study with control animals consuming slightly less (1.48 ± 0.20 kg) than treatment animals (1.51 ± 0.22 kg) on average.
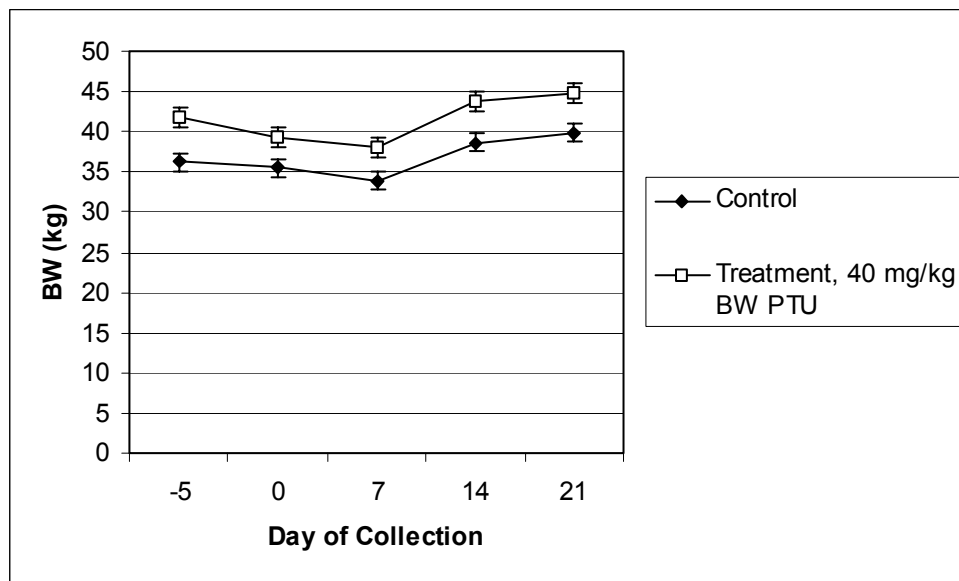
Serum

Prior to treatment triiodothyronine levels for control and treatment animals were similar (0.89 ± 0.05 ng/mL and 0.96 ± 0.02 ng/mL respectively). Thyroxine levels were comparable in control animals at 48.5 ± 3.28 ng/mL and treatment animals at 49.5 ± 7.13 ng/mL. Strong correlations were observed between T_3 and T_4 in the control group (r=0.68; P<0.0001). Similar correlations were seen for the treatment group (r=0.72; P<0.0001). Further analysis revealed a significant date effect for T_3 (P<0.001) with hormone levels decreasing over time with no significant treatment (P=0.91) or treatment by date interaction (P=0.26) between groups. A date by treatment interaction (P=0.0016) was observed for T_4. Hormone levels for the control group increased over time (T_3=0.89 ng/mL (Begin) to 1.05 ng/mL (End); T_4=48.5 ng/mL (Begin) to 60.5 ng/mL (End)) (Figure 10) while decreasing in the treatment group (T_3=0.96 ng/mL (Begin) to 0.87 ng/mL (End); T_4=49.5 ng/mL (Begin) to 33.83 ng/mL (End)) (Figure 11).
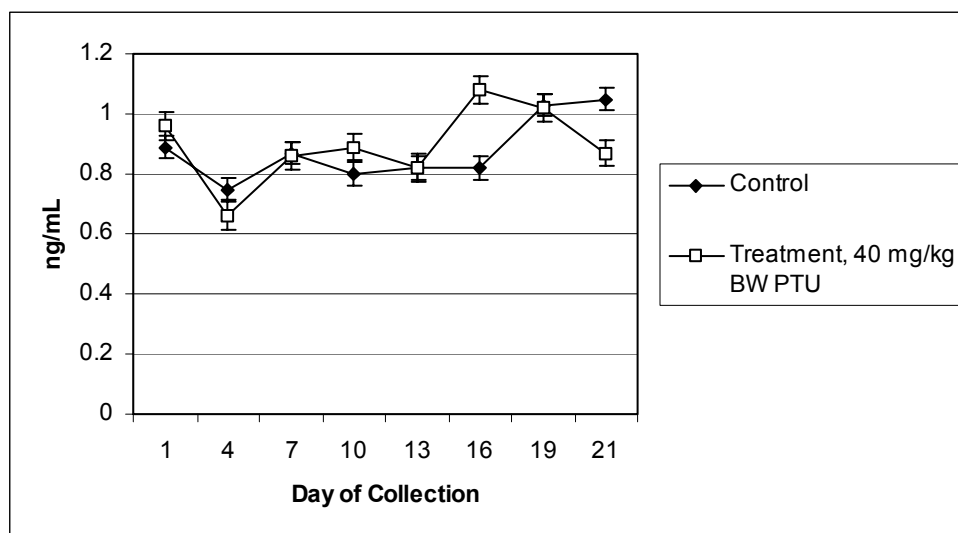
Figure 10. T$_3$ (ng/mL) analysis from sheep (n=12) serum collections from sheep receiving either 0.0 or 40 mg/kg BW 6-N-propyl-2-thiouracil (PTU) for 21 days.
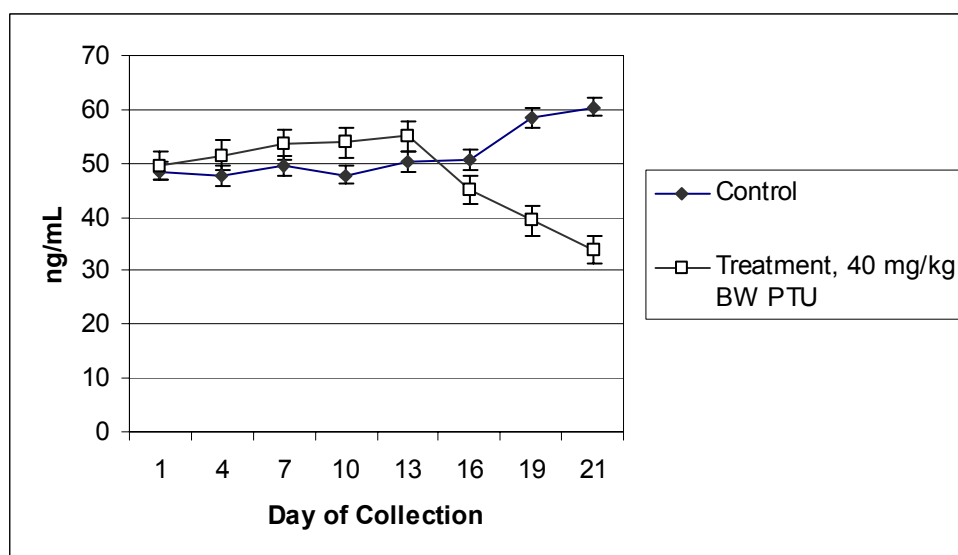
Figure 11. Serum T$_4$ (ng/mL) analysis from sheep (n=12) receiving either 0.0 or 40 mg/kg BW 6-N-propyl-2-thiouracil (PTU).

Generic E. coli

A date effect was observed for the shedding of generic *E. coli* (P=0.005) due to initial decreasing then increasing log counts. There was also a significant treatment effect (P=0.1). In general, the treatment group shed at higher levels throughout the study period (Figure 12) with slightly more than a log CFU difference between groups at the last collection point (control=3.8 $\pm$ 0.59CFU/gram of feces ($\log_{10}$); treatment=4.9 $\pm$ 0.43 CFU/gram of feces ($\log_{10}$)). No treatment by date interaction was observed (P=0.49).
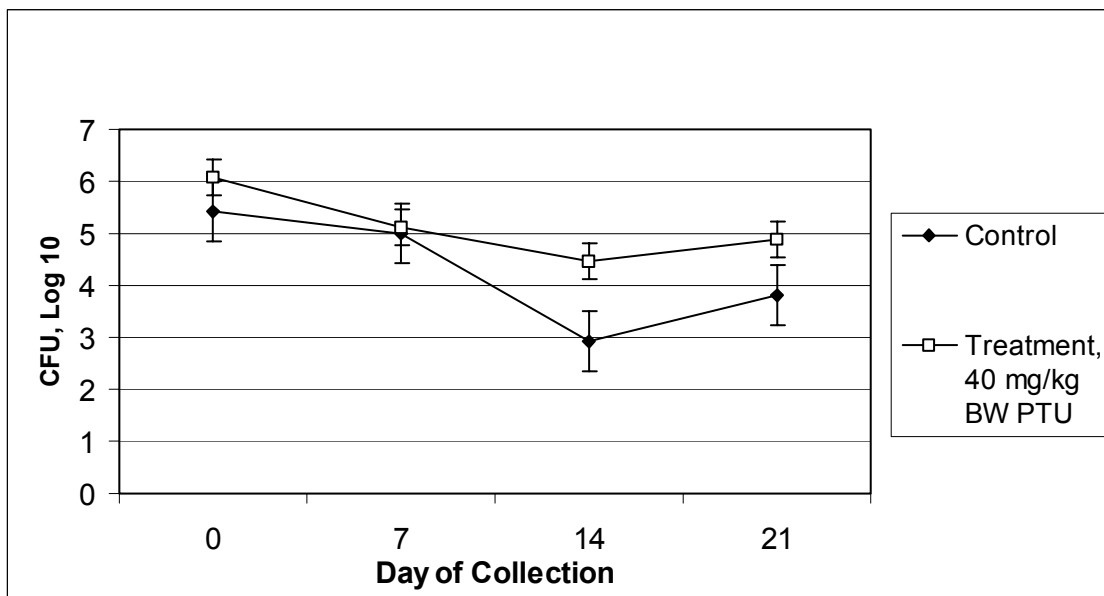
Figure 12. Fecal shedding of generic *E. coli* for sheep (n=12) receiving 0.0 or 40 mg/kg BW 6-N-propyl-2-thiouracil (PTU).

E. coli *O157:H7*

Analysis indicated a date effect, but no treatment by date effect and while log CFU's decreased over time, there was no significant difference between treatment groups. Further MANOVA analysis indicated a multivariate response between T_3 and T_4 (p=0.03). In addition, T_4 and *E. coli* O157:H7 shedding have a multivariate treatment (p=0.0005) and date effect (P=0.0174) but no significant interaction. A date trend (P=0.065) in O157:H7 shedding (Figure 13) and T_3 was observed, however no significant treatment or treatment by date interaction. A treatment effect (p=0.0004) and a date effect (p=0.0359) were observed for T_3, T_4 and bacterial log counts.

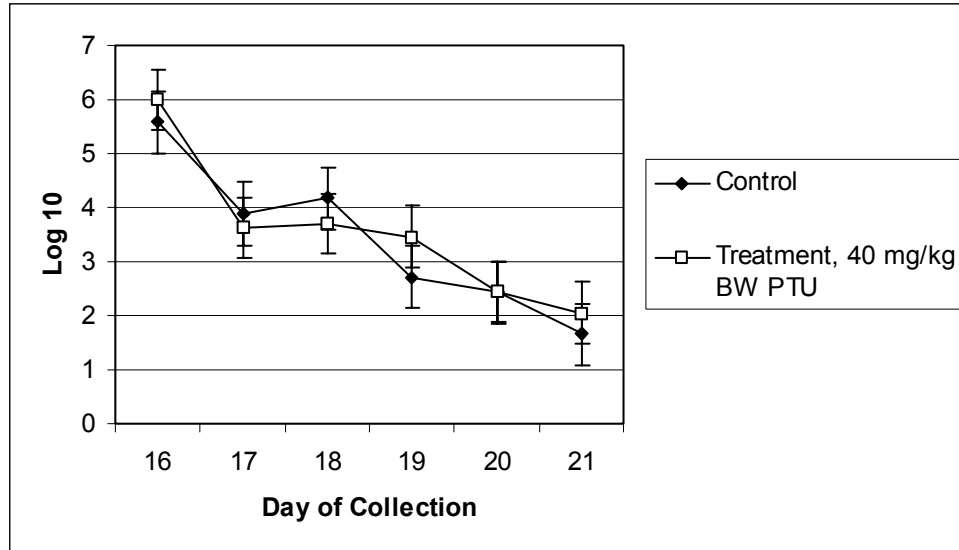Figure 13. Fecal shedding of *E. coli* O157:H7 for sheep (n=12) receiving either 0.0 or 40 mg/kg BW 6-N-propyl-2-thiouracil (PTU).

DISCUSSION

E. coli *O157:H7*

Sheep and cattle are considered major reservoirs for *E. coli* O157:H7 and are typically asymptomatic while shedding these pathogens into the environment (Kudva et al., 1996; Hancock et al., 1997; Bach et al., 2002). Naturally infected animals shed bacteria into the environment at levels lower than that of experimental infection and in a transient nature. Kudva et al. (1996) while sampling healthy, free ranging sheep, detected levels of 1 CFU/10 g of feces on average. Robinson et al. (2004) reported average levels of 10^3 CFU/1 g of feces in naturally infected cattle. High inoculation doses used for experimental infection can increase the amount of O157:H7 bacteria shed in the feces of an animal and thus increase the probability of detection for tracking purposes. Animals typically remain asymptomatic throughout infection even when exposed to inoculation doses as high as 10^{10} CFU of *E. coli* O157:H7 (Cray and Moon, 1995; Brown et al., 1997; Wales et al., 2001; Cornick et al., 2002). In addition, bacterial shedding has been shown to dramatically decrease in the first two weeks following experimental infection with *E. coli* O157:H7 (Cray and Moon, 1995; Brown et al., 1997; Cornick et al., 2002). These findings are consistent with results seen in both experiments 1 and 2 with sheep rapidly shedding bacteria following infection and remaining asymptomatic throughout the study period.

The seasonal patterns displayed by *E. coli* O157:H7, with levels being highest in the summer months and lowest in the winter months is inversely correlated with seasonal

fluctuations in thyroid hormone levels. At commercial abbatoirs and at various

production settings, *E. coli* O157:H7 is isolated at higher rates from May to September

in both sheep and cattle (Kudva et al., 1996; Hancock et al., 1997; Chapman et al., 2001;

Barkocy-Gallagher et al., 2003). To estimate the overall herd prevalence of *E. coli*

O157:H7 before reaching slaughter, fourteen dairy cattle herds were sampled monthly

for one year. A seasonal pattern with the majority of samples testing positive in the

summer months was reported by Hancock et al. (1997). The same trend has been seen in

free range ewes with 31% testing positive in June, 5.7% positive in August, and 0%

testing positive in November (Kudva et al., 1996). An intermittent shedding pattern for

this bacteria has also been documented (Kudva et al., 1996; Hancock et al., 1997;

Chapman et al., 2001; Barkocy-Gallagher et al., 2003). Variability of *E. coli* O157:H7

shedding within an individual animal has been shown. Robinson et al. (2004) found

bacterial shedding variation between 20 and 90% in calves sampled 5 times per day for 5

days and up to twice daily for 15 days.

Isolation of bacteria from content and tissue samples was not consistent across

experiments. For each experiment, bacterial isolation from tissue samples yielded no

statistical differences between control and treated animals. However, *E. coli* O157:H7

was detected more often in tissue samples from animals in experiment 2 than samples

from experiment 1. Grauke et al. (2002) found that with longer infection times, bacterial

detection decreased in both tissue and content samples from the gastrointestinal tract.

For sheep experimentally infected for 22 days, *E. coli* O157:H7 was isolated from fecal

samples of only half of the sheep sacrificed and not detected in any other tissue or

content samples. In contrast, researchers were able to isolate bacteria from all tissue samples and from fecal as well as lower ileum, cecum, and colon content samples from sheep experimentally infected for only 7 days (Grauke et al., 2002). These experimental results are consistent with findings from both experiments. The sheep from experiment 1 were experimentally infected up to 25 days. One sheep tested positive for bacteria in content samples and only three tested positive in enriched tissue samples. Content and tissue samples from experiment 1 displayed a clear trend with O157:H7 bacteria absent in the rumen and increasing toward the terminal end of the gastrointestinal tract with levels highest in rectal samples. This finding is consistent with previously reported data (Cookson et al., 2002; Grauke et al., 2002; Naylor et al., 2003). Grauke et al. (2002) experimentally infected sheep and found during harvest when *E. coli* O157:H7 was present, it was most often present in the lower gastrointestinal tract, cecum, and ascending colon. Samples obtained from sheep in experiment 2 at the time of harvest displayed many more positives than negatives in both tissue and content samples collected from the rumen, ileum, colon and rectum. In addition, unlike experiment 1, bacteria was detected in content samples from the rumen, ileum, colon and rectum and did not display the trends previously recorded. However, these findings are consistent with Grauke et al. (2002) for sheep experimentally infected for a short time. While Grauke et al. (2002) did not detect bacteria in rumen content samples, they did detect it in rumen tissue samples of sheep infected for a short time. This data helps to support the findings of experiment 2 and the lack of a clear trend of bacterial levels increasing toward the terminal end of the gastrointestinal tract.

The established inconsistent shedding pattern of *E. coli* O157:H7 (Cookson et al., 2002) makes it difficult to detect differences across time throughout either experimental group. However, a variety of factors could help explain the differences in detection of bacteria between experiment 1 and 2. 1) Different bacterial strains were used for experimental infection for each study, 2) the sheep from experiment 1 were mature sheep while younger, growing lambs were used for experiment 2, 3) the sheep from experiment 1 were infected for a much longer time period than the sheep in experiment 2 and 4) the diets between the two experiments varied slightly.

Two different strains of bacteria were used for these experiments. Sheep in experiment 1 were inoculated with *E. coli* O157:H7 strain 2029 selected for resistance to rifampicin and naladixic acid while experiment 2 sheep were infected with *E. coli* O157:H7 strain 933 was selected for resistance to novobiocin and naladixic acid. Both strains were selected for resistance to antibiotics for ease of detection following sampling. It is possible that the strains became slightly attenuated during this process which might contribute to possible differences in the bacteria's ability to establish infection. Cornick et al. (2000) evaluated different pathogenic *E. coli* serotypes and their ability to persistently infect sheep. Animals were experimentally infected and evaluated for 60 days post-innoculation. The two *E. coli* O157:H7 strains evaluated (strain 86-24 and strain 3081) differed in persistence of infection *in vivo*. By day 60 of infection, strain 86-24 was recovered from 2 of the 6 infected sheep. On the other hand, at the same time point, strain 3081 was recovered from 4 of six infected sheep. Wales et al. (2001) reported similar results when evaluating the "hardiness" of different *E. coli*

O157:H7 strains in sheep. Bacterial strains EC157 (streptomycin resistant), 140065 (naladixic acid resistant), 218 (rifampicin resistant) and 222 (nalidixic acid/rifampicin resistant) were combined in a cocktail and sheep were orally inoculated. Marked differences in recovery of bacterial strains were reported perhaps due to inter-strain competition, or perhaps due to decreased ability of some strains to colonize the gastrointestinal tract of the ovine host. Interestingly, the nalidixic acid/rifamicin resistant strain was recovered at lower levels than the rifamicin or nalidixic acid resistant strains (Wales et al., 2001). Incidentally, the strain used in experiment 1 was also a rifampicin/naladixic acid resistant strain.

Another distinct difference between the sheep of each experiment was age. Mature 12-16 month old sheep were used for experiment 1, while growing 5-7 month old lambs were the experimental unit for experiment 2. The ability of *E. coli* O157:H7 to colonize the sheep used in either experiment could have been affected by the very nature of immunity. Two types of immunity are responsible for protection of the gastrointestinal tract, innate and adaptive immunity. It is likely that both the mature and the growing lambs had an established innate immune system that would provide for a certain level of protection against invading organisms. Innate immunity includes those defense mechanisms such as epithelial cells, mucus, and phagocytic cells capable of consuming bacterial cells. Adaptive immunity, on the other hand, is based primarily on antibodies developed either from exposure to certain antigens either through natural infection or through vaccination. This type of immunity provides a stronger immune response against invading organisms, and is enhanced via repeated exposure to infection

or antigens through vaccination protocols (McClure, 2000). It can be assumed that the older sheep used for experiment 1 would have a more strongly developed adaptive immune system based upon exposure. In addition, the natural commensal bacteria of the gastrointestinal tract play an important role in immunity. It is documented that the gut flora change as animals mature in order to provide a stronger level of protection as well as to enhance the overall function of the gastrointestinal tract especially for ruminant animals (Draksler et al., 2002). The well established microbial flora of the mature sheep in experiment 1 could have inhibited colonization of *E. coli* O157:H7 simply by out competing it. Significant differences have been observed between *E. coli* O157:H7 infection of calves versus adult cattle (Cray and Moon, 1995). Animals were experimentally infected with a 10^{10} CFU dose of *E. coli* O157:H7. At the time of necropsy, all animals were histologically normal. However, through the course of the experiment, calves shed higher numbers of the bacteria and shed them for a longer period of time than the adult cattle. The authors concluded that the differences in rumen microbe development between the calves and adult cattle was most likely responsible for some of the observed differences in shedding patterns (Cray and Moon, 1995).

Duration of infection also differed between experiments. For experiment 1, some sheep were infected up to 25 days before harvest. The ability to detect bacteria both in fecal and gastrointestinal tissue and content samples has been shown to decrease as infection time increases (Grauke et al., 2002). At the time of harvest, experiment 1 sheep were shedding at an average rate of .87 CFU/g of feces (Log_{10}) whereas experiment 2 sheep at the time of harvest were shedding nearly a log count higher at 1.86 CFU/g of

feces (Log_{10}). The consistent decline in bacterial shedding for both control and treated animals in experiment 1 caused isolation to be more difficult and contributed to fewer differences across treatments. However, it was clearly demonstrated across both experiments 1 and 2 that enrichment of samples increased the likelihood of detecting bacteria.

Lastly, the sheep in both studies were maintained on slightly different diets. In experiment 1, sheep were fed a coastal grass hay and 1.13 kg per head per day. Experiment 2 sheep were fed an 80% concentrate, 20% forage diet at 3.8% of body weight. Sheep in experiment 2 received a higher concentrate diet which has been shown to increase fecal shedding of *E. coli* O157:H7. Lema et al. (2002) examined the effects of varying levels of acid-detergent fiber (ADF). Diets were formulated with ADF levels in increments of 5 from 5% to 35%. The 5% ADF diet was formulated to simulate a pure concentrate diet while the 35% ADF diet was to represent a forage diet. Feed consumption was similar across groups, however, fecal shedding of bacteria differed. Treatment groups receiving the 10% ADF to 35% ADF all had similar pathogen shedding levels. However animals recieving the 5% ADF, or high concentrate, diet, bacterial shedding was significantly higher (Lema et al., 2002). Similar results were seen in calves receiving either a high grain or high roughage diet (Tkalcic et al., 2000). Calves receiving high grain diets displayed increased fecal bacterial shedding as compared to calves maintained on high forage diets. Conflicting results were reported by Kudva et al. (1995) and Kudva et al. (1997). These studies reported increased and sustained levels of *E. coli* O157:H7 fecal shedding following experimental infection of

sheep maintained on high forage diets. However, bacterial levels in these studies were shown to increase when sheep were abruptly switched from a high grain to a high forage diet or when feed was abruptly withheld. It is unknown whether the high forage diet or the abrupt diet changes were responsible for the observed shedding patterns.

Generic **E. coli**

Generic *E. coli,* or total fecal coliforms, were evaluated in experiment 2 to determine if PTU treatment was able to affect other naturally occurring *E. coli* of the gastrointestinal tract. Sheep fecal samples were evaluated every seven days beginning on d 0. On d 0, *E. coli* populations were slightly lower in both groups compared to those reported for cattle on high grain diets. Diez-Gonzalez et al. (1998) reported log counts of 6.8 log cells/g of feces for cattle on moderate grain diets and 6.9 log cells/g of feces for cattle on high grain diets. The sheep from experiment 2 maintained on a high grain diet exhibited average *E. coli* populations of 5.8 log cells/g of feces. By day 21 of treatment, counts in both groups had declined, however, control group shedding declined much more rapidly and to a lower level than that observed for treated animals. Control group shedding decreased to 3.8 log cells/g of feces while treated levels remained at 4.9 log cells/g of feces. The treatment effect (P=0.1) observed indicates a possible direct interaction between PTU treatment and generic *E. coli* shedding.

Body Weight and Intake

Weight loss has been reported for sheep receiving PTU. Hernandez et al. (2003) reported decreased body weight gains for sheep receiving 20 mg/kg BW PTU as compared to controls and body weight losses for sheep receiving 40 mg/kg BW PTU. In

addition, Wells et al. (2003) reported lower body weight for young lambs receiving either 20 or 40 mg/kg BW PTU as compared to controls. However, Wells et al. (2003), did not see differences in weight across treatments until 3 weeks post treatment. Similarly, Hernandez et al. (2003) reported differences in weight 8 weeks post treatment. Body weights were recorded during both experiments to monitor animal health. With the exception of six sheep in experiment 1, all sheep lost weight over the course of the experiment. Control sheep tended to lose more weight on average over time than treated animals (Control: 3.7 kg; Treated: 1.9 kg). It is unknown why sheep exhibited lower end weights than beginning weights for experiment 1. All sheep consumed the experimental ration entirely and appeared in good health. The indicated weight loss could be due in part to the collection method. Sheep were all weighed prior to the acclimation phase of the trial and again on the day of harvest. Prior to the experiment, sheep were all maintained on pasture and the noticeable difference in beginning and end weight could be due to initial loss of gut fill due to the change in diet. This initial loss was also seen in experiment 2, but only for the first two weeks of the trial after which weights in both groups quickly rebounded. The difference seen in the two studies could be due to the difference in sheep types (the growing lambs used for experiment 2 versus the mature sheep of experiment 1) or perhaps due to the difference in feeding regimens. Sheep in experiment 2 were maintained on an 80% grain 20% forage ration with amount fed increasing as consumption increased. All of these factors could have contributed to sheep in experiment 2 displaying weight gains rather than weight losses.

Serum

All animals were housed indoors with controlled photoperiod of 16h light and 8h dark in order to simulate long day length as experienced during the summer months when thyroid hormones are low and *E. coli* O157:H7 is typically high. Sheep in both experiments were treated with the antithyroidal compound, PTU and serum T_3 and T_4 were quantified. Propylthiouracil is able to create a hypothyroid state by decreasing the release of T_4 and T_3 from the thyroid gland or inhibiting the conversion of T_4 to T_3 in the peripheral circulation or both (Achmadi and Terashima, 1995). The release of T_4 and T_3 from the thyroid is reduced because PTU directly affects thyroid utilization of iodine. In the periphery, PTU acts by inhibiting the type I 5'-monodeiodination. This type I monodeiodinase is responsible for activating the reverse T_3 (rT_3), an inactive intermediate in the conversion of T_4 to active T_3 (Villar et al., 1998).

In these experiments, at least one thyroid hormone was affected by treatment. The 20 mg/kg of BW PTU dose used for the first experiment did not have a significant effect on serum T_4 levels, however, T_3 levels decreased over time in the PTU treated group. The higher dose of 40 mg/kg of BW PTU used for experiment 2 decreased both thyroid hormones in the treatment group, however the effects on T_4 were more profound and began to decline earlier during treatment. Hernandez et al. (2003) found that with 20 mg of PTU/kg of BW a much longer time period was necessary to lower serum T_4 to desired levels than with the higher 40 mg dose. Ewes were treated with either 20 or 40 mg/kg BW PTU and serum T_4 was evaluated over time. It is unknown what effect on T_3 the two different PTU doses had because this hormone was not measured, however, it

was determined that at least 30 d of treatment were necessary to lower serum T_4 to levels below 20 ng/mL. Forty mg/kg BW PTU was able to elicit this response in approximately 18 days (Hernandez et al., 2003). It is possible based on these results that sheep from experiment 1 could have exhibited differences in T_4 levels across treatments had dosing continued beyond 15 days. It is hypothesized that the dose of PTU administered may affect hormone circulation in different ways. Low doses of PTU are thought to act on circulating levels of T_4 and T_3 directly and inhibit the type I 5'-monodeiodinase, thereby decreasing circulating T_3 levels (Villar et al, 1998). On the other hand, high PTU doses are thought to act directly on the thyroid gland and reduce circulating levels of T_4 (Villar et al., 1998). Villar et al. (1998) demonstrated differences in thyroid function with varying PTU doses in goats. Different groups of goats were orally administered low doses of PTU including 1.1, 2.2, 4.4, 8.8, and 17.5 mg/kg BW and a high group was given 35 mg/kg BW. Goats receiving the high PTU dose exhibited increased thyroid gland size (goiter) indicating a hypothyroid state and decreased type I 5'monodeiodinase activity (Villar et al., 1998). High doses of PTU tend to affect younger lambs more than mature sheep as indicated by greater decreases in circulating T_4 levels (Wells et al., 2003).   When administering 20 and 40 mg/kg BW PTU to six month old ewes, serum T_4 levels were reduced below 20 ng/mL in only seven days. Indicating that in younger animals, a smaller PTU dose may be necessary over a shorter time period to elicit a strong response in lowered thyroid hormone levels. In cattle, low PTU doses have been shown to inhibit type I 5'-monodeiodination in extrathyroidal tissues and not directly in the thyroid gland (Rumsey et al., 1985; Elsasser et al., 1992).

Therefore, lower PTU doses are more likely to elicit decreases in serum T_3 leaving T_4 virtually unaffected. This effect was clearly seen in experiment 1 where a lower PTU dose of 20 mg/kg of BW was administered resulting in only decreased T_3 levels.

Thyroid Hormones and **E. coli** *O157:H7*

For both experiments as expected, thyroid hormone levels decreased following PTU treatment. Experiment 1 sheep displayed a correlation between T_3 levels and *E. coli* O157:H7 shedding. As hormone levels decreased in treated animals, bacterial shedding increased as compared to controls. Similar results were seen for experiment 2 with T_4 and *E. coli* O157:H7 displaying a significant multivariate treatment (p=0.0005) and date (p=0.0174). Additionally, as hormone levels decreased in treated animals, generic *E. coli* shedding increased as compared to control animals. For both experiments, correlations were seen between decreasing thyroid hormone levels and increased fecal shedding of both generic *E. coli* and *E. coli* O157:H7 in experimentally infected sheep.

SUMMARY AND CONCLUSIONS

A thorough understanding of the complexity of pathogen carriage and shedding in the ruminant is necessary due to the continuing contamination of the human food supply. Results from these experiments suggest that correlations do exist between both thyroid hormones and *E. coli* O157:H7 shedding. However, small sample size and noisy data make a clear interpretation difficult. The generic *E. coli* data does show increased bacterial shedding with decreasing T_4 levels. It is clear that chemically suppressing thyroid hormone levels affects bacterial shedding, but the extent is unclear. Further investigations are needed to evaluate the exact role of thyroid hormones in the gastrointestinal tract of ruminants as well as the possible role thyroid hormones may have in the life cycle of bacteria like *E. coli* O157:H7.

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VITA

SASHA BROOKE SCHROEDER

October 21, 1980	Born in San Jose, California
May 1999	Graduated from Sharyland High School, Mission, Texas
May 2003	B. S., Animal Science, Texas A&M University, College Station, Texas
September 2003-March 2005	Graduate Assistant USDA-Food and Feed Safety Research Unit
April 2005-August 2005	Graduate Assistant-Department of Animal Science, Texas A&M University, College Station, Texas

FIELD OF STUDY

Major Field: Animal Science (Food Safety)

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PERMANENT ADDRESS
2471 TAMU
Kleberg Center, Room 114
College Station, TX 77843-2471